Impaired immunity in seals exposed to bioaccumulated environmental contaminants

Immuunsuppressie in zeehonden blootgesteld aan
in de voedselketen geaccumuleerde milieuvervuilende stoffen

PROEFSCHRIFT

Ter verkrijging van de graad van doctor

aan de Erasmus Universiteit Rotterdam

op gezag van de Rector Magnificus

Prof. Dr. P.W.C. Akkermans M.A.

en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op

vrijdag 23 juni 1995 om 16.00 uur

door

Ritske Ludolf de Swart

geboren te Doetinchem

Promotiecommissie

Promotores:	Prof. Dr. A.D.M.E. Osterhaus
	Prof. Dr. J.G. Vos
Overige leden:	Prof. Dr. E. Claassen
	Prof. Dr. M.C. Horzinek
	Prof. Dr. W. Seinen

CIP GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Swart, Ritske Ludolf de

Impaired immunity in seals exposed to bioaccumulated environmental contaminants / Ritske Ludolf de Swart
Rotterdam: Erasmus Universiteit Rotterdam
- Met lit. opg. - Met samenvatting in het Nederlands.
ISBN 90-9008419-3
Trefw.: immuuntoxicologie / organochloorverbindingen / zeehonden

The author of this thesis is employed as a research biologist by the Seal Rehabilitation and Research Centre in Pieterburen. The scientific advisory committee of this centre initiated and coordinated the studies described.

The work was also carried out at the former Laboratory of Immunobiology (head Prof.Dr. A.D.M.E. Osterhaus) of the National Institute of Public Health and Environmental Protection (RIVM), Bilthoven, and at the Department of Virology (head Prof. Dr. A.D.M.E. Osterhaus) of the Erasmus University Rotterdam, The Netherlands.

The studies presented in this thesis were supported by a grant from the Nederlandse Aardolie Maatschappij (NAM).

Illustraties:	Hilda Snoeyer, Groningen De schetsen die gebruikt zijn voor de titelpagina's van de hoofdstukken zijn voorstudies voor de tekeningen van de omslag. Voorzijde: "Zonder titel", 40 x 65.5 cm, 1995. Achterzijde: "Zonder titel", 45 x 65.5 cm, 1995. Beide houtskool op papier.
Ontwerp:	Piet Sebens, Groningen
Drukwerk:	Febodruk B.V., Enschede

Voor Hetty en mijn familie

Abbreviations

Ab	antibody
AFC	antibody forming cells
Ag	antigen
Ah-receptor	aryl hydrocarbon-receptor
AIDS	acquired immunodeficiency syndrome
ALAT	alanine aminotransferase
AP	alkaline phosphatase
APCs	antigen presenting cells
ASAT	aspartate aminotransferase
5-BCIP	5-bromo-4-chloro-3-indolyl phosphate
ß-HCH	ß-hexachloro-cyclohexane
CDV	canine distemper virus
CM	culture medium
Con A	concanavalin A
cpm	counts per minute
CTLs	cytotoxic T lymphocytes
DDA	dimethyldioctadecyl-ammonium bromide
DDT	dichlorodiphenyl-trichloro-ethane
DMSO	dimethylsulfoxide
DMV	dolphin morbillivirus
DTH	delayed type hypersensitivity
EDTA	ethylenediamine tetra-acetic acid
ELISA	enzyme linked immunosorbent assay
FBS	fetal bovine serum
FCS	fetal calf serum
F protein	fusion protein
GGT	gamma glutamyl transpeptidase
Hb	haemoglobin
HCB	hexachlorobenzene
Hct	haematocrit
H&E	haematoxylin and eosin
HIV	human immunodeficiency virus
H protein	haemagglutinin protein
HPLC	high pressure liquid chromatography
^{3}H-Trd	tritium-labeled thymidine
Ig	immunoglobulin
IL-2	interleukin-2
im	intramuscularly
IU	international units
KLH	keyhole limpet haemocyanin
LD	lactate dehydrogenase
LF	flocculation unit
LGLs	large granular lymphocytes

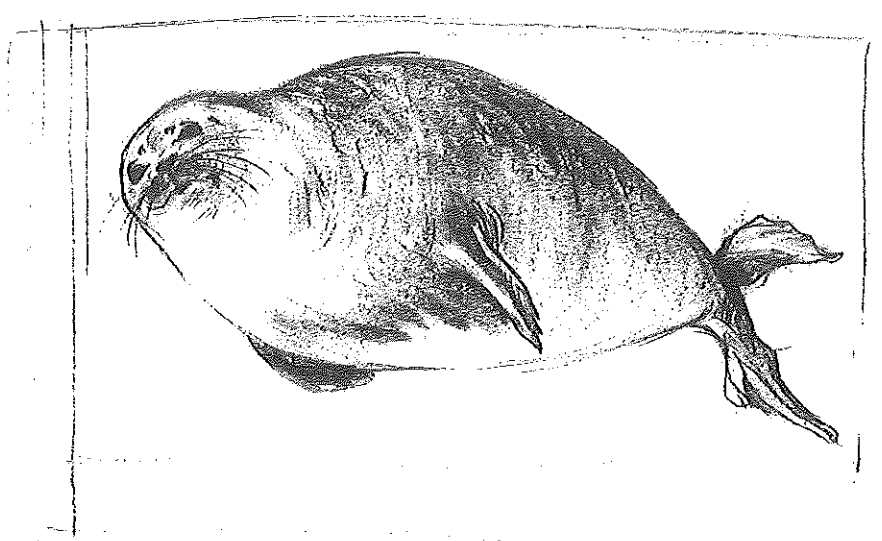
LPS	lipopolysaccharide from *Salmonella typhimurium*
MCH	mean corpuscular haemoglobin
MCHC	mean corpuscular haemoglobin concentration
MCMV	mouse cytomegalovirus
MCV	mean corpuscular volume
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
M protein	matrix protein
MV	measles virus
NK cells	natural killer cells
NP	nucleoprotein
Ova	ovalbumin
PBMC	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PCBs	polychlorinated biphenyls
PCDDs	polychlorinated dibenzo-*p*-dioxins
PCDFs	polychlorinated dibenzofurans
PDV	phocid distemper virus
PHA	phytohaemagglutinin-M
PHAH	polyhalogenated aromatic hydrocarbons
PMV	porpoise morbillivirus
P protein	phosphoprotein
PPRV	peste-des-petits-ruminants virus
RPV	rinderpest virus
PWM	pokeweed mitogen
RBC	red blood cell
rhIFN	recombinant human interferon-γ
rhIL-2	recombinant human interleukin-2
RV	rabies virus antigen
SD	standard deviation
SE	standard error
SI	stimulation index
SPF	specific pathogen free
TBTO	bis(tri-n-butyltin-oxide)
TCDD	2,3,7,8-tetrachloro-dibenzo-*p*-dioxin
TCDF	2,3,7,8-tetrachloro-dibenzofuran
TEFs	toxic equivalency factors
TEQ	TCDD toxic equivalents
T_h cells	T helper cells
TMB	tetramethylbenzadine
TT	tetanus toxoid
WBC	white blood cell

Contents

Chapter 1

General introduction

In part taken from: Morbilliviruses and morbillivirus diseases of marine mammals



Rik L. de Swart, Timm C. Harder, Peter S. Ross, Helma W. Vos and Albert D.M.E. Osterhaus

Infectious Agents and Disease, in press

To date, the serious and global threat of environmental contaminants to the delicate balance of ecosystems is not only recognized by the scientific community, but also by the public at large. In the late sixties, the organochlorine pesticide DDT was shown to be the cause of eggshell thinning in raptorial and fish-eating birds, which led to reduced reproduction rates and declining population sizes[16,90]. In subsequent years, polyhalogenated aromatic hydrocarbons (PHAH), including pesticides, polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) (figure 1), as well as other xenobiotics, were shown to be ubiquitously present in the environment[111,167,209]. Their hydrophobic nature and resistance to enzymatic breakdown resulted in accumulating levels towards the top of the food chain. Marine top predators, including seals, sea lions, dolphins and carnivorous whales, were shown to carry high body burdens of these contaminants[3,121,129,196,198,199].

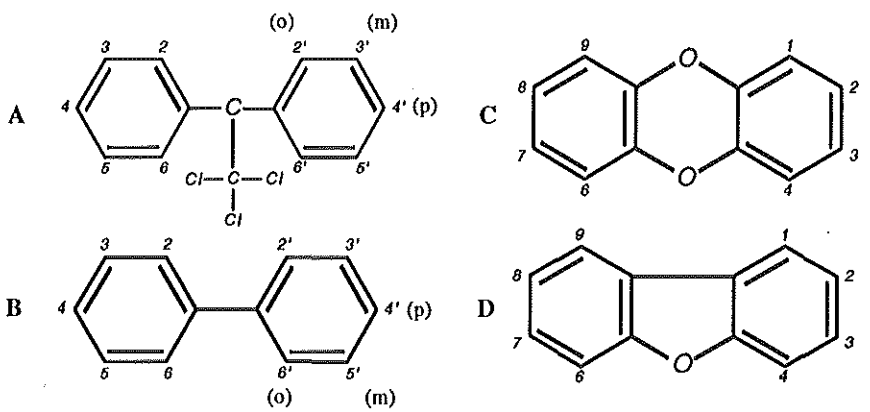


Figure 1: Molecular structure of DDTs (A), PCBs (B), PCDDs (C) and PCDFs (D). Numbers indicate position numbering of chlorine substitutions. Chlorine substitution positions of DDTs and PCBs are also indicated by "o" (ortho; positions 2,2',6,6'), "m" (meta; positions 3,3',5,5') and "p" (para; positions 4,4') (see diagram).

The association of bioaccumulated organochlorine contaminants in marine mammals with adverse biological effects has been the subject of a considerable number of scientific papers over the last decades (for review see 96). Reproductive toxicity has been reported in several species, including California sea lions (*Zalophus californianus*), ringed seals (*Phoca hispida*), grey seals (*Halichoerus grypus*) and harbour seals (*Phoca vitulina*)[1]. In a prospective feeding study, female harbour seals fed fish from the highly polluted Dutch Wadden Sea displayed a lower reproductive success than seals fed less contaminated fish from the Atlantic Ocean[163]. In the same study, the seals fed contaminated fish also proved to have reduced serum levels of retinol (vitamin A) and thyroid hormones[28], both biomarkers generally associated with PHAH contamination[159]. In Pacific Dall's porpoises (*Phocoenoides dalli*), reduced serum testosterone levels were shown to correlate with body burdens of

organochlorines[192]. Retrospective studies concerning the developmental biology of grey seals and harbour seals from the Baltic Sea demonstrated skull irregularities which were absent in specimens from before the sixties, and were suggested to be associated with increased levels of organochlorine contaminants[12,136]. A high incidence of tumours in beluga whales (*Delphinapterus leucas*) inhabiting the Gulf of St. Lawrence in Canada was suggested to be related to pollution with PHAH and non-halogenated polycyclic aromatic hydrocarbons and other environmental chemicals[46,130].

In the late eighties and early nineties, mass mortalities amongst seals and dolphins led to speculation about a causative role of environmental pollution in these events. Although established and newly recognized morbilliviruses were identified as the primary cause of these outbreaks, a contributory role of environmental contaminants causing immunosuppression was seriously considered.

The immune system as a target for toxic chemicals: a special place for marine top-predators?

The immune system

A major function of the mammalian immune system is to provide protection against invading pathogens. It consists of a complex network of interacting cells and molecules, and may somewhat arbitrarily be divided into an innate and an adaptive arm. The innate immune system functions as a non-specific first line of defence. Its main objective is to prevent pathogens from entering into the body or, if this fails, to limit the replication and spread of the pathogen. This creates more time for the adaptive arm of the immune system to raise a specific response. In the innate immune response to virus infections, important roles are played by cytokines and natural killer (NK) cells. Cytokines are soluble factors produced by different cell types involved in the immune response, and may alter the susceptibility of host cells to virus infection (e.g. interferon-α), or enhance the efficiency of different effector cell types of the immune system (e.g. interferon-γ)[14]. A more general and extremely important function of cytokines is to act as intercellular signalling molecules, which eventually results in an integrated immune response. NK cells are defined as leucocytes that have the capacity to recognize autologous virus-infected cells or tumour cells in a non major histocompatibility complex (MHC) restricted manner, as opposed to T lymphocytes (see below, for review see 232). NK cell activity is closely associated with large granular lymphocytes (LGLs), a lymphocyte subpopulation phenotypically different from B lymphocytes and T lymphocytes which mediate the adaptive immune response. Although the role of NK cells in the immune response has long been neglected, their function in the early stages of primary virus infections is becoming more and more appreciated. Their importance is illustrated by the fact that patients with rare NK cell deficiencies tend to suffer from severe virus infections[15].

The key features of the adaptive immune response are its specificity and immunological memory. Three different types of molecules are involved in the recognition of antigens: B cell receptors (immunoglobulins), T cell receptors and MHC molecules. The recognition by antigen receptors on individual B or T cells is restricted to one specific antigenic determinant: upon encounter with the antigen the cells are stimulated to proliferate, which eventually provides the host with a large pool of lymphocytes recognizing the same

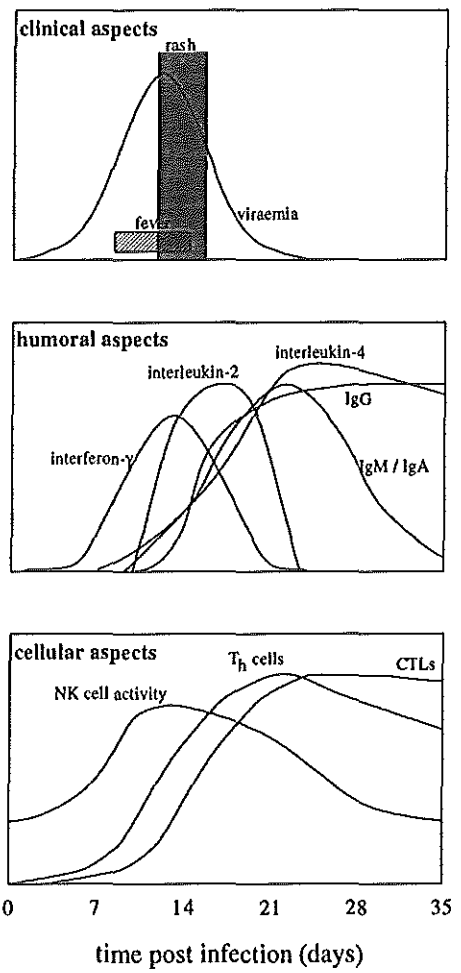


Figure 2: Schematic diagram of measles virus infection in primates, as an example of a morbillivirus infection in mammals: clinical aspects and kinetics of humoral and cellular immunological parameters. T_h cells and CTLs include both effector and memory cells. Adapted from reference 80, with additional information from references 54, 68, 203, 205, 206, 215, 218, 220 and 222.

antigen. Following this clonal expansion the cells may differentiate into effector cells or memory cells. Memory cells are formed following first encounter with a certain antigen (primary immune response), and will elicit a stronger and more rapid response to subsequent encounter with the same antigen (secondary immune response).

B cells mediate the humoral immune response: their effector cells (plasma cells) produce soluble immunoglobulins (Ig) with the same specificity as the B cell receptor on the lymphocyte from which they originated. These Ig circulate through the body and bind to, and in some cases neutralize, the pathogen that elicited their production. Different Ig isotypes are produced depending on the stage and location of the immune response. IgM is produced early in primary responses but has relatively low antigen affinity and will not persist long after infection. IgG is produced slightly later in primary responses, but is the major circulating Ig in secondary responses. It has higher affinity for the antigen and persists longer after infection. IgA is the main Ig isotype involved in mucosal immunity: it may be secreted across mucosal surfaces as secretory IgA.

T cells mediate the cellular immune response and have two major effector functions: T helper (T_h) cells act as regulators of the immune response while cytotoxic T cells (CTLs) may kill cells expressing or presenting foreign antigens, including virus-infected host cells. T cells do not recognize antigens directly but need processing and presentation by antigen presenting cells (APCs): the T cell receptor recognizes peptides originating from within host cells bound to MHC molecules. Uptake and processing of antigen from the circulation by specialized APCs may lead to presentation of peptides by MHC class II, which can be

recognized by T_h cells. Infection of any host cell with intracellular parasites such as viruses may lead to presentation of viral peptides by MHC class I molecules, which may be recognized by CTLs.

Both humoral and cellular immune responses are of importance in virus infections. Serum antibodies may neutralize infectious virus and limit cell to cell spread, thus preventing repeated infection with the same virus. CTLs may have a special role in infections with viruses that have the capacity to spread between cells without entering circulation, like morbilliviruses. T_h cells are especially important due to their regulatory function: if T_h cell function is impaired, the functioning of most of the other effector cells in the system is also affected. This is best illustrated by the pathogenesis of human immunodeficiency virus (HIV) infection: progression towards the disease (AIDS) is associated with declining numbers and malfunctioning of T_h cells leading to impaired immunocompetence, which eventually results in secondary infections and occurrence of tumours. In figure 2, a simplified diagram of the kinetics of the different cells and molecules involved in the immune response to measles virus is shown as an example of a mammalian immune response to morbilliviruses.

Due to its complex nature, it is difficult to assess the overall responsiveness of the immune system in the framework of immunotoxicological studies. However, a tiered approach has been developed to evaluate the potential immunotoxicity of chemicals[213]. The first tier of this screening consists of the evaluation of a limited number of functional immunological parameters. If this first tier provides indications of immunotoxicity, a larger number of *ex vivo/in vitro* and *in vivo* immune function assays is applied to study the function of the individual cell types and effector mechanisms involved in the immune response. Finally, host resistance to infectious agents may be evaluated in laboratory animals.

Some of the functional assays used in this tiered screening may be adapted for use in marine mammals. *Ex vivo/in vitro* assays are usually carried out with peripheral blood mononuclear cells (PBMC). NK cell function may be assessed by measuring the ability of PBMC to lyse tumour cell lines or virus-infected cells in a non-MHC restricted manner[174]. The lymphoproliferative capacity of B and T cells, important during clonal expansion, can be measured following stimulation of PBMC by mitogens or antigens[49]. Mitogen stimulation *ex vivo/in vitro* mimics the stimulatory action of antigens, inducing proliferation of certain lymphocyte subsets irrespective of their antigen specificity. Antigens can be used to stimulate memory cells *ex vivo/in vitro* which are present in body fluids after a primary immune response to the same antigen *in vivo*. Apart from proliferative assays, B cell function may be assessed by measuring serum Ig levels or numbers of Ig-producing cells present in PBMC (spot-ELISA)[49,107,108]. Specific T cell functions other than lymphoproliferative capacity that can be studied include *ex vivo/in vitro* antigen-specific lymphoproliferative responses and *in vivo* delayed type hypersensitivity (DTH) responses (mediated by T_h cells)[173], and *ex vivo/in vitro* cytolysis of virus infected cells by CTLs present in e.g. PBMC (not yet described for seals). Additional information on the state of the immune system may be obtained from measuring cytokine levels in circulation or in PBMC cultures[109].

Immunotoxicology

Laboratory animal studies have shown that the mammalian immune system can be a sensitive target of environmentally occurring toxic chemicals, including PHAH, hexachlorobenzene, non-halogenated polycyclic aromatic hydrocarbons, organotin compounds

and heavy metals[122,223,225]. Depending on the nature of the chemical and the type of exposure (acute or chronic), different compartments of the immune system may become functionally impaired, in some cases leading to a decreased host resistance. In the case of PHAH, which are thought to be the most important immunotoxic xenobiotics present in the environment at present, chronic exposure is predominantly associated with impaired cell-mediated immune responses. One of the toxic effects of these compounds first described was a pronounced thymus involution[225]. During perinatal development of the immune system, the thymus plays an important role in the maturation of T lymphocytes. Disruption of the thymic micro-environment as a result of perinatal PHAH exposure may therefore strongly influence cell-mediated immune responses.

Of the different immunotoxic PHAH, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) is the most potent and best studied immunotoxicant. The mechanism of toxicity of TCDD is thought to be receptor mediated[93]. Polyhalogenated aromatic hydrocarbons consist of different congeners, which are identified by the number and position of their chlorine substitutions, and differ in their biological toxicity. Although not all possible congeners of PCDDs (n=75), PCDFs (n=135) and PCBs (n=209) occur in the environment, the mixture of PHAH present in marine mammal tissues is complex. The most important and best studied mechanism of PHAH toxicity is mediated by binding to a cytosolic protein, the aryl hydrocarbon (*Ah*) receptor[179,180]. Upon binding, the organochlorine/receptor complex is transported into the nucleus, where it acts as a transcription regulator of a gene complex. Since the majority of the adverse biological activities of PHAH is thought to be mediated by their binding to this receptor, the toxic action of a mixture of different PHAH congeners is at least partially additive. TCDD is the PHAH with the highest affinity for the *Ah* receptor. Toxicity of other congeners may therefore be expressed relative to the toxicity of this congener, in TCDD toxic equivalents (TEQ)[179]. Using this method, it is possible to estimate the cumulative toxicity of a mixture of environmentally occurring PHAH on the basis of levels and known *Ah* receptor affinity of the most important toxic congeners.

Immunotoxicity resulting from chronic exposure to environmental chemicals accumulated through the food chain has not yet been subject of extensive studies in humans or wildlife. Although in the case of PHAH immunotoxicity of different congeners may be additive, interactions with and between other xenobiotics present in the environment are still unpredictable. From an immunotoxicological point of view, marine mammals may be of special interest. Certain species have a high daily consumption of fatty fish: harbour seals for instance consume approximately 5 kg fish per day. In certain contaminated areas, this results in levels of potentially immunotoxic chemicals which are among the highest recorded in free-ranging animals. It is therefore not surprising that the recent high incidence of mass mortalities among marine mammals led to speculation about the involvement of contaminant-induced immunosuppression.

The cause of mass mortalities amongst marine mammals: virus infections or environmental pollution?

Morbillivirus infections in pinnipeds

In 1988, an apparently contagious disease spread among harbour and grey seal

populations in northwestern Europe. Disease symptoms included respiratory, gastro-intestinal and neurological disorders, and were often complicated by parasitic, bacterial or viral secondary infections. Among harbour seals, the disease was accompanied with mortality levels of more than 60% in some areas, while in grey seals mortality was relatively low. On the basis of serological data[157], in combination with the clinical signs and pathological lesions found in infected seals which appeared to be quite similar to those observed in dogs with canine distemper, it was concluded that a morbillivirus closely related if not identical to canine distemper virus (CDV) was the primary cause of the outbreak[153]. Virus isolation and characterization studies demonstrated that the virus was not identical to CDV, but should be considered a new member of the genus *Morbillivirus*, now referred to as phocine distemper virus (PDV)[19,42,125,215]. Koch's postulates were fulfilled when seals proved to be protected from fatal challenge infection by vaccination with an inactivated CDV vaccine, and the virus could be re-isolated from the sham vaccinated animals in this experiment[156,220].

Members of the genus *Morbillivirus* of the family *Paramyxoviridae* are negative strand RNA viruses, which are known to be the causative agents of serious illnesses in their respective mammalian host species: measles virus (MV) is the causative agent of measles in humans, CDV of distemper in carnivorous species including canids (e.g. dogs, foxes), mustelids (e.g. ferrets, mink) and felids (e.g. lions, tigers). Rinderpest virus (RPV) and peste-des-petits-ruminants virus (PPRV) may cause serious disease in large and small ruminants (e.g. cattle, goats, sheep, for review see 17).

After the identification of PDV as the causative agent of the northwestern European epizootic, a similar outbreak amongst Baikal seals (*Phoca sibirica*) in Siberia (Lake Baikal), which started in 1987, could also be attributed to infection with a morbillivirus[78,155]. Initially, it was suspected that an epizootiological link existed between this virus, which was provisionally called PDV-2, and the northwestern European virus. PDV-2, like PDV, could be isolated from organ materials, and caused mild clinical symptoms in SPF dogs[215]. However, comparison of PDV, PDV-2 and CDV with respect to biological, morphological, protein chemical and antigenic properties confirmed that PDV was a morbillivirus different from CDV, while PDV-2 was a strain of CDV[215,221].

Morbillivirus infections in cetaceans

When in 1988 harbour porpoises (*Phocoena phocoena*) that had stranded along the Irish coasts were shown to be morbillivirus antigen positive[105], and the virus involved was subsequently isolated from animals stranded along both the Irish and Dutch coasts[132,218], it was assumed that the virus isolated would be identical to PDV. This notion changed when from 1990 onward large numbers of striped dolphins (*Stenella coeruleoalba*) started to wash ashore along the coasts of the western part of the Mediterranean Sea[59,208]. Virus isolates were compared with PDV and CDV, and with the above mentioned virus isolates from porpoises. It was shown that the dolphin and porpoise viruses were quite similar, but both were distinct from PDV and CDV. They were subsequently named dolphin morbillivirus (DMV) and porpoise morbillivirus (PMV)[207,218]. Sequence analysis studies suggested that they form a distinct lineage more closely related to the ruminant morbilliviruses (RPV and PPRV) than to the carnivore morbilliviruses[9]. A phylogenetic tree of morbilliviruses, based on sequence analysis of the F genes, shows the relationship of the newly discovered viruses with the established members of the genus (figure 3).

Origin and epizootiology of morbilliviruses of marine mammals

The origin of PDV was the subject of extensive speculation during and after the outbreak in 1988. Initial speculation about a possible link with CDV infections in sled dogs in Greenland, or with the PDV-2 (CDV) outbreak amongst seals in Siberia, could readily be dismissed after characterization of PDV as a new member of the genus. However, interspecies transfer of the virus is still considered the most likely cause of the outbreak. In sero-epizootiological studies, the presence of a virus closely related, if not identical, to PDV was shown in several North American marine mammal populations from the early eighties onward[63,64,152,177]. The virus could have been transmitted by Arctic harp seals (*Phoca groenlandica*), a species that is in contact with both the North American and European seal populations and has been shown to be infected with a PDV-like virus[56,191]. Interestingly, a mass migration of harp seals southwards to continental European waters had been observed in the period directly preceding the 1988 outbreak.

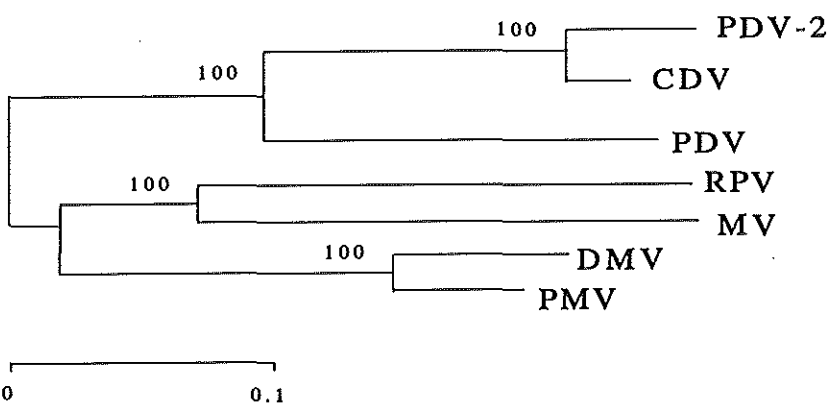


Figure 3: Unrooted phylogenetic tree of morbillivirus fusion protein encoding regions. Branch lengths are drawn in proportion to genetic distances as indicated by a bar. Numbers at nodes represent bootstrap percentages: values greater than 95% indicate a statistically significant separation of the respective cluster from the rest of the tree. Taken from reference 48.

Since virus characterization studies showed that PDV-2, the virus isolated from Baikal seals, should be considered a strain of CDV, the origin of this virus was probably a local terrestrial carnivore. Speculation that the origin might have been a vaccine strain of CDV used to vaccinate local dogs had to be dismissed since nucleotide sequences of PDV-2 P genes showed a closer relationship to European wild type strains than to the vaccine strain used in that area[127]. A similar event may have taken place in the Antarctic region in 1955, when a mass mortality amongst crabeater seals (*Lobodon carcinophagus*) was possibly related to a simultaneous distemper outbreak amongst recently imported sled dogs. Support for this assumption came from serological studies showing the presence of CDV neutralizing antibodies in these animals[10].

Serological studies have shown that morbilliviruses similar to DMV and PMV are enzootic amongst many cetacean populations[218]. Recently, retrospective immunohistochemical

studies suggested the involvement of a morbillivirus in an epizootic amongst bottlenose dolphins (*Tursiops truncatus*) along the U.S. Atlantic coast in 1987[119]. Based on its phylogenetic distinctness from other morbilliviruses, it may be concluded that it is unlikely that the PMV/DMV lineage has a recent origin in terrestrial animals (see figure 3). Common viral ancestors may have acquired access to phylogenetically related animal species on the basis of biological similarities: CDV and PDV may share a common ancestor, which is probably also the case for the PMV/DMV and the RPV/PPRV lineages.

Host factors in viral disease

The identification of morbillivirus infections as primary cause of the mass mortalities amongst marine mammals did not end speculation about a possible involvement of environmental contaminants in the severity and extent of the outbreaks[65,158]. Since both the pinniped and the cetacean morbilliviruses had been shown to be enzootic in other populations of marine mammals (see above), infection with these viruses apparently does not necessarily lead to extensive disease or mass mortalities. Viral pathogenicity is not only governed by viral determinants, but also by host determinants of resistance. Mims and White described the course of a virus infection as "a race between the ability of the virus to multiply, spread and make its exit from the body, and the ability of the host to curtail and control these events"[135]. Disease generally results from high levels of virus replication in target organs, while a timely control of virus replication by the immune system may lead to an asymptomatic subclinical infection and development of immunity.

A factor which strongly contributes to the pathogenicity of morbilliviruses is the fact that they infect cells of the immune system, and cause a dysfunctioning of the host defence mechanisms[112,131]. The primary target cells of morbilliviruses are macrophages in the lungs and the upper respiratory tract. From there, the virus circulates through the bloodstream in infected monocytes and/or lymphocytes[25,69,103]. During this cell-associated viraemia the virus is efficiently spread to other target organs, including lymph nodes, spleen, kidneys and the central nervous system. Morbillivirus viraemia is often accompanied by a lymphopenia: the absolute numbers of lymphocytes in circulation and in the lymphoid organs are strongly reduced. The virus-induced immunosuppression often leads to the occurrence of secondary viral, bacterial or parasitic infections, which are in many cases the direct cause of death. This phenomenon implies that a rapid and efficient immune response to morbillivirus infections may be even more critical than in response to certain other viruses, since the outcome of the battle between virus and immune system may be decided in the early stages of the infection. The marine mammal populations struck by the recent morbillivirus-related mass mortalities had not been previously exposed to the virus, leading to primary, and thus relatively slow, immune responses in the affected animals (see above).

During the PDV outbreak among European seals in 1988, both harbour seals and grey seals were affected, but mortality rates in grey seals were relatively low, emphasizing the importance of host factors in disease development. These host factors may include species determined factors like differences in cellular receptors for viruses[135], genetic factors (e.g. MHC variability[18,146]), co-infections (e.g. secondary infections[137]), population density[116] and immune responsiveness. The latter may be influenced by a large number of factors, including nutritional state[37], age[126], hormones, pregnancy, lactation[71] or stress[106,160], as well as exposure to toxic chemicals[225].

Indications of a contributory role of environmental chemicals

Several researchers have attempted to link environmental contaminant levels to the morbillivirus outbreaks, or to other diseases of marine mammals inhabiting polluted areas. Hall *et al.* reported significantly higher PHAH levels in harbour seals that had died along the UK coasts during the 1988 PDV outbreak, as compared to animals that had survived the outbreak[83]. Similarly, Aguilar and Borrell reported increased PHAH levels in striped dolphins that had died during the DMV outbreak in the early nineties, when compared to samples from free-ranging animals[3]. However, in both studies it was not possible to draw firm conclusions about a causative role of these toxic chemicals in the respective epizootics, since it was impossible to control for factors such as sampling methods, physical condition and virus exposure. In the dolphin study, liver lesions were reported in almost all DMV affected animals[60]. However, it was unclear whether these lesions resulted from high PHAH body burdens or from the virus infection, or were already present before the infection, thus leading to impaired liver function resulting in increased PHAH levels. In another study, levels of PHAH in bottlenose dolphins affected by the possibly morbillivirus related mass mortality[119] were also shown to be high, although it was impossible to make comparisons with PHAH levels in animals that had survived the outbreak[113].

Olsson *et al.* studied the relationship between disease and environmental contaminants in seals from the Baltic Sea[149]. Although grey seals and ringed seals inhabiting this region were found to suffer from impaired adrenal functions, epidemiological studies could not link these observations to levels of PHAH. On the basis of results of chemical residue analyses, as well as pathological findings (especially the absence of thymus involution), the authors suggested the involvement of DDT and PCB metabolites (methyl sulfones) rather than *Ah* receptor-binding PHAH in the disease complex. Kuiken *et al.* compared PHAH body burdens between harbour porpoises that had died of infectious or parasitic disease and animals that had died of acute physical trauma (e.g. drowning in fishing nets), but did not find significant differences[114].

Taken together, it may be concluded that epidemiological approaches to study the involvement of environmental contaminants have not been very successful. In one case an experimental approach was followed to study the influence of PCBs on host resistance of seals against PDV infection. Six harbour seals were fed fish spiked with PCBs for a period of several weeks, while four animals were fed relatively uncontaminated fish. All animals were subsequently challenged with PDV, resulting in similar mortality rates in both groups (4/6 and 2/4, respectively)[84]. The short conditioning period did however not lead to high PCB body burdens in the contaminant-exposed animals, and no conclusions could be drawn as to the influence of the administered PCBs.

We decided to use a different approach to address the same problem. In our opinion the question should not be whether environmental contaminants, in particular PHAH, can cause immunosuppression in seals: immunotoxicity has been observed in every mammalian species studied thusfar, but the effects proved to be largely dependent on levels of exposure, with sensitivities differing considerably between species. The most important question should therefore be, whether the contaminant levels and mixtures currently present in the environment influence immune function in the species concerned.

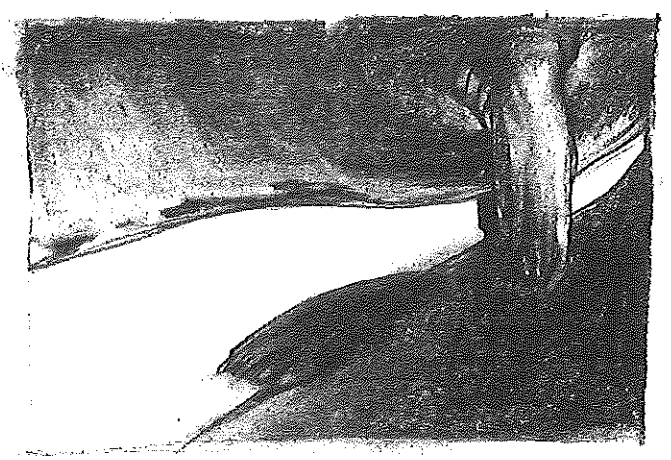
Outline of this thesis

We have addressed the question whether environmentally occurring contaminant mixtures can influence immune function in seals in a controlled experiment, in which young harbour seals after an adaptation period of one year were fed fish contaminated through the food chain of different marine regions. During a 2½ year period, two groups of 11 harbour seals each were fed herring from the heavily polluted Baltic Sea or the relatively uncontaminated Atlantic Ocean. During the feeding study, blood samples were taken at regular intervals in order to compare functional immunological parameters. Results of this study are presented in this thesis and elsewhere[172].

The development of a set of functional immunological assays is described in chapter 2: methods routinely used in immunological studies on other species were adapted for use in the harbour seal. In chapter 3, an overview of the differences in haematological and *ex vivo/in vitro* immunological parameters is presented. These differences are reported in more detail in the chapters 4, 5 and 6. In chapter 7, *in vivo* differences in immune function between the two groups are studied, by analyzing DTH and serum antibody responses. In chapter 8, an experiment is described in which the seals were experimentally fasted for a period of two weeks at the end of the study. Levels of PHAH in the herring fed during the feeding study, and in seal blubber and blood before the start of the fasting experiment are reported. During fasting, mobilization of organochlorine contaminants from the blubber and concurrent effects on immunological parameters were measured. In chapter 9, the results of the experiments are summarized and discussed in the context of the recent mass mortalities among marine mammals.

Chapter 2

Mitogen and antigen induced B and T cell responses of peripheral blood mononuclear cells from the harbour seal (Phoca vitulina)



Rik L. de Swart, Ruud M.G. Kluten, Carla J. Huizing, Lies J. Vedder, Peter J.H. Reijnders, Ilona K.G. Visser, Fons G.C.M. UytdeHaag and Albert D.M.E. Osterhaus

Veterinary Immunology and Immunopathology 37: 217 - 230 (1993)

Abstract

In vitro assays were developed for studies concerning the functioning of the immune system of the harbour seal (*Phoca vitulina*). Proliferative responses of peripheral blood mononuclear cells (PBMC) were measured after stimulation with different concentrations of the mitogens concanavalin A (Con A), pokeweed mitogen (PWM), phytohaemagglutinin (PHA) or lipopolysaccharid from *Salmonella typhimurium* (LPS). Con A and PWM induced strong proliferative responses, while PHA and LPS induced comparatively low proliferative responses. Responses of mitogen stimulated PBMC to recombinant human interleukin-2 (rhIL-2) and *in vitro* immunoglobulin production by mitogen stimulated PBMC were measured to discriminate between stimulation of T cells and B cells. It was found that Con A and PHA stimulate phocine T cells, PWM stimulates both T cells and B cells and LPS predominantly stimulates phocine B cells.

Antigen-specific immune responses were measured after immunization of seals with an inactivated rabies vaccine and/or with tetanus toxoid. Antigen-specific proliferation of PBMC and the presence of antigen-specific antibody forming cells were demonstrated for both antigens in the PBMC of immunized animals. The responses measured *in vitro* correlated well with the development of specific serum antibody titers to these antigens.

Introduction

The mechanisms underlying the recent mass mortalities among seals in North Western Europe and in Siberia are not fully understood. We showed that in both cases the primary cause was infection with morbilliviruses closely related to canine distemper virus[153,155]. However, it cannot be excluded that other factors may have influenced the severity and extent of these outbreaks[158]. Most of the discussion has focused on the possible suppression of the immune system by environmental contaminants.

A wide range of chemicals can influence the immune system of mammalian species, in some cases leading to immunosuppression and elevated susceptibility to infectious diseases[183]. Among these immunotoxic chemicals are persistent pollutants like PCBs and DDT, that tend to accumulate in the food chain and are found at high concentrations in the fat of seals and other marine mammals[162,199]. The major target for most of these immunotoxic chemicals appears to be the cellular immune system[229], which also plays an important role in the clearance of morbillivirus infections[205].

To assess whether indeed immunosuppression by environmental xenobiotics under natural conditions may have a significant impact on the resistance to infections in the harbour seal (*Phoca vitulina*), information on the functioning of the immune system of this species is needed. Studies on the humoral immune system and histology of lymphoid organs of several species of marine mammals[35,142,182] suggest gross similarities to the immune system of terrestrial mammals. Information concerning the cell mediated immunity of marine mammals is limited to lymphocyte transformation studies. Mitogen-induced proliferative responses of lymphocytes from several dolphin species have been described using different techniques [39,115,140]. Ross *et al.*[176] measured Con A-induced proliferation of harbour seal PBMC as an

indicator of cellular immune function.

In this report, we describe the establishment of a series of functional *in vitro* immunoassays which can be used to assess different aspects of the functioning of the immune system of the harbour seal.

Materials and Methods

Animals

Two female (Nos. 2514 and 2519) and three male (Nos. 2515, 2517 and 2518) harbour seals, caught as newborns in the Moray Firth (northeastern Scotland) in 1989 were held in captivity as previously described[163]. At the time the experiments were carried out the animals were between 1 and 2 years old.

To protect the animals from currently circulating PDV-1 infections, they were all vaccinated three times intramuscularly with the CDV-iscom vaccine that was previously shown to induce protection against PDV-1 infection in seals[220]. No signs of illness were observed during the course of the experiments. Three of the animals (Nos. 2514, 2515 and 2517) were immunized four times with an inactivated rabies vaccine (three doses with 3 week intervals, one dose 9 months later), and three animals (Nos. 2517, 2518 and 2519) were immunized twice with tetanus toxoid (TT) adsorbed to aluminium phosphate (3 months interval), both produced at the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands, as human vaccines.

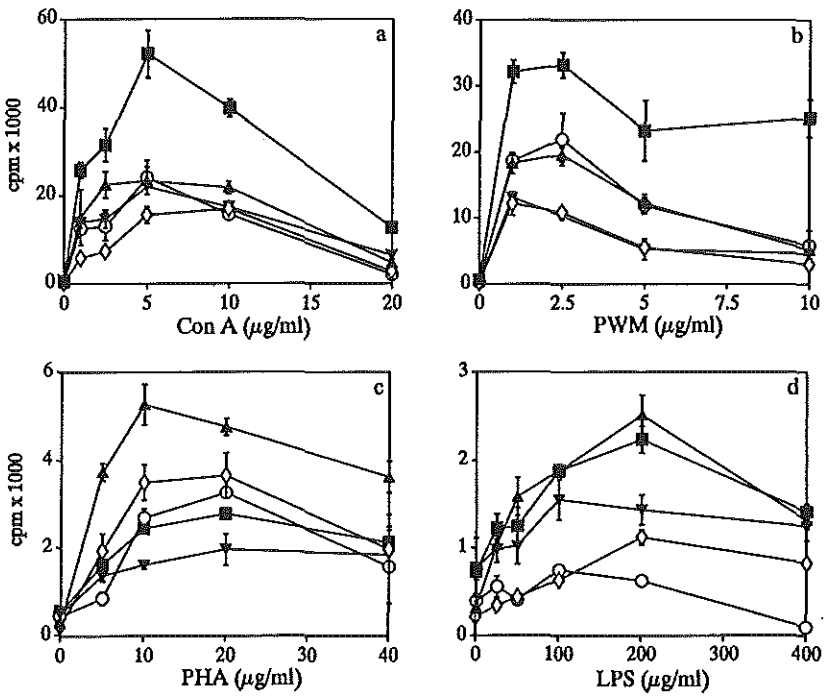
Isolation of peripheral blood mononuclear cells

Blood samples were taken from veins in the hind flippers or from the extradural vein into heparinized evacuated blood collection tubes (Becton-Dickinson, Rutherford, NJ), and kept at 4°C until further processing (2-8 h later). After dilution 1:2 in RPMI-1640 (Gibco, Grand Island, NY) with heparin (10 IU/ml, Organon Teknika, Boxtel, The Netherlands), PBMC were isolated by density gradient centrifugation on Ficoll-Isopaque (1.077 g/ml) for 30 min at 600 g. PBMC were subsequently washed once in RPMI-1640 containing heparin (10 IU/ml), once in RPMI-1640 containing heparin and 3% heat inactivated fetal bovine serum (FBS, Bocknek Laboratories, Toronto, Ont.), and twice in RPMI-1640 containing 3% FBS. Aggregates of platelets were removed by a short centrifugation at 150 *g*. PBMC were stored overnight on ice in RPMI-1640 containing 20% FBS.

Mitogen and antigen induced proliferation

PBMC were cultured in round-bottomed culture plates (10^5 cells/well, Greiner Labor Technik, Nurtingen, Germany) at 37°C in a humidified atmosphere of 5% CO_2 in air, in RPMI-1640 supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), 2-mercapto ethanol (2.10^{-5} M) and 10% FBS (further referred to as culture medium, CM). Where indicated Con A (1-20 µg/ml, Flow Laboratories), PWM (1-10 µg/ml, Flow Laboratories), PHA-M (2.5-40 µg/ml, Boehringer, Mannheim, Germany), LPS from *Salmonella typhimurium* (25-400 µg/ml, Sigma Chemicals, St. Louis, MO), rabies virus antigen (RV) (5-20 µg/ml) or TT (1-20 flocculation units (LF)/ml) were added. PBMC were cultured for 3-5 days. During the last 16 h cultures were pulsed with 0.5 µCi tritium labelled thymidine (^{3}H-Trd) per well and cell associated radioactivity was counted. Results are

Figure 1: Proliferative responses of PBMC from five healthy young harbour seals after mitogen stimulation. PBMC from seals 2514 (■-■), 2515 (O-O), 2517 (▼-▼), 2518 (◊-◊) and 2519 (▲-▲) were stimulated with Con A (a), PWM (b), PHA (c) or LPS (d), and cultured for 72 h (c) or 96 h (a,b,d). Results are shown in cpm x 1000 ±SD.



expressed as the mean counts per minute (cpm) ± standard deviation (SD) of triplicate wells, or as stimulation index (SI), defined as the mean cpm of three stimulated wells divided by the mean cpm of three control wells.

Human IL-2 induced proliferation

To measure the proliferative response to recombinant human interleukin-2 (rhIL-2, Boehringer), PBMC were stimulated for 48 h with one of the mitogens at concentrations giving optimal proliferative responses, washed to remove lymphokines produced in culture, and cultivated in rhIL-2 free CM for 24 h. Cells were washed again and were cultured for 48 h at a density of $5x10^4$ cells per well in CM containing different concentrations of rhIL-2. ^{3}H-Trd was added during the last 16 h of culture and cell associated radioactivity was counted.

ELISAs

To measure total or antigen (Ag)-specific protein A binding antibody titers 96-wells ELISA-plates (Costar, Cambridge, MA) were coated with Protein A (Boehringer, 100 ng per well), RV (600 ng per well) or TT (0.5 LF per well) in PBS for 2 h at 37°C. Plates were washed three times with water containing 0.05% Tween-80 (Merck, Munich, Germany) and

then blocked with PBS/0.05% Tween-20 /5% NaCl/1% Boseral (Organon Teknika) for 1 h at 37°C. Again plates were washed three times with water/Tween and incubated with serial threefold dilutions of plasma samples from the different seals (starting dilution 10^{-1}) for 45 min at 37°C. After washing three times, the plates were incubated with horseradish peroxidase conjugated protein A (Amersham International, Amersham, UK) for 45 min at 37°C. Finally plates were coloured using 3,3',5,5'-tetremethylbenzidine as a substrate and extinctions were measured at 450 nm. Titers were determined by measuring the dilution at which the extinction was more than twice the extinction of the same dilution of a plasma sample from the same seal taken before immunization.

Table 1: Stimulation indices of mitogen stimulated PBMC, corresponding to data shown in figure 1.

Seal number	Con A ($5\ \mu g\ ml^{-1}$)	PWM ($2.5\ \mu g\ ml^{-1}$)	PHA ($20\ \mu g\ ml^{-1}$)	LPS ($200\ \mu g\ ml^{-1}$)
2514	97	62	5	3
2515	87	79	7	2
2517	83	39	4	2
2518	68	48	17	5
2519	38	32	17	8

Spot-ELISAs

To determine total or Ag-specific protein A binding antibody forming cells (AFC) after *in vitro* stimulation of PBMC, a spot-enzyme linked immunosorbent assay (spot-ELISA) was carried out as described earlier for human PBMC[184,202]. Briefly, PBMC were stimulated with an antigen or one of the mitogens at a concentration of $2x10^6$ cells per well in 1 ml of CM in 24-wells flatbottom plates (Greiner Labor Technik). After 5 days cells were washed twice and resuspended in 200 µl CM. 96-wells ELISA-plates (Flow laboratories) were coated with Protein A (100 ng per well, Boehringer), RV (2500 ng per well) or TT (0.5 LF per well), and blocked with CM. Eight serial dilutions of the cell suspension were added to the wells and cultured overnight. The next day the plates were washed, incubated with alkaline phosphatase conjugated protein A (Sigma) and spots were coloured by adding 5-bromo-4-chloro-3-indolyl phosphate (5-BCIP, Sigma) in low melting point agarose. The total numbers of spots were then counted and the numbers of AFC per 10^6 PBMC were calculated.

Results

Mitogen-induced proliferation of PBMC

Proliferative responses of PBMC from the five healthy young harbour seals were determined after stimulation with several concentrations of the mitogens Con A, PWM, PHA

and LPS as shown in figure 1. Optimal concentrations of these mitogens were approximately 5 µg/ml for Con A, 1-2.5 µg/ml for PWM, 10-20 µg/ml for PHA and 100-200 µg/ml for LPS. The stimulation indices at these concentrations are shown in table 1. These data indicate that Con A and PWM in particular are powerful mitogens for inducing proliferative responses of PBMC of harbour seals. These optimal ^{3}H-Trd incorporation levels were found 3-4 days after stimulation with Con A, PWM or PHA, or 4-5 days after stimulation with LPS, RV or TT (data not shown).

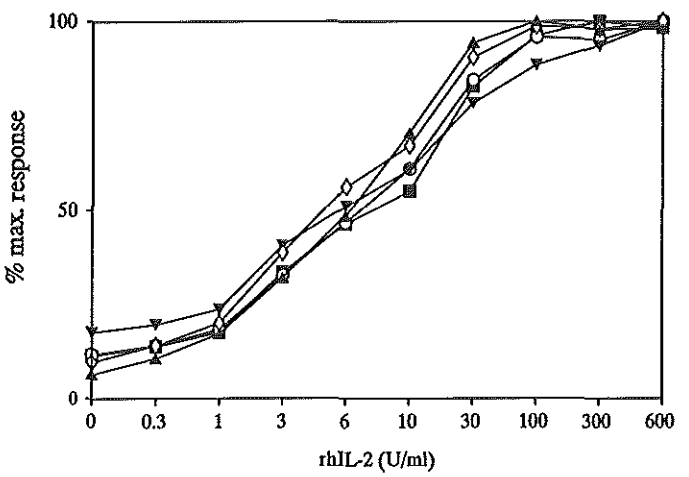


Figure 2: rhIL-2 induced proliferative responses of Con A-stimulated PBMC from seals 2514 (■-■), 2515 (○-○), 2517 (▼-▼), 2518 (◇-◇) and 2519 (▲-▲). Results are shown as percentages of maximal proliferative responses. Maximal responses were 119493±2460 cpm (No. 2514), 129953± 3151 cpm (No. 2515), 93292±4015 cpm (No. 2517), 109404±6290 cpm (No. 2518) and 69623±2760 cpm (No. 2519).

Discrimination between T cell and B cell activation by mitogens

During the course of our experiments we noticed that rhIL-2 could induce proliferation of Con A stimulated phocine PBMC. PBMC from five seals were stimulated with Con A, and washed on days 2 and 3 to remove endogenously produced lymphokines. This resulted in the emergence of large, blastoid cells, that were subsequently cultured for 2 days in the presence of different concentrations of rhIL-2. Strong proliferative responses of these cells were induced by rhIL-2 (figure 2).

As IL-2 interacts with the IL-2 receptor that is normally expressed on the surface of activated T cells, this response can be used to evaluate the capability of mitogens to activate phocine T cells. PBMC of four seals (Nos. 2514, 2515, 2517 and 2519) were stimulated with the mitogens Con A, PHA, PWM and LPS, at the optimal concentrations described above. The proliferative response of mitogen stimulated PBMC from these four seals to a saturating dose of rhIL-2 (250 IU/ml) is shown in figure 3a. Con A stimulated PBMC and, to a lesser extent, PHA- and PWM-stimulated PBMC showed significant proliferative responses to rhIL-2, whereas non stimulated controls and LPS-stimulated PBMC showed almost no responses. From these results we conclude that Con A, PHA and PWM stimulate phocine T cells.

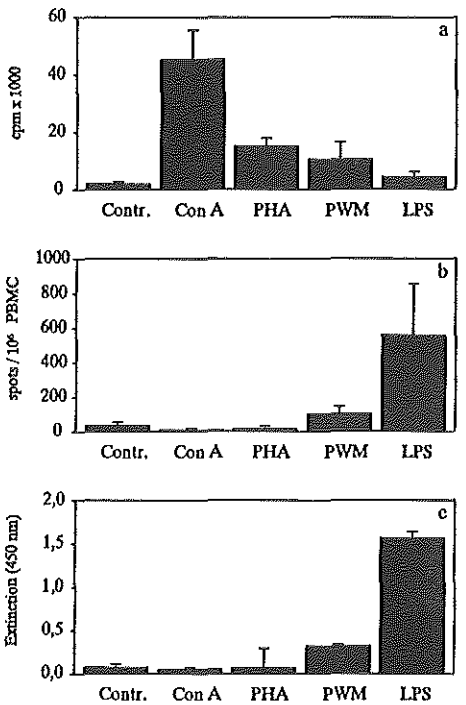


Figure 3: T and B cell responses of mitogen stimulated PBMC.
a. rhIL-2 induced proliferative responses of phocine PBMC. Proliferative responses of PBMC from four harbour seals (Nos. 2514, 2515, 2517 and 2519) were calculated by subtracting background proliferation from the maximal responses. The average response of the four animals is shown in cpm x 1000 ± SD. b. Spot-ELISA to detect AFC that produce protein A binding immunoglobulins in mitogen stimulated phocine PBMC. Results are shown as the mean number of spots per 10^6 PBMC from five animals ± SD. c. Protein A-ELISA to detect total immunoglobulin concentrations in supernatants of the mitogen stimulated phocine PBMC cultures shown in figure 3b. The average extinction at 450 nm ± SD of supernatants from PBMC from five different seals is shown for each mitogen.

To evaluate which mitogens can stimulate phocine B cells, a protein A spot-ELISA was used to enumerate AFC in mitogen stimulated PBMC. In addition a protein A ELISA was used to quantify the immunoglobulin concentrations in supernatants from these cultures. Figures 3b and 3c show that LPS and PWM stimulated antibody production by phocine B cells *in vitro*, and that Con A and PHA did not. LPS was more powerful than PWM in this respect: not only the number of AFC formed (figure 3b), but also the total production of protein-A binding immunoglobulins by B cells in culture was higher (figure 3c). From these results we conclude that LPS and PWM stimulate phocine B cells *in vitro*.

Antigen-specific proliferative responses

To measure Ag-specific immune responses, three seals (Nos. 2514, 2515 and 2517) were immunized three times with RV, and boosted 9 months later. In parallel, three seals (Nos. 2517, 2518 and 2519) were immunized twice with TT. This implies that seal No. 2517 was immunized with both RV and TT. The development of protein-A binding serum antibody titers against RV and TT was followed over a period of more than 1 year. High titers were found 1-2 months after vaccination, which gradually declined in the subsequent months (figure 4)

Figure 4: Ag-specific serum antibody titers after immunization with RV or TT. above. Three harbour seals, No. 2514 (■-■), No. 2515 (O-O) and No. 2517 (▼-▼) were immunized four times with RV. Seals No. 2518 (◊-◊) and No. 2519 (▲-▲) were not immunized with RV. Immunizations are indicated on the time scale by numbered arrows. RV-specific serum antibody titers were determined using an RV-ELISA. below. Three harbour seals, No. 2517 (▼-▼), No. 2518 (◊-◊) and No. 2519 (▲-▲) were immunized twice with TT. Seals No. 2514 (■-■) and No. 2515 (O-O) were not immunized with TT. Immunizations are indicated on the time scale by numbered arrows. TT-specific serum antibody titers were determined using a TT-ELISA.

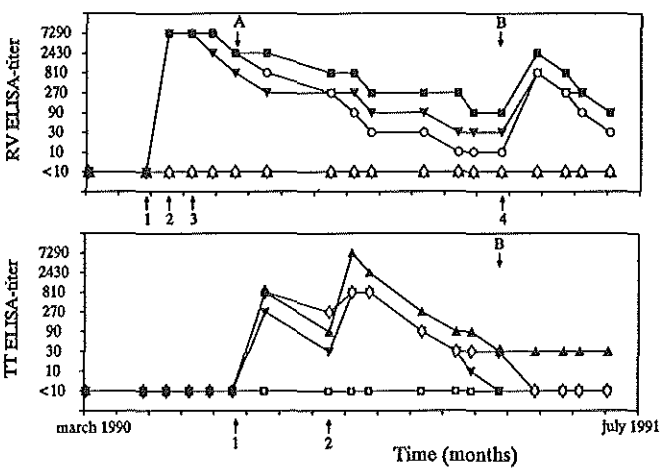


Table 2: RV- and TT-specific proliferative responses (cpm±SD) of PBMC from five healthy young harbour seals. Seals were immunized four times with an inactivated rabies vaccine (Nos. 2514, 2515 and 2517) and/or twice with tetanus toxoid (Nos. 2517, 2518 and 2519) (see figure 4). RV-specific proliferative responses of PBMC were measured (A) 6 weeks and (B) 9 months after the third rabies immunization. TT-specific proliferative responses of PBMC were measured 5 months after the second TT-immunization (B)

Seal number:	2514	2515	2517	2518	2519
Immunized with:	RV	RV	RV/TT	TT	TT
A					
Control	2506±1021	3766±770	867±90	1601±71	3378±227
RV (5 μg ml^{-1})	7219±1248[1]	14320±4192	3776±390	1910±452	3242±637
RV (10 μg ml^{-1})	8889±1934	15222±3987	3394±679	1953±405	4560±443
B					
Control	1731±30	2314±303	1961±144	1140±578	994±182
RV (5 μg ml^{-1})	2358±225	3850±2258	3313±424	1686±569	1188±132
RV (10 μg ml^{-1})	2492±690	2989±652	3680±674	1747±524	985±108
TT (10 LF ml^{-1})	1972±611	2141±605	2891±472	9761±1627	6464±515
TT (20 LF ml^{-1})	2267±665	2558±606	3686±451	11777±881	7562±427

[1]Responses exceeding 2.5 x background proliferation are underlined.

In vitro RV- and TT-specific proliferative responses of PBMC were measured after culturing PBMC from the different animals in the presence of different concentrations of RV or TT. Six weeks after RV immunization significant RV-specific proliferative responses of PBMC were measured in PBMC from all three RV-immunized seals (Nos. 2514, 2515 and 2517), and not in both seals that had not been immunized with RV (Nos. 2518 and 2519) (table 2A). Eight months later, when RV-specific antibody titers had dropped considerably (see figure 4a), no significant RV-specific proliferative responses could be measured in PBMC from either of the five seals (table 2B).

TT-specific proliferation of PBMC was measured 5 months after the second TT-immunization. PBMC from two TT-immunized seals (Nos. 2518 and 2519) showed significant TT-specific proliferative responses. The TT-immunized animal that was also immunized with RV (No. 2517), did not show a TT-specific proliferative response (table 2B). By the time this assay was carried out this animal had also lost its TT-specific serum antibody titer (see figure 4b). PBMC from both non TT-immunized seals (Nos. 2514 and 2515) showed no significant TT-specific proliferative responses (table 2B).

Antigen-specific B cell responses

RV- and TT-specific *in vitro* B cell responses were measured in a spot-ELISA, that was carried out 9 months after the third RV-immunization and 5 months after the second TT immunization. PBMC were stimulated for 5 days with PWM or different concentrations of RV or TT, and RV- and TT-specific AFC were enumerated on RV- and TT-coated ELISA plates.

RV-specific protein-A binding AFC were found in all RV-immunized animals after PWM stimulation of PBMC, but in only one animal (No. 2517) after *in vitro* stimulation with RV (table 3A). RV-specific AFC were also found in PBMC from seal No. 2519 that was not immunized with RV, but their numbers were not influenced by *in vitro* stimulations.

Table 3: Ag-specific Spot-ELISA, carried out 9 months after the third RV-immunization of seal Nos. 2514, 2515 and 2517, and 5 months after the second TT-immunization of seal NOs. 2517, 2518 and 2519. After five days of *in vitro* stimulation the numbers of (A) RV-specific or (B) TT-specific AFC per $2x10^6$ PBMC were enumerated.

Seal number:	2514	2515	2517	2518	2519
Immunized with:	RV	RV	RV/TT	TT	TT
A (RV coat)					
Control	2	3	3	3	18
PWM(2.5µg ml^{-1})	30[1]	24	30	3	18
RV (0.1µg ml^{-1})	3	2	62	6	24
B (TT coat)					
Control	2	3	27	2	11
TT (0.2 LF ml^{-1})	5	5	24	26	32

[1]responses exceeding 2.5 x background proliferation are underlined.

Induction of TT-specific AFC after *in vitro* stimulation with TT was demonstrated in two TT-immunized seals (Nos. 2518 and 2519). The third TT-immunized seal (No. 2517) showed high numbers of TT-specific AFC in both control and TT-stimulated PBMC (table 3B).

Discussion

In the present paper we have shown the establishment of *in vitro* mitogen and antigen induced responses of PBMC from the harbour seal, which may be used to study functional aspects of the immune system in this species.

Mitogenic lectins are known for their lymphocyte stimulating capacities in many different species, and are routinely used to evaluate changes in immune function. However, there is a certain degree of variation in the response to different mitogen among species. Thusfar the effects of mitogens on lymphocytes of aquatic mammals in general and their discriminative action on T and B cell populations in particular, have had little or no attention. Therefore, we tested the widely used mitogens Con A, PWM, PHA and bacterial LPS for their capacity to stimulate phocine PBMC, and showed that Con A and PWM in particular induce strong proliferative responses of PBMC from harbour seals. Previously it had been described that especially Con A, and to a lesser extent PWM and PHA could induce proliferative responses of PBMC from bottlenose dolphins (*Tursiops truncatus*)[39,115]. Ross *et al.*[176] showed that PBMC from harbour seals respond to Con A, but did not compare this response with that obtained with other mitogens.

Different mitogens stimulate discrete subsets of lymphocytes. Con A, PHA and PWM are known to stimulate T cells in several mammalian species[138]. Our finding that phocine PBMC stimulated with Con A, PHA or PWM respond to rhIL-2 indicates that these lectins are also T cell mitogens for the harbour seal. PWM and LPS are generally known to stimulate mammalian B cells[5,79]. In agreement with this, we demonstrated that both LPS and PWM induce immunoglobulin production by phocine PBMC *in vitro*.

In vitro mitogen assays are based on polyclonal stimulation of lymphocyte populations, and are generally accepted as a way to evaluate lymphocyte function. However, measurement of *in vivo* and *in vitro* Ag-specific immune responses may give more information about the functioning of the immune system as a complex network of interacting cell populations, because antigen presenting cells and B and T cell receptors are involved. In addition, the levels and persistence of the serum antibody responses after immunization with primary antigens can be used as parameters for the *in vivo* functioning of the immune system.

Therefore, we developed a model in which the immune response of immunized harbour seals to RV and TT can be studied. As these antigens can be considered to be primary antigens for seals, interference with memory cells may be ruled out. Resulting serum antibody titers were higher than those generally found in man after vaccination with these antigens. This may be explained by the relatively high concentrations of serum immunoglobulins found in seals[32]. The persistence of these titers proved comparable with other mammalian systems, where antibody titers to inactivated vaccines generally decline considerably within 1-2 years.

Ag-specific proliferation of PBMC *in vitro* was measured after stimulation with RV or TT. RV-specific responses could be measured in RV-immunized animals 6 weeks after the third RV immunization, but 8 months later these *in vitro* responses could no longer be

demonstrated. This implies that at that time numbers of circulating Ag-specific lymphocytes may have dropped to undetectable levels. TT-specific proliferative responses of PBMC were measured 5 months after the second TT immunization. Significant proliferative responses were measured in two TT-immunized animals (Nos. 2518 and 2519), but not in the third TT-immunized seal (No. 2517). This last animal, which had been immunized with both TT and RV, had also lost its TT-specific serum antibody titer by the time this assay was carried out. This suggests that the response to the TT immunization of this animal had been less efficient than that of the other two animals. In conclusion it may be stated that the *in vitro* Ag-specific proliferative responses show a good correlation with the presence of serum antibody titers against the respective antigens.

In vitro RV-and TT-specific B cell responses were measured in a spot-ELISA. After stimulation with PWM RV-specific AFC were detected in PBMC from RV-immunized animals (Nos. 2514, 2515 and 2517), but not in PBMC from animals that were not immunized with RV (Nos. 2518 and 2519). Enumeration of AFC specific for either RV or TT after *in vitro* stimulation of PBMC with the respective antigen preparations resulted in variable responses. Ag-specific AFC were only found in PBMC of one RV-immunized seal (No. 2517) and two TT-immunized seals (Nos. 2518 and 2519). It is not clear at present why in the PBMC of the other two RV-immunized seals (Nos. 2514 and 2515) no RV-specific B cells could be demonstrated after RV stimulation *in vitro*. This in despite of the fact that these cells were demonstrable after *in vitro* PWM stimulation of PBMC. This could indicate that the antigen recognition by B lymphocytes in this *in vitro* system is inefficient. Therefore we are considering the use of *in vitro* PWM or LPS stimulation rather than Ag-specific stimulation to demonstrate Ag-specific B cells in circulating blood in future immunotoxicological experiments. The inability to demonstrate TT-specific B cells in PBMC from seal No. 2517 after TT stimulation *in vitro*, may be due to the relatively high background in this animal and/or the virtual absence of the relevant cells in circulation. The latter is not unlikely since TT-specific serum antibody levels had dropped to undetectable levels.

We recently started an immunotoxicological experiment, in which two groups of young harbour seals are fed fish containing different levels of contaminants. During a three year period the functional assays described in this paper will be used to monitor and compare the functioning of the immune system of the seals in both groups, in combination with additional functional *in vitro* and *in vivo* immuno-assays (e.g. mixed lymphocyte reaction, measurement of natural killer activity and delayed type hypersensitivity responses). These experiments may clarify to what extent environmental xenobiotics influence the functioning of the immune system of the seal.

Acknowledgements

The authors thank Paul Thompson and Harry Ross for their support in obtaining the animals, Piet van Leeuwen (IFN) and all employees of the SRRC for their help in collecting blood samples, Rob van Binnendijk, Kees Siebelink and Roger van der Heijden for technical advices and Ms. Conny Kruyssen for preparing the manuscript. This work was supported by the Nederlandse Aardolie Maatschappij (NAM).

Chapter 3

Impairment of immune function in harbour seals (Phoca vitulina) feeding on fish from polluted waters

Rik L. de Swart, Peter S. Ross, Lies J. Vedder, Helga H. Timmerman, Siem H. Heisterkamp, Henk van Loveren, Joseph G. Vos, Peter J.H. Reijnders and Albert D.M.E. Osterhaus

AMBIO 23: 155 - 159 (1994)

Abstract

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 harbour 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 contaminants accumulated through the food chain.

Introduction

Marine mammals inhabiting polluted coastal areas are known to accumulate high levels of environmental chemicals[129,162,199], 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[77]. 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[12,85,86,136]. 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[192]. 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[163].

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[228]. These chemicals include polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs)[225], hexachlorobenzene (HCB)[223], dieldrin[73], ß-hexachloro-cyclohexane (ß-HCH)[41], and dichlorodiphenyl-trichloroethane (DDT)[7]. 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[78,155,217], striped dolphins (*Stenella coeruleoalba*)

in the Mediterranean Sea from 1990 onward[59,217], and harbour seals in northwestern Europe in 1988[157,217], has lead to extensive speculation about the possible contribution of environmental pollutants to these outbreaks of infectious diseases, by causing an impairment of immune function[83,148,158,181,188]. In addition, morbillivirus infections have been observed in seals inhabiting less polluted areas without causing any evident mortality[56,177].

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 harbour 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 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 harbour 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[20], 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 (7 females and 4 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 Centre in Pieterburen, in two similar basins with approximately 40 m^3 water and haul-out platforms of approximately 24 m^2.

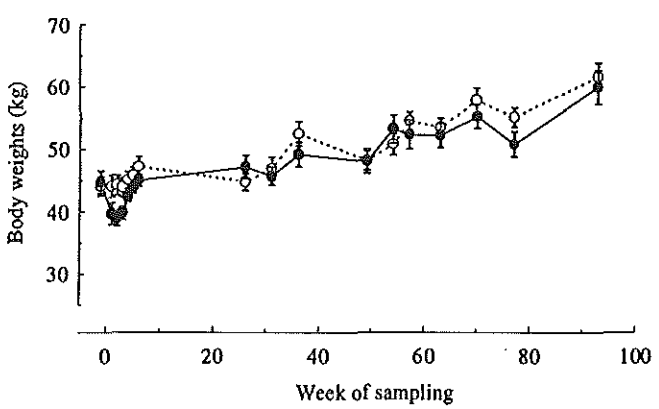


Figure 1: Mean body weights of harbour seals feeding on herring from the Baltic Sea (closed symbols) or the Atlantic Ocean (open symbols) over the course of the feeding experiment. Each group consists of 7 females and 4 males. Vertical error bars indicate standard errors of the means. Week 0 repre-sents the start of the feeding experiment (September 1991).

Diets were similar as regards overall quality, and were both supplemented weekly with a 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 basis of extractable fat. In addition to analyses performed as described previously[23], congener-specific analyses of PCDDs, PCDFs and coplanar PCBs were carried out using previously described methods[118,211]. 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.*[214], and the proposed TEFs for coplanar and mono-ortho coplanar PCBs as given by Safe[179], 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[62]. Vitamin A concentrations measured in this way showed a good correlation (r^2=0.89) with retinol concentrations measured in plasma (Dr. A. Brouwer, personal communication) using methods previously described[28]. 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 leucocyte subsets. Samples were kept shielded from direct day light 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[49], 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[212]. 10^6 YAC-1 cells were labeled with 100 µCi ^{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[49]. 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, 2.5 µg/ml, 20 µg/ml and 100 µg/ml, respectively)[49]. Proliferation was quantified by measuring the incorporation of ^{3}H-labeled 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.

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 and in ng TEQ day.

Compounds	Estimated daily intakes			
	Group 1		Group 2	
	µg day^{-1}	ng TEQ day^{-1}	µg day^{-1}	ng TEQ day^{-1}
sum 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.[3]	6	n.a.
Dieldrin	491	n.a.	54	n.a.
ß-HCH	17	n.a.	<5	n.a.
sum DDT	497	n.a.	102	n.a.

[1]Estimated daily intakes of sum PCBs in µg/day are based on total PCB concentrations in lipids, determined as described by Boon *et al.*[23]. Estimated daily intakes of PCBs in ng TEQ/day 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.*[211], and mono-ortho substituted PCBs with IUPAC numbers 118, 156 and 189, determined as described by Boon *et al.*[23]; [2]Estimated daily intakes of PCDDs and PCDFs both in µg/day and in ng TEQ/day are based on 17 2,3,7,8-chlorine substituted congeners only, determined as described by Liem *et al.*[118]; [3]n.a. = not applicable.

Statistical analysis

Accumulated longitudinal data were analyzed with ANOVA split plot analyses with time, sex and diet as factors[133], 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 figures represent standard errors of means (figure 1) or ratios (figure 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 (figure 1).

As shown in table 1, estimated daily intake of organochlorines was two to eight 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.

Comparison of haematological data

Vitamin A levels proved to be significantly lower in serum of the seals of the first group ($P<0.01$, figure 2), confirming results of a previous experiment with a similar setup in which reproductive disorders had been observed[28,163]. 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 (figure 2).

Comparison of immunological data

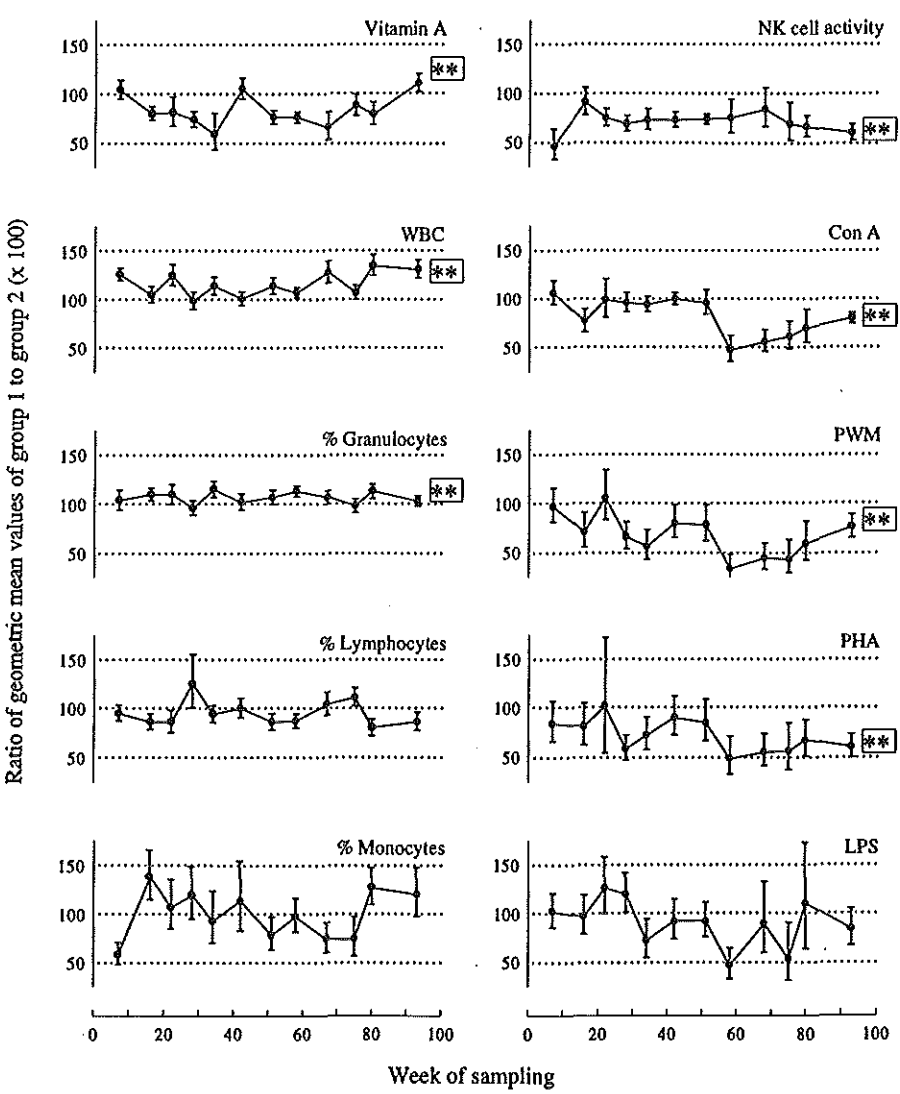
For comparison of immune function in the seals of both groups, peripheral blood mononuclear cells (PBMC) were isolated, and a series of *in vitro* functional immunological assays was carried out. Natural killer (NK) cell activity, as determined by a chromium release assay with the YAC-1 tumour cell line as target, was significantly lower in PBMC from the seals of the first group ($P<0.01$, figure 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 ($P<0.01$, figure 2). No significant differences were found in responses to LPS stimulation. Sex-related differences in the reduction of lymphocyte proliferation were observed, 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 harbour 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[29,159]. Furthermore, in a previous study in which harbour seals were fed with fish containing different levels of contaminants, levels of vitamin A and thyroid hormone levels were shown to be significantly reduced in seals feeding on polluted fish[28].

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[232]. 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[49]. T cells, especially cytotoxic T lymphocytes (CTLs), are known to be of crucial importance in the clearance of virus infections[72], which has also been documented for morbillivirus infections[204]. 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[212,225], and reduced proliferative responses of

Figure 2: Differences in means of serum vitamin A levels, haematological and immunological values between harbour 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.



lymphocytes to mitogens have been observed after exposure to PCBs, PCDDs, PCDFs, Dieldrin, and ß-HCH[41,73,225].

The observed increased 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[26]. The results of a sampling carried out 21 weeks before the start of the experiment (see legend of figure 2) make a genetic bias in immunological responsiveness of the seals in one of the groups unlikely.

It remains difficult to prove that 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[145]. 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.

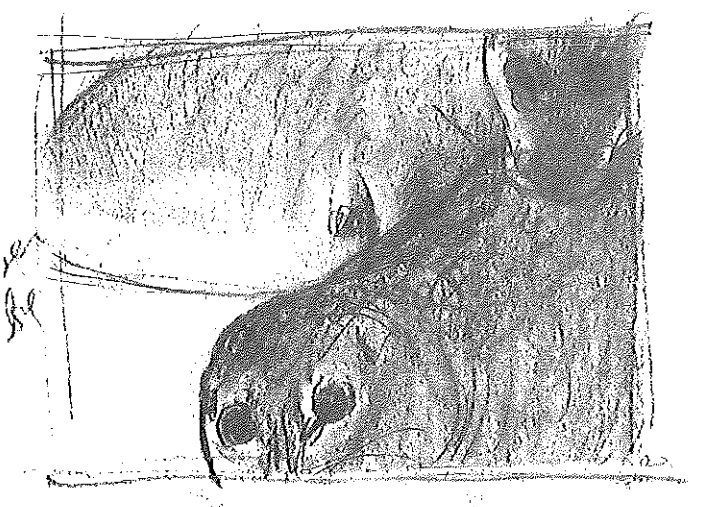
These results add malfunction of the immune system to previously identified biological effects of the contaminants that accumulate in the food chain, showing again that their present levels are a tangible threat to mammals inhabiting the marine ecosystem.

Acknowledgements

We thank Paul Thompson, Harry Ross, Bram Brouwer, Fons UytdeHaag, Jaap Scholtens, Djien Liem, Elze de Ruiter, Ilona Visser, the Martini Hospital in Groningen and all collaborators of the SRRC and the DLO-IFN for their contribution to these studies. We kindly acknowledge Conny Kruyssen for help in preparing the manuscript.

Chapter 4

Suppression of natural killer cell activity in harbour seals (Phoca vitulina) fed Baltic Sea herring



Peter S. Ross, Rik L. de Swart, Helga H. Timmerman, Lies J. Vedder, Henk van Loveren, Joseph G. Vos, Peter J.H. Reijnders and Albert D.M.E. Osterhaus

Submitted for publication

Abstract

Mass mortalities among marine mammal populations in recent years have raised questions about a possible contributory role of contaminants accumulated through the marine food chain. While viruses were shown to be the primary cause of the outbreaks, an immunotoxic action by organochlorine chemicals in affected animals could not be ruled out. We carried out a 2½-year immunotoxicological experiment in which two groups of 11 harbour seals each were fed herring from either the relatively contaminated Baltic Sea or the relatively uncontaminated Atlantic Ocean. Seals in the Baltic Sea group accumulated 3-4 times higher levels of *Ah* receptor-mediated 2,3,7,8-TCDD toxic equivalents in blubber than did their Atlantic counterparts following two years on the respective diets. Blood was sampled a total of 17 times during the course of the experiment for immunological evaluation, during which time the natural cytotoxic activity of peripheral blood mononuclear cells isolated from seals fed Baltic Sea herring declined to a level approximately 25% lower than that observed in seals fed Atlantic herring ($p<0.01$). Natural killer (NK) cell activity has not been previously described for a marine mammal species. We characterized the natural cytotoxic activity of harbour seal PBMC, and found this to be interleukin-2 responsive, sensitive to antibody anti-asialo GM1, and it was higher against a virus-infected target cell, like NK cells described for other mammals. As NK cells are leucocytes which play an important role in the first line of defence against viruses, the observed impairment of NK cell activity in the seals feeding on the Baltic Sea herring suggests that exposure to contaminants may have an adverse effect on the defence to virus infections in seals inhabiting polluted waters in Europe. This may therefore have affected the severity of the infections, the survival rates and the spread of infection during epizootics.

Introduction

Morbillivirus-induced mass mortalities among marine mammal populations in recent years have led to extensive speculation about the possible contributing role of organochorine pollutants. In 1988, approximately 20,000 harbour seals (*Phoca vitulina*) died when a newly identified morbillivirus spread rapidly through the populations inhabiting the coasts of northwestern Europe[57,153,217]. Despite the isolation and characterization of the responsible virus, called phocine distemper virus (PDV), pollutants could neither be implicated nor ruled out as contributing factors. Additional mass mortalities among bottlenose dolphins (*Tursiops truncatus*) in the Gulf of Mexico in 1987-88 and striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea in 1990-91 were also induced by morbilliviruses[119,208]. The high trophic status of many marine mammal species predisposes these animals to accumulating high concentrations of lipophilic organochlorines, including polychlorinated biphenyls (PCBs), DDT and, to a lesser extent, polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs)[188,189,197].

Environmentally-occurring levels of organochlorines have been correlated with biological effects in seals. Harbour seals fed contaminated fish from the Dutch Wadden Sea in an earlier experiment had significantly lower reproductive success[163] and vitamin A and

thyroid hormone levels[28]. Organochlorine chemicals have been linked to skeletal lesions among grey[12] and harbour seals[136] in the Baltic Sea. PCBs have been correlated with pathological lesions which inhibited reproduction in ringed seals in the Baltic Sea[85]. While contaminants have not been conclusively shown to have played a contributory role in virus epizootics[2,83,188], the high organochlorine chemical burdens of marine mammals in many areas makes these species potentially vulnerable to the adverse effects of pollution. Evidence from studies of laboratory animals suggests that PCBs, PCDDs, and TCDFs are potent immunosuppressants[225]. In preliminary findings, we observed a reduction in T lymphocyte responses and natural killer cell activity of PBMC isolated from harbour seals fed herring from the Baltic Sea as compared to controls[53], as were *in vivo* specific delayed-type hypersensitivity and antibody responses to the antigen ovalbumin[173].

Natural killer cells are lymphocytes which represent an important first line of defence against both virus infections and tumour cells (for reviews see 117,232). They have been characterized as large granular lymphocytes in mammals, which migrate directly to peripheral lymphoid organs and blood following development in the bone marrow. While NK cells have been defined by surface markers in rodents (e.g. monoclonal antibody 3.2.3 in the rat[36]) and humans (e.g. antibodies against $CD16^{+}CD56^{+}$[147]), much of the research on these lymphocytes has relied upon a functional definition. Routine testing for natural killer cell activity in different animal species involves tumour cell-directed cytotoxicity assays. To date, however, NK cell activity has not been characterized for a marine mammal species. Natural killer cells have been shown to lyse infected cells in different viral models, without major histocompatibility complex (MHC) restriction and independent of prior exposure to the virus. They are therefore vital in non-specific immunological defence against certain pathogens which the animal has not previously encountered, and play a role in limiting the spread of infection while a more effective specific antibody and cellular response is mounted[232]. The latter specific responses require a minimum of 4-5 days to begin an effective clearance of virus, and initial defence against a viral infection therefore relies upon the non-specific responses, of which the natural killer cells play a vital role.

In an attempt to determine whether contaminants at environmentally-occurring levels adversely affect immune function in harbour seals, we undertook a 2½-year captive feeding experiment. We extend here our previous findings of impaired T lymphocyte responses and NK cell activity in harbour seals fed Baltic Sea herring expressed as a percent of control[53], by describing the specific natural cytotoxic activity of peripheral blood mononuclear cells isolated from both the Atlantic and Baltic groups of seals. Since natural killer cells have not been described for harbour seals, we first undertook a series of assays to determine the feasibility of measuring natural cytotoxicity of leucocytes isolated from harbour seals, and to characterize this activity in harbour seals from comparative knowledge of NK cells in the other mammals.

Materials and Methods

Captive seal feeding experiment

Twenty-two recently weaned harbour seals were captured on the relatively unpolluted north east coast of Scotland in 1990 and housed as described elsewhere[53]. Briefly, seals were fed relatively uncontaminated herring from the North Atlantic Ocean for a period of one year

prior to the start of the feeding experiment. The seals were matched for weight and sex and subsequently divided at random between two groups of 11. The two groups were then fed their respective diets of herring originating from either the North Atlantic Ocean or the Baltic Sea, commencing in late September 1991. A supplement of vitamins was added weekly to the seal diets to compensate for losses during storage at -20°C. The nutritional quality of the two diets and the similar clinical chemistry profiles and weight gain patterns of the two groups of seals suggest that, other than the differences in intakes of contaminants, the two groups of seals were comparable[50,53].

Sampling

Blood was drawn every 6-9 weeks from the epidural vein of the seals for tests of immune function. Heparinized Vacutainers (Becton Dickinson, New Jersey, USA), kept at room temperature prior to sampling, were used for the collection of approximately 40 ml blood.

Blubber biopsies were taken aseptically from the 22 seals at week 104 of the experiment as described previously[173]. Briefly, 200 mg of blubber was sampled by inserting a 6 mm biopsy plug (Codman and Shurtleff, Randolph, USA) into a small incision in the skin on the dorsal surface of the seal, approximately 10 cm lateral to the spinal column. Samples were stored in glass vials at -20°C until analysis.

Isolation of leucocytes

Peripheral blood mononuclear cells (PBMC) were isolated from heparinized whole blood which was diluted 1:2 with cell culture medium (RPMI 1640 (Gibco, Life Technologies, Paisley, Scotland) containing penicillin (100 IU/ml), streptomycin (100 µg/ml) and L-glutamine (2 mM) and 10 IU/ml sodium heparin (Organon Teknika, Boxtel, The Netherlands) on a Lymphoprep (Nycomed Pharma, Oslo, Norway) 1.077 g/ml density gradient at room temperature within six hours of sampling as previously described[49]. Briefly, PBMC isolated following density gradient separation were washed in cell culture medium with the following added to respective washing steps: twice with 10 IU/ml sodium heparin; followed by 10 IU/ml sodium heparin plus 3% heat-inactivated fetal calf serum (FCS; Bockneck Laboratories, Guelph, Canada); and twice in 3% FCS only. Following overnight storage at 0°C in culture medium containing 20% FCS, PBMC were washed once in culture medium containing 10% FCS. The cell pellet was resuspended, counted using a haemocytometer, and adjusted to a standard concentration of 1×10^7 cells/ml in culture medium containing 3% FCS.

Natural killer cell assays

YAC-1 tumour cells of murine origin were used as targets for cytotoxicity in routine experiments. Briefly, 1×10^6 YAC-1 cells were radiolabeled with 100 µCi ^{51}Cr in 100 µl cell culture medium containing 10% FCS for 45 minutes at 37°C, washed five times in culture medium containing 10% FCS, and viable cells counted using Trypan blue dye exclusion. Natural killer cell assays consisted of a co-incubation of 1×10^6, 5×10^5, and 2.5×10^5 seal PBMC (effector cells) with 1×10^4 radiolabeled YAC-1 target cells in a final well volume of 200 µl in 96 well round bottom plates (Costar, Cambridge, USA) for six hours at 37°C in a humidified, 5% CO_2 atmosphere. Three effector:target cell ratios were therefore tested for

each seal at each routine sampling (100:1, 50:1, and 25:1). The specific release of ^{51}Cr by YAC-1 target cells reflected the natural cytotoxic activity of the PBMC, and was calculated as the (radioactive counts in the supernatant minus the spontaneous release by YAC-1) divided by (the maximal release by YAC-1 minus the spontaneous release by YAC-1).

In a parallel series of experiments, we characterized the activity of the effector cells responsible for natural cytotoxicity in our seal experiments. For these purposes, PBMC from the seals fed North Atlantic herring were used, and standard six hour cytotoxicity assays using an effector:target ratio of 100:1 were undertaken with an additional treatment during the assay:

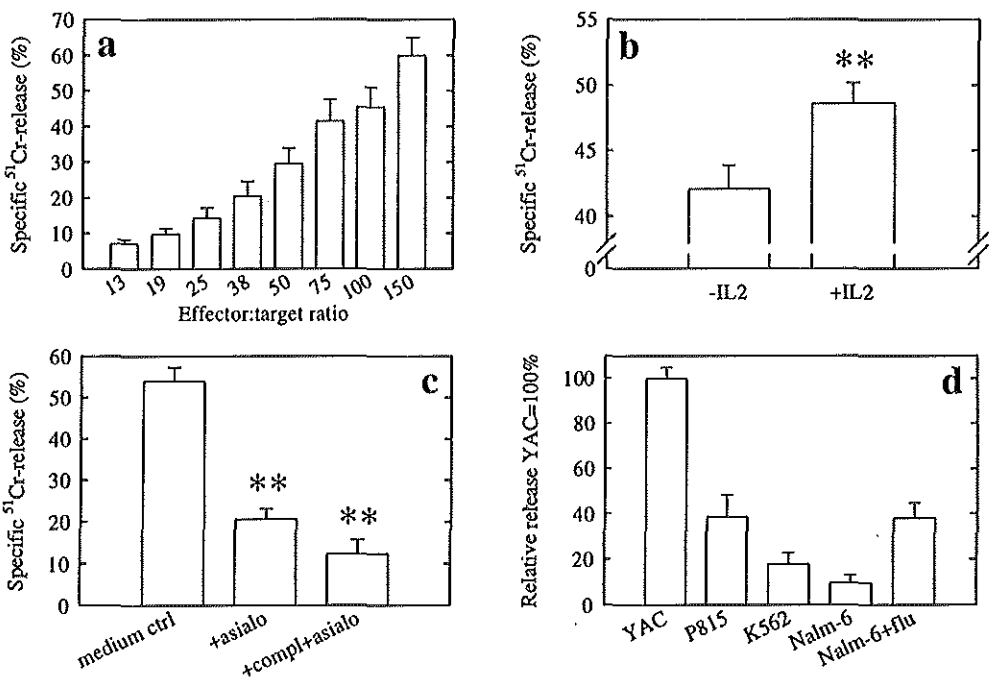
a) a co-incubation of 100 IU/ml recombinant human Interleukin-2 (rhIL-2; EuroCetus, Amsterdam, The Netherlands);
b) a co-incubation with culture medium, a 1:20 final dilution of anti-asialo GM1 antibodies (Wako Chemicals, Neuss, Germany), or a mixture of a 1:20 final dilution of anti-asialo GM1 plus a 1:60 final dilution of rabbit complement (Cedarlane Laboratories, Hornby, Canada);
c) 200 U/ml final concentration of recombinant human gamma-Interferon (rhIFN) in a co-incubation during the assay, or separately in a 15-minute pre-incubation and subsequent washing of effector cells immediately prior to the assay;
d) using P815 mastocystoma, K-562 human myeloid leukemia, Nalm-6 pre-B human leukemia, and Nalm-6 infected with Influenza A virus H1N1 (PR8 strain) in a 90 minute infection without FCS prior to the assay, as replacement targets for the YAC-1 cells.

For the purposes of immunotoxicological monitoring of the two groups of study seals, the natural cytotoxic activity of isolated PBMC was measured against YAC-1 target cells in a six-hour assay under identical sampling and culture conditions at each blood sampling. Following two preliminary experiments prior to the start of the feeding experiment using standard four-hour cytotoxicity tests, it was decided to use six-hour tests in order to enhance the sensitivity of the system. There were no significant differences in the natural cytotoxic activity of PBMC between the two groups in the two experiments undertaken prior the start of the feeding experiment (Atlantic 17.3±SE 5.8 vs Baltic 23.9±SE 5.8; Atlantic 19.3±SE 2.7 vs Baltic 13.8±SE 4.5). During the course of the feeding experiment, blood was sampled 15 times for assay. Statistical analysis was undertaken using a split plot ANOVA with time, sex and diet as factors. Natural cytotoxic activity was averaged for blood samples taken during the entire feeding period and these data were correlated against indicators of contaminant burden in seal blubber: total TEQ for dioxins and furans; TEQs for planar PCB congeners; and TEQs for mono- and di-ortho PCB congeners.

Electron microscopy

We assessed the extent to which platelets may have interfered with cytotoxicity assays. For this, a qualitative evaluation of PBMC samples isolated from seal blood was undertaken using standard techniques of scanning electron microscopy i) during winter (February) and summer (July) for four animals; and ii) using PBMC from seals with high cytotoxic activity or low cytotoxicity in the two groups. We have previously encountered problems in the density gradient isolation of seal PBMC as a consequence of platelet activation (unpublished

Figure 1: natural cytotoxicity assays were undertaken using PBMC from harbour seals: a) using YAC-1 target cells at three effector:target cell ratios; b) with recombinant human interleukin-2 (rhIL-2); c) with medium only, the antibody anti-asialo GM1, or a mixture of complement and anti-asialo GM1; d) using four different target cells, as compared to the standard YAC-1 tumour cell (using a corrected YAC=100%). In all experiments, 6-hour cytotoxicity assays were undertaken using PBMC from 11 seals of the Atlantic group and ^{51}Cr-labeled target cells. Means±SE are plotted and significance is indicated by asterisks where appropriate using paired *t* tests ($p<0.01$).



observations), and such an evaluation was carried out in order to exclude a methodological artefact in observed cytotoxicity results. For scanning microscopy, 10 x 10^6 PBMC were washed in phosphate buffered saline, fixed (4% formaldehyde (containing 10-14% methanol) and 1% glutaraldehyde solution, buffered with 0.1 M sodium cacodylate-HCl), and mounted on 0.1 M poly-L-lysine coated glass microscope cover slides.

Determination of contaminant levels

The intake of organochlorines was estimated at 288 ng TEQ/seal/day in the Baltic Sea group, compared to 29 ng TEQ/seal/day in the Atlantic group[53].

Blubber biopsy samples were used for congener-specific analyses of planar (IUPAC numbers 77, 126 and 169) and mono- (IUPAC numbers 118, 156, and 189) and di-ortho (IUPAC number 180) PCBs. Analyses were undertaken using methods described elsewhere[23,211]. All 2,3,7,8 chlorine-substituted dioxin (n=7) and furan (n=10) congeners were measured using methods described elsewhere[118]. Concentrations of these congeners were used to calculate 2,3,7,8-TCDD Toxic Equivalents (TEQs) in seal blubber using the Toxic

Equivalent Factors (TEFs) described for PCBs by[4] and for dioxins and furans by[214]. Individual values for all 22 seals are presented here, while means of the two groups have been presented elsewhere[173].

Results

Natural cytotoxic activity in harbour seal PBMC

The specific ^{51}Cr release by YAC-1 target cells following co-incubation with different concentrations of seal PBMC showed characteristic patterns of natural cytotoxicity, as higher effector:target ratios resulted in a high specific release as compared to lower effector:target ratios (figure 1a). The specific release observed during preliminary experiments using YAC-1 cells was relatively low, but this was resolved by increasing assay incubation time from four to six hours. The sensitivity of the YAC-1 cells to lysis made this cell line a suitable target for use in the routine assays during the course of the feeding study.

The natural cytotoxic activity of seal PBMC was significantly enhanced when the assay was undertaken in a co-incubation with rhIL-2 (paired *t* test, $p<0.01$; figure 1b). Conversely, the antibody anti-asialo significantly reduced the natural cytotoxic activity of seal PBMC, and complement plus anti-asialo almost completely eliminated cytotoxic activity (paired *t* test, $p<0.01$; figure 1c). Recombinant human Interferon had no effect on cytotoxic activity, whether assayed in a co-incubation during the assay, or following a pre-incubation and subsequent washing of effector cells (results not shown). While YAC-1 cells proved to be highly sensitive to the cytotoxic activity of seal PBMC, other cell lines were somewhat less so (figure 1d). P815 cells exhibited lower specific release, though there was considerable inter-animal variability. K562 cells were relatively insensitive to lysis by seal PBMC. While Nalm-6 cells were also insensitive as target cells, the same cells infected with Influenza A proved to be relatively sensitive targets.

Contaminant burdens in seal blubber

Following two years on the different diets, the mean toxicological burden of total TEQs in blubber differed between the two seal groups by a factor of approximately 3.4, with mono- and di-ortho PCB congeners accounting for the majority of the total TEQ composition

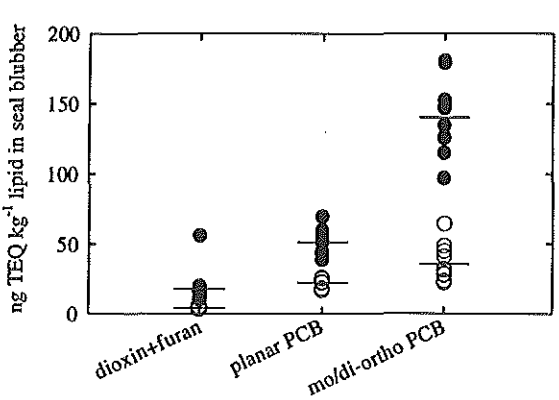


Figure 2: contaminant burdens in blubber sampled from individual seals fed Atlantic herring (open symbols) or Baltic herring (closed symbols) following two years on the respective diets. Contaminants were grouped here as i) dioxin (PCDD) and furan (PCDF) TEQs; ii) planar PCB TEQs; and iii) mono- and di-ortho PCB TEQs. Means of the two groups are indicated by a solid dash.

(figure 2). Mean natural cytotoxic activity of the PBMC of all seals during the entire feeding study was significantly correlated with the contaminant burden of seal blubber, whether measured against TEQ PCDD and PCDF (r=-0.32), TEQ planar PCBs (r=-0.49), TEQ mono and di-ortho PCBs (r=-0.56), total TEQ (r=-0.55), or concentration PCB (r=-0.68).

Monitoring of natural cytotoxicity of PBMC during the feeding experiment

There was considerable temporal variation in the cytotoxic activity of the seal PBMC, but seals fed the Baltic Sea herring had consistently and significantly reduced natural cytotoxic cell activity as compared to the seals fed the Atlantic Ocean herring over time, a trend which began within four months of the start of the feeding experiment (figure 3). The effect of pollution (diet group) was significant at the $p<0.01$ level (split-plot ANOVA). Natural cytotoxic cell activity was identical in both groups in the first six-hour assay, carried out 16 weeks following the start of the feeding experiment (atlantic 26.0±SE 3.3 vs Baltic 25.1±3.5;

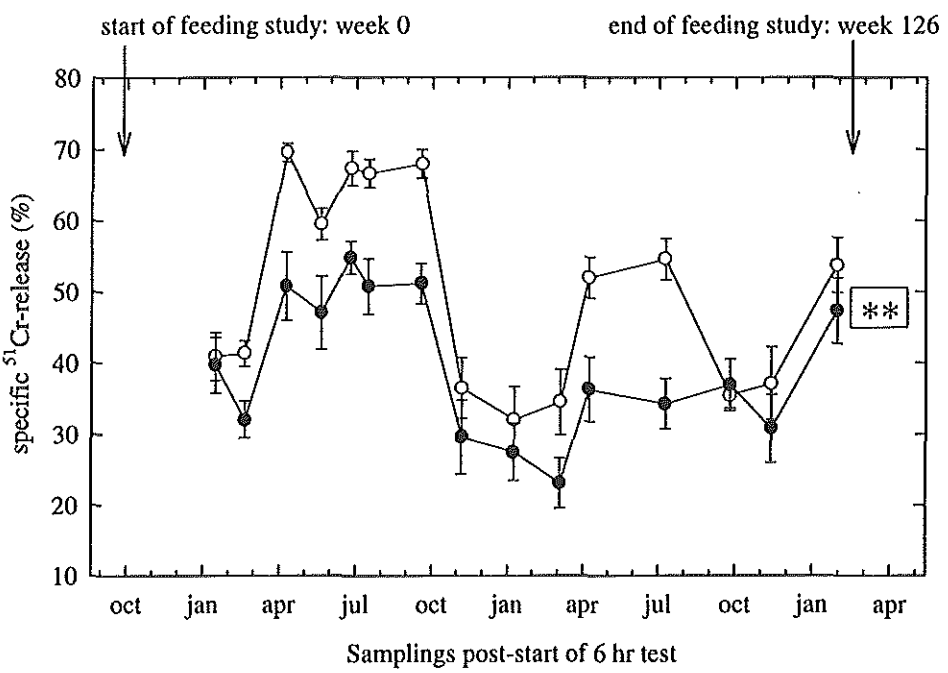


Figure 3: natural cytotoxic activity of PBMC isolated from harbour seals fed herring from either the relatively contaminated Baltic Sea (solid symbols) or the relatively uncontaminated Atlantic Ocean (open symbols). Activity was measured as the specific release of ^{51}Cr from YAC-1 target cells in a six-hour co-incubation with seal PBMC at an effector:target ratio of 100:1. Data points represent the means±SE of 11 seals. Differences between the two groups during the course of the feeding experiment were significant as measured by a split plot analysis of variance ($p<0.01$).

see figure 3), after which it declined in the Baltic group. There were no sex-related differences. An apparent seasonal pattern emerged in the responses of the seals in the two groups, with natural cytotoxic cell activity in winter being approximately half of that observed during the summer months for both groups of seals. There were no discernible differences in platelet presence or activation in the microscope preparations between summer and winter, or between low and high cytotoxic responders.

Discussion

The characterization of the effector cells responsible for target cell-directed cytotoxicity from our harbour seals suggests that they have similar functional properties to natural killer cells described in other mammals, including mice[40]; rats[47]; chickens[104]; dogs[110]; cattle[31]; pigs[165]; horses[38]; and humans[89,200]. The classic pattern of natural killer cell-induced cytotoxicity, as observed at different effector:target ratios[117] was apparent when PBMC from our seals were co-incubated with radiolabeled YAC-1 tumour cells (i.e. higher numbers of effector cells, higher release of ^{51}Cr by radiolabeled YAC-1 target cells). We have shown the effector cells in seals which lysed YAC-1 target cells to be IL-2 responsive, a trait shared by NK cells in other mammals studied[88]. The antibody anti-asialo GM1, when combined with complement, virtually eliminated cytotoxic activity from seal PBMC in a co-incubation. Anti-asialo GM1 is an antibody which binds to the cell surface glycolipid GM1 of NK cells and some macrophages in several mammalian species, including mice[102], and has been shown to suppress or abolish NK cell activity when administered *in vivo*[102] or *in vitro*[97]. Human gamma-Interferon did not enhance or block natural cytotoxicity, indicating a non-responsiveness of seal leucocytes, which may be expected for this non-autologous cytokine. Pre-incubation with autologous interferon leads to enhanced NK cell cytotoxicity in mice[58], but can also protect virus-infected target cells against lysis[30]. Since NK cells in mammals comprise a population of leucocytes which are generally defined by their functional properties, our observations support the concept that our assay system indeed detects the activity of NK cells in harbour seals.

The lower natural killer cell activity in seals of the Baltic group suggests that contaminants accumulated in the marine food chain are immunotoxic, and impair such activity by a mechanism which remains to be elucidated. Suppression of natural killer cell activity has been demonstrated in laboratory animals exposed to 3.9 µg/g diet methyl mercury[98]; to 20 mg/kg diet bis(tri-n-butyltin)oxide (TBTO) or 150 mg/kg hexachlorobenzene (HCB)[212]; and to 50 mg Aroclor 1254 (PCB)/kg in feed over a ten-week period[195]. Exposure to 2,3,7,8-TCDD has led to both an increase[75] or to no change[234] in baseline natural killer cell activity in rodents, though in the former study, an Influenza virus-associated increase in NK cell activity was suppressed in exposed compared to control animals. Host resistance to pathogens can be reduced by organochlorine chemicals as evidenced by higher virus titers and increased mortality following pathogenic challenge[94,120] (for review see 227).

The seasonal variation in NK cell activity in our harbour seals may be of interest in the continuing debate surrounding the complex immune system-pollution-disease matrix in the PDV epizootic. Seasonal cycles in immune responses including antibody production[124] and lymphocyte proliferation responses to mitogens[21] have been demonstrated, though to our knowledge, this has not been previously established for NK cell activity. Seasonality in

immune function is hypothesized to be mediated via photoperiod length-induced changes in the release of melatonin by the pineal gland[124]. Since no discernible differences were found in platelet presence or state of activation in PBMC samples from winter and summer or from seals with low and high cytotoxic activity, as examined using electron microscopy, an artefact introduced by platelets interfering with NK-target binding was ruled out as a possible factor in the seasonal patterns in cytotoxicity and in differences between the two groups of seals. Taken together, it may be speculated that the influence of both contaminants and season may represent significant factors affecting the outcome of NK cell-mediated virus infections in marine mammals.

Natural killer cell deficiencies have led to increased susceptibility to virus infections in different situations. A human patient suffering from recurring and life-threatening virus infections including varicella, hepatitis, cytomegalovirus, and herpes simplex, lacked functional NK cells, but all other immune parameters examined were normal[15]. NK-deficient young mice[24] and beige mice[185] are less resistant to mouse cytomegalovirus (MCMV). Stein-Streilen and Guffee[190] showed that the *in vivo* administration of anti-asialo to mice and hamsters resulted in diminished survival rates following challenge with Influenza virus. Welsh *et al.*[231] found that mice administered anti-asialo GM-1 had higher mortality rates and virus titers following MCMV infection as compared to control mice. Our observation that Influenza A-infected Nalm-6 cells were more effectively lysed than their control counterparts supports the notion that NK cells in harbour seals play a role in early responses to viral infections, as has been shown in other mammals[33].

While it is difficult to assess the *in vivo* significance of the observed decline in NK cell activity in the seals fed the Baltic Sea herring in our study, it is clear that diminished NK cell activity can have serious clinical repercussions. In the case of the PDV-epizootic among harbour seals in 1988, a virus was introduced to an immunologically naive population. Under normal circumstances, non-specific immunological defences, particularly NK cells, would respond in an attempt to eliminate or slow the spread of the virus in the first 2-4 days of infection. Specific T and B cell responses would then follow in order to clear the virus 5-8 days following infection and maintain protection against future encounters with the same virus by immunological memory. If both a first line of defence against virus infections (NK cells), as well as specific T cell responses, were less functional as a consequence of contaminant-induced immunosuppression, pollution could well have contributed to the severity and extent of the outbreak. Our study seals were not perinatally exposed to the high contaminant diet, and accumulated a mean contaminant burden during the course of the feeding study which was lower than that observed in many seal populations of Europe. This suggests that free-ranging populations of seals in Europe may be at a higher risk to the effects of contaminant-induced immunosuppression than our captive seals. The multitude of factors that affect the outcome of a virus epizootic (e.g. population density, social behaviour, immunological memory) make it impossible to directly relate contaminant levels to mortality rates in an event such as the 1988 PDV outbreak. As such, contaminants may be expected to play a role in, but not directly govern, the course and outcome of a virus epizootic.

It is impossible to identify any particular contaminant in the Baltic Sea herring which led to the suppression of NK cell activity, owing to the presence of hundreds of different PCB, PCDD and PCDF congeners, as well as numerous other potentially immunotoxic chemicals. The correlation analysis suggests only an inverse relationship between NK cell

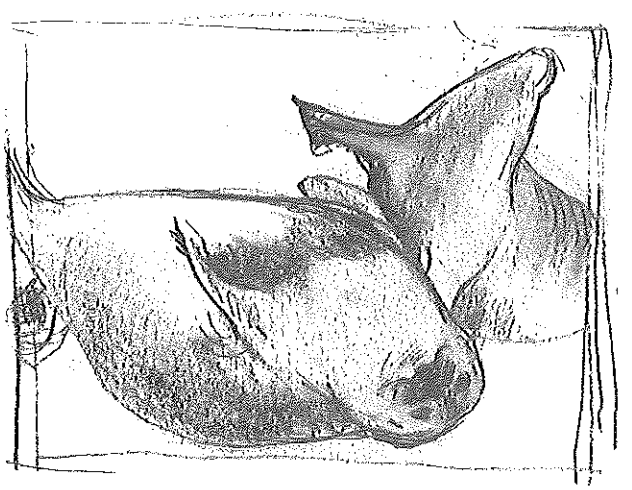
activity and environmental "contaminants", since the Baltic Sea herring had elevated levels of all compounds measured. However, the available evidence suggests that the Ah-receptor mediated PCBs, PCDDs and PCDFs have the most immunotoxic potential[225] and may, through an additive effect, contribute to a complex immunotoxic mixture of contaminants in the Baltic Sea food chain. Since mono- and di-ortho PCBs largely contributed to the TEQ profile in the seal blubber, we have speculated that they may be, to a large extent, responsible for the observed impairment in T lymphocyte mediated specific immune responses[173], and may also affect the NK cell activity reported here. The more rapid appearance of an impairment in NK cell activity (four months) than T lymphocyte responses (one year) may be due to the ontogeny of these two leucocyte subpopulations. While both NK cells and T lymphocytes or their precursors originate in the bone marrow, NK cells are fully functional once they enter the bloodstream, whereas T lymphocytes must first migrate to the thymus and mature there. In addition, NK cells have a rapid turnover[201] compared to T lymphocytes[134], and this may result in an earlier systemic manifestation of an immunotoxic mechanism taking place in the bone marrow and/or thymus. Since both cell types play vital roles in fighting virus infections, several marine mammal species may be under increased threat of infection by pathogens in industrialized coastal areas.

Acknowledgements

The authors gratefully acknowledge the assistance of the many individuals, without whom this study would not have been possible: all the staff of the Seal Rehabilitation and Research Centre, Siem Heisterkamp, Djien Liem, Trudy Riool, Harry Ross, Elze Ruiter, Koos Teppema and Paul Thompson. Research was supported in part by a Natural Sciences and Engineering Research Council of Canada award to PSR and a Nederlandse Aardolie Maatschappij grant to RdS.

Chapter 5

Impaired cellular immune response in harbour seals (Phoca vitulina) fed environmentally contaminated herring



Rik L. de Swart, Peter S. Ross, Helga H. Timmerman, Helma W. Vos, Peter J.H. Reijnders, Joseph G. Vos and Albert D.M.E. Osterhaus

Clinical and Experimental Immunology, in press

Abstract

In a 2½ year immunotoxicological study, two groups of captive harbour seals (*Phoca vitulina*) were fed herring from the heavily polluted Baltic Sea or from the relatively uncontaminated Atlantic Ocean. Blood samples were collected at regular intervals, and functional immunological parameters were monitored. T cell mitogen- and mixed lymphocyte-induced proliferative responses of peripheral blood mononuclear cells (PBMC) obtained from seals fed Baltic herring were significantly reduced over the course of the experiment. Upon immunization with rabies virus antigen (RV) and tetanus toxoid (TT), specific proliferative responses of PBMC from the seals fed Baltic herring were also significantly reduced. Impairment of T cell-mediated immune responses became especially apparent during the second year on the respective diets, and correlated significantly to 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin toxic equivalent levels in blubber biopsies taken from the seals after two years on the respective diets. Humoral immune responses, including LPS-induced lymphoproliferative responses, *ex vivo/in vitro* immunoglobulin production by PBMC, as well as RV-, TT- and poliovirus-specific serum antibody responses following immunization, remained largely unaffected.

We conclude that suppression of the cellular immune response in the seals fed Baltic herring was induced by the chronic exposure to immunotoxic environmental contaminants accumulated through the food chain. Since cellular immune responses are known to be of crucial importance in the clearance of morbillivirus infections, these results suggest that environmental pollution-related immunosuppression may have contributed to the severity and extent of recent morbillivirus-related mass mortalities among marine mammals.

Introduction

Studies in laboratory animals have shown that the mammalian immune system can be adversely affected by a variety of chemical agents[123,178,225]. In most cases, these studies focused on acute immunotoxicity caused by relatively high exposure levels of the chemical studied. Little information is available about immunotoxic effects of chronic exposure of wildlife to mixtures of environmental chemicals.

Potentially immunotoxic chemicals including polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs), hexachlorobenzene (HCB), dieldrin, β-hexachloro-cyclohexane (β-HCH), and dichlorodiphenyl-trichloroethane (DDT) are present in abundance in the marine environment. As top predators, seals and dolphins inhabiting coastal waters of industrialized regions are known to accumulate high levels of some of these xenobiotics[101,121,199], and may therefore be at particular risk.

The possible adverse effects of environmental chemicals on immune function in marine mammals became the subject of speculation in recent years, when morbillivirus infections led to mass mortalities among harbour seals (*Phoca vitulina*) in Europe, Baikal seals (*Phoca sibirica*) in Siberia (Lake Baikal), and striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea[217]. Recently, retrospective evidence has been presented for an involvement of a morbillivirus in an epizootic amongst bottlenose dolphins (*Tursiops truncatus*) along the

Atlantic coast of the U.S.A. in 1987[119]. The outbreak of phocine distemper virus (PDV) infection among European harbour seals in 1988 killed an estimated 20,000 animals, with mortality rates up to 60% in certain areas.

The mechanism of the most extensively studied group of immunotoxic chemicals, 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) and related compounds, including PCDDs, PCDFs and PCBs, is thought to be mediated by binding to a cytosolic protein, the aryl hydrocarbon (*Ah*-) receptor[92,186]. Toxicity of these chemicals is therefore largely dependent on their stereochemical resemblance to TCDD, the chemical with the highest affinity for this receptor. Based on this resemblance, the toxicity of a complex mixture of different dioxin-, dibenzofuran- and PCB-congeners can be expressed in TCDD toxic equivalents (TEQ)[179,180]. In all mammalian species studied thusfar, TCDD-like chemicals induce thymus atrophy and impairment of T cell mediated immune responses, especially following perinatal exposure, although species sensitivities differ markedly[93,225].

In order to assess the impact of ambient levels of environmental contaminants on immune function in seals, we conducted a prospective feeding study under semifield conditions. During a period of 2½ years, two groups of young harbour seals were fed herring contaminated through the food chain of the heavily polluted Baltic Sea or herring originating from the relatively uncontaminated Atlantic Ocean. Significantly higher levels of lipophilic environmental contaminants in the seals fed Baltic herring were found in blubber biopsies taken from the seals after two years on their respective diets. Blood samples were collected at regular intervals, and functional immunological assays were carried out. Previously we reported impaired *ex vivo/in vitro* natural killer (NK) cell and lymphocyte functions and *in vivo* delayed type hypersensitivity (DTH) responses in the seals fed Baltic herring[53,173]. Haematological studies showed increased white blood cell (neutrophils) and red blood cell counts in these animals[52,53]. Here we report effects of the different diets on cellular and humoral immune responses of these animals.

Materials and Methods

Seals

22 harbour seals were caught as weaned pups from the north-east coast of Scotland, and fed relatively unpolluted herring for an adaptation period of one year. The seals were matched for weight and gender and divided over two groups, which were fed herring from the heavily polluted Baltic Sea or from the relatively uncontaminated Atlantic Ocean for a period of 2½ years[52,53,173]. The animals (seven females and four males in both groups) were housed at the Seal Rehabilitation and Research Centre in Pieterburen in two basins with haul-out platforms. At the beginning of the experiment (week 0) the animals were approximately 15 months old. At the end of the experiment all 22 seals were fed Atlantic herring for a period of six months, after which they the animals were released in the North Sea.

Immunizations

Six months before the start of the feeding study (week -24, -23, -21), all animals were immunized three times with an inactivated rabies virus vaccine (adjuvanted with aluminium phosphate). They received a booster immunization at week 65. The seals were immunized twice with TT adsorbed to aluminium phosphate at week 33 and 50 following the start of the

feeding study. At weeks 93 and 103, the animals were immunized with a trivalent poliomyelitis vaccine, containing killed poliovirus type 1 (Mahoney), type 2 (MEF), and type 3 (Saukett), which was adjuvanted with aluminium phosphate. All immunizations were given intramuscularly. The vaccines had all been produced at the National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands, for human use.

Diets

Composition and vitamin supplementation of herring diets have been described previously[52,53,173]. Estimated daily intakes of potentially immunotoxic xenobiotics analyzed in the fish diets (PCBs, PCDDs, PCDFs, HCB, dieldrin, β-HCH and DDT) were three to more than ten times higher in the seals feeding on Baltic herring. Estimated daily intakes of dioxin-like organochlorines were 29 and 288 ng TEQ per day for the seals feeding on Atlantic or Baltic herring, respectively[53].

Blood sampling

Blood samples were taken at 21 and 11 weeks before, and 7, 16, 22, 28, 34, 42, 51, 58, 67, 75, 80, 93, 104, 111 and 121 weeks following the start of the feeding study into heparinized Vacutainer tubes (Becton-Dickinson), and kept refrigerated during transport to the laboratory. All serological assays were carried out using heat inactivated plasma (30 minutes, 56°C).

Mitogen and antigen induced proliferation of PBMC

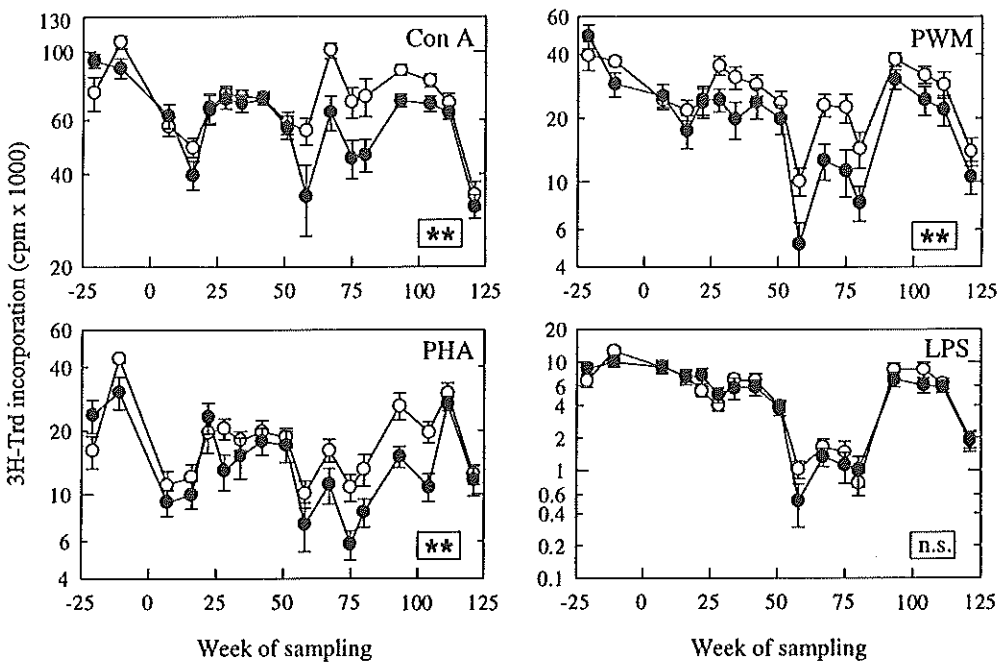
Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation as described previously[49,53]. All samples were coded prior to processing, and assays were carried out double blind. Isolated PBMC were stored overnight in RPMI-1640 medium containing 20% fetal bovine serum (FBS). The following day PBMC were counted in duplicate using a haemocytometer, and cell concentrations were adjusted to 2×10^6 PBMC per ml in RPMI-1640 medium supplemented with penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutamine (2 mM), 2-mercapto ethanol (2.10^{-5} M) and 10% FBS (further referred to as culture medium, CM).

Proliferative assays were carried out in round-bottomed culture plates (10^5 PBMC/well) as described previously[49]. PBMC were stimulated with the mitogens Con A (5 µg/ml), PWM (2.5 µg/ml), PHA (20 µg/ml) or LPS (100 µg/ml), or with rabies virus antigen (RV, 15 µg/ml) or tetanus toxoid (TT, 20 LF/ml). PBMC were cultured in triplicate for four days (Con A, PWM, PHA), five days (LPS, RV) or six days (TT) before harvesting, and were pulsed with 0.5 µCi tritium-labeled thymidine (^{3}H-Trd) per well during the last 16 hours of culture. Unstimulated control cultures were included for each animal for each day of harvesting, and control counts were subtracted from stimulations before statistical analysis.

Mixed lymphocyte reactions

The harbour seal lymphosarcoma cell line PV1.P1 (ATCC CRL 6526) was used to develop a one-way mixed lymphocyte reaction assay (MLR). This cell line of lymphoblastoid morphology was originally isolated from the pleural fluid of a harbour seal from the west coast of the U.S.A. The cells were cultured in CM in 25 cm^2 culture flasks. Shortly before the addition of stimulator cells to seal PBMC, PV1.P1 cells were counted and gamma

Figure 1: mitogen-induced proliferative responses of PBMC obtained from harbour seals fed on Atlantic herring (open circles) or Baltic herring (closed circles), measured as ^{3}H-Trd incorporation after subtraction of controls. Data shown are mean cpm±SE of eleven animals per group (7 females and 4 males each). Asterisks indicate a significant difference between the two groups over time (repeated measures ANOVA, $p<0.01$). n.s.: not significant.



irradiated (3000 rad). Optimal concentration of stimulator cells and culture period were determined in separate experiments (not shown). Results are shown as ^{3}H-Trd incorporation of PBMC (10^5 per well in round-bottomed plates) cultured for six days after stimulation with $6x10^3$ irradiated PV1.P1 cells per well.

In vitro immunoglobulin production

Ex vivo/in vitro immunoglobulin production by PBMC was measured as previously described[49]. Briefly, PBMC were cultured in CM in 24-wells plates ($2x10^6$ cells/well) as control cultures or stimulated with the mitogens PWM (1 µg/ml) or LPS (100 µg/ml). Six days later culture supernatants were frozen at -20°C until analysis in a protein A sandwich ELISA.

Serological assays

RV- and TT-specific antibody titers were measured in plasma using direct enzyme linked immunosorbent assays (ELISAs) as previously described[49]. Briefly, plates were coated with the respective antigens and blocked with bovine serum albumin. After incubation with

serial dilutions of seal plasma (in duplicate per sample), bound antibodies were detected using horseradish peroxidase labeled protein A. Previously, we have shown that protein A predominantly binds phocine IgG[175]. Results are shown as 50% titers (sample dilution at which extinction at 450 nm is reduced to 50% of the maximal signal). Poliovirus type-specific neutralizing plasma antibody titers were determined by a routinely used microneutralization test as previously described[61].

Statistical analysis

Longitudinal data were analyzed using a repeated measures ANOVA model (BMDP module 5V), with sex and diet as between subject grouping factors and time as within factor. The method of restricted maximum likelihood was used to estimate parameters of the coefficients of the model. For the covariance matrix of the residuals a first order autoregressive structure was assumed.

Correlations between proliferative responses and TEQ burdens

Total TEQ levels in blubber biopsies taken from all seals at week 104 have been described previously[173], and were natural log-transformed prior to correlation analyses. Proliferative responses measured after the first seven, respectively the last seven blood samplings, were averaged following natural log-transformation.

Results

Mitogen-induced proliferation

Mitogen-induced proliferative responses of PBMC collected from the seals in both dietary groups are shown in figure 1. Proliferative responses of PBMC obtained from seals feeding on Baltic herring after stimulation with the T cell mitogens Con A, PWM and PHA were significantly lower than the same responses from seals feeding on Atlantic herring ($p<0.01$). Proliferative responses of PBMC upon stimulation with the B cell mitogen LPS were not different between the two groups.

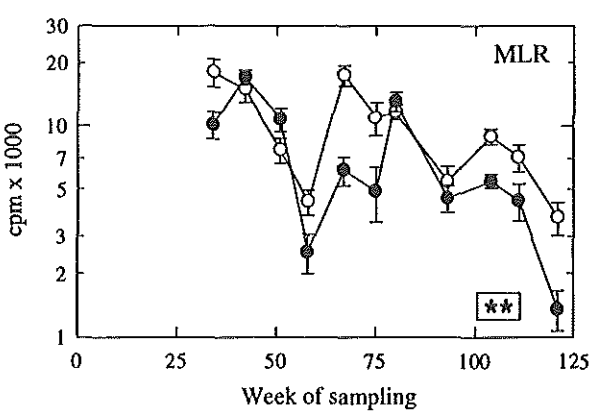


Figure 2: mixed lymphocyte reactions (MLR) of PBMC obtained from harbour seals fed on Atlantic herring (open circles) or Baltic herring (closed circles), measured as ^{3}H-Trd incorporation after subtraction of controls (mean cpm ± SE). Asterisks indicate a significant difference between the two groups over time (repeated measures ANOVA, $p<0.01$).

The impairment of T cell mitogen-induced proliferative responses was especially evident during the second part of the experiment. Proliferative responses measured during the first half of the experiment did not show a significant correlation with total TEQ levels in blubber biopsies taken from the seals after two years on the respective diets, while mean responses to the mitogens Con A, PWM and PHA from the last seven blood samplings showed a significant inverse correlation to these blubber contaminant levels (Con A: $r=-0.72$, $p<0.01$; PWM: $r=-0.44$, $p<0.05$; PHA: $r=-0.56$, $p<0.01$). No significant correlation was found with LPS-induced proliferative responses.

Mixed lymphocyte responses

In order to measure a non-specific immunological response which results from the complex sequence of events involved in antigen-processing and -presentation, an MLR assay was carried out using a lymphosarcoma cell line from harbour seal origin as stimulator cells. Since this assay was developed during the feeding

Figure 3: specific serum antibody titers (plots A and C) and proliferative responses (plots B and D) of seals fed on Atlantic (open circles) or Baltic (closed circles) herring, following immunization with the primary antigens rabies virus antigen (RV, plots A and B) or tetanus toxoid (TT, plots C and D). Immunizations are indicated by arrows. Data shown are means±SE. Asterisks indicate a significant difference between the two groups over time (repeated measures ANOVA, ** $p<0.01$, * $p<0.05$), n.s. not significant.

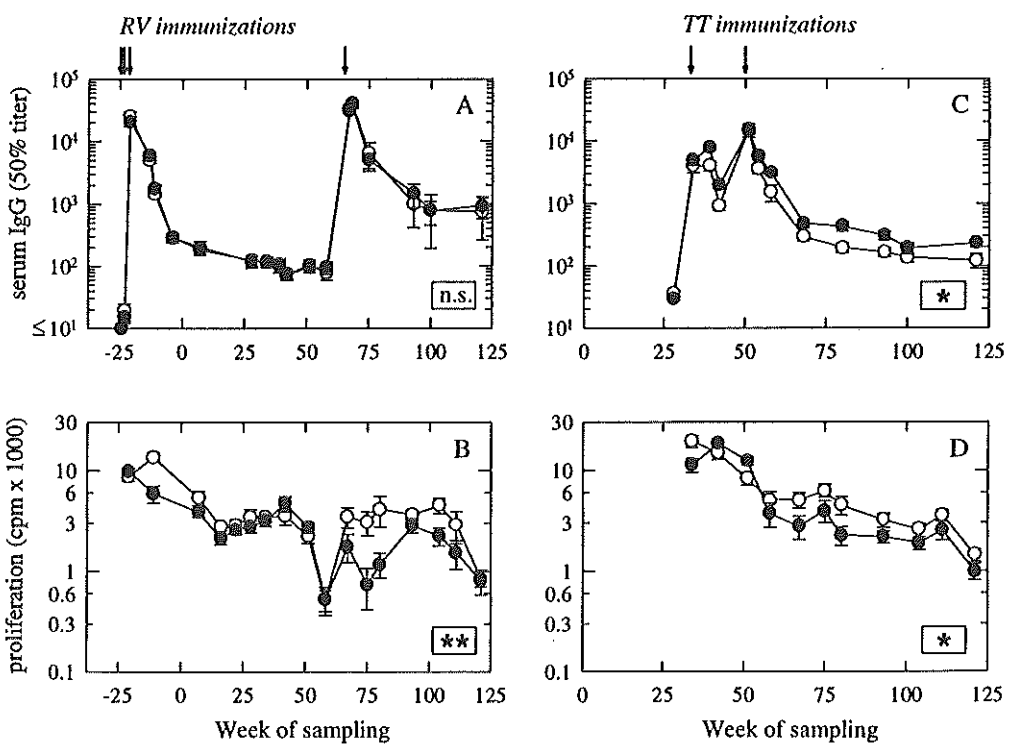
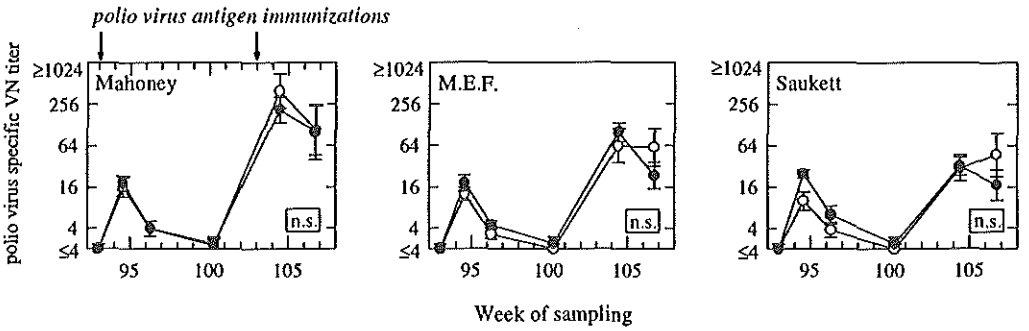


Figure 4: virus neutralizing serum antibody titers against polioviruses type 1 (Mahoney), type 2 (M.E.F.) and type 3 (Saukett) in seals fed on Atlantic (open circles) or Baltic (closed circles) herring, after immunization with trivalent poliovaccine. Immunizations are indicated by arrows. Data shown are means±SE. n.s.: not significant.



study and all proliferative assays were carried out on freshly isolated cells, results of this assay were only available from week 34 onward. As shown in figure 2, MLR responses of seals feeding on Baltic herring were significantly lower ($p<0.01$). Mean MLR responses from the last seven blood samplings correlated inversely with total TEQ levels in blubber biopsies ($r=-0.55$, $p<0.01$).

Antigen specific responses after immunization with primary antigens

In order to measure immunological responses following *in vivo* immunization, the seals in both groups were vaccinated with different primary antigens. Six months before the start of the feeding study (weeks -24, -23 and -21), all animals were vaccinated three times with an inactivated rabies virus vaccine. A booster vaccination was given 65 weeks following the start of the experiment. Antigen-specific serum IgG titers were not different in the two groups (figure 3A). However, proliferative responses of PBMC after *ex vivo/in vitro* stimulation with RV were significantly lower in seals feeding on Baltic herring (figure 3B, $p<0.01$). Mean RV-induced proliferative responses from the last seven blood samplings showed a significant inverse correlation with total TEQ levels in blubber biopsies ($r=-0.45$, $p<0.05$).

During the feeding study the seals were immunized with TT (weeks 33 and 50). Tetanus toxoid-specific serological responses were significantly higher in seals fed Baltic herring (figure 3C, $p<0.05$), but *ex vivo/in vitro* proliferative responses of PBMC obtained from the same animals were significantly lower (figure 3D, $p<0.05$).

At a later stage, the animals were also immunized with a trivalent polio vaccine. Virus neutralizing serum antibody titers against poliovirus type 1 (Mahoney), type 2 (M.E.F.) and type 3 (Saukett) were not significantly different when the seals of both groups were compared (figure 4). No poliovirus-specific proliferative responses could be measured using a concentrated antigen preparation, as has been observed previously using human PBMC[202].

In vitro immunoglobulin production

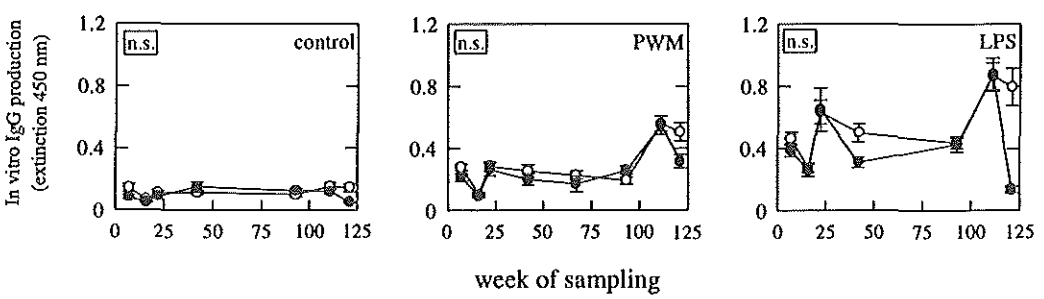
Ex vivo/in vitro non-specific Ig production was measured in culture supernatant of PBMC six days upon stimulation with the B cell mitogens PWM and LPS and shown in figure 5. No significant differences were found in total IgG production.

Discussion

As top-predators in the marine food chain, seals and dolphins carry some of the highest burdens of immunotoxic chemicals in the natural environment. In the present paper we have reported results obtained in studies on immune function in seals feeding on fish with different levels of naturally accumulated contaminants. These extend our previous observations, which indicated an impairment of *ex vivo/in vitro* NK cell activity and T cell mitogen-induced lymphoproliferative responses and *in vivo* delayed type hypersensitivity (DTH) responses in seals fed on Baltic Sea herring[53,173]. Here we have presented B and T cell mitogen induced-proliferative responses for both groups of seals, data on MLR- and primary antigen-induced proliferative responses, as well as data on *ex vivo/in vitro* and *in vivo* humoral immune responses. Both non-specific and antigen-specific T cell mediated immune responses were impaired, whereas humoral immune responses proved to be largely unaffected in the seals fed polluted herring from the Baltic Sea.

The major advantage of the "semifield" setup of this experiment, is that the results can be directly extrapolated to harbour seals inhabiting the Baltic Sea, from where the polluted herring originated. The inherent limitation of our approach is that the chemical or group of chemicals responsible for the observed effects can not be conclusively identified. However, when the estimated daily intakes of potentially immunotoxic contaminants by the seals are considered, one suspect group of chemicals for the observed effects consists of the *Ah*-receptor binding organochlorines. The immunotoxic potency of TCDD and related compounds (including dioxins, furans and some PCBs) has been well established[93,225]. The estimated daily intakes of this group of contaminants in TEQ was approximately ten times higher in the group of seals feeding on Baltic herring[53]. Over the course of the experiment, the estimated cumulative intake was 270 and 27 µg TEQ per animal, or approximately 5 and 0.5 µg TEQ per kg body weight, for the seals in the Baltic and the Atlantic group, respectively. TCDD has been reported to affect immune responses in laboratory animals at

Figure 5: *ex vivo/in vitro* total IgG production by PBMC obtained from harbour seals fed on Atlantic herring (open circles) or Baltic herring (closed circles), after stimulation with PWM or LPS (top plot: medium control). Data shown are protein A binding antibodies in culture supernatants six days after stimulation, shown as means±SE of optical densities at 450 nm. n.s.: not significant.



short-term doses as low as 0.5 µg/kg, including impaired lymphoproliferative responses[93,225]. In general, chronic exposure to *Ah*-receptor mediated organochlorines has been associated with alterations in cell-mediated immune responses, while acute exposure to high doses has been reported to affect humoral immune responses[225].

Other immunotoxic mechanisms could also have played a role in the observed impairment of immune function in seals. A range of non *Ah*-receptor binding environmental xenobiotics have been identified as potentially immunotoxic[178,233]. In addition, many contaminants are present in the natural environment which may have an, as yet unknown, immunotoxic potential.

Contrary to our previous observations of reduced serum IgG responses following immunization with the primary antigen ovalbumin[173], we found no suppression of humoral immune responses. *Ex vivo/in vitro* IgG production by PBMC and antigen-specific serum antibody levels following immunization with RV or polioviruses were unaffected. In light of the suppressed lymphoproliferative responses to TT, serum IgG levels following immunization with this antigen were unexpectedly higher in seals fed Baltic herring. In immunotoxicological studies using laboratory animals, this phenomenon has only been described following exposure to HCB, especially when using TT as model antigen[223]. One explanation for the absence of reduced serum antibody responses in this study, as opposed to our previous observations, may be related to the different adjuvants used for the respective antigens. TT- and poliovirus vaccines were adjuvanted with aluminium phosphate, which has been described as a particularly potent inducer of humoral immune responses[81,230], while dimethyldioctadecyl-ammonium bromide (DDA), the adjuvant to the ovalbumin immunizations, is an adjuvant which is particularly potent in inducing cell-mediated immune responses[91]. It may be speculated that the impaired cellular responses of the seals feeding on Baltic herring contributed to a reduced T helper response following the ovalbumin/DDA immunization, which then led to lower humoral immune responses in these animals.

Both the Atlantic and Baltic herring that were used to feed the seals were originally destined for human consumption. In humans, certain consumer groups may also be at risk to the effects of immunotoxic chemicals accumulated through the marine food chain. In a Scandinavian study, serum levels of chlorinated dioxins and dibenzofurans were shown to correlate strongly with the consumption of fatty fish from the Baltic[194], and indications of immunological differences were reported in the high fish consumption group[193]. However, considering the differences in fish intake between humans (mean intake in high consumer group in Scandinavian study: 1.2 kg/week) and seals (mean intake by seals feeding on Baltic herring in this study: 39.2 kg/week), immunotoxic effects induced by environmental lipophilic contaminants may be more likely in marine mammals.

Whether or not dioxin-like organochlorine contaminants are responsible for the immunosuppression observed in our Baltic fish consuming group of seals cannot be conclusively demonstrated. However, our results show that the consumption of fish contaminated through the marine food chain leads to an impairment of immune function in harbour seals. The impaired proliferative capacity of T cells may especially alter the immune response during systemic viral infections, when rapid clonal expansion of specific lymphocytes is essential for an effective immune response. Since perinatal exposure to immunotoxic chemicals, especially TCDD-like organochlorines[225], generally leads to more pronounced adverse effects than adult exposure, seals born in contaminated marine regions may suffer

from a more pronounced contaminant-related immunosuppression than our Baltic group of animals, which were born in a relatively unpolluted area and fed uncontaminated herring during their first year in captivity. In addition, seals and dolphins inhabiting contaminated marine regions often have higher burdens of immunotoxic chemicals than the animals in the present study.

The impaired T cell-mediated immune responses and unaffected humoral responses are especially interesting in light of the recent morbillivirus-related marine mammal epizootics. Although neutralizing antibodies may be effective in preventing infection, cytotoxic T lymphocytes (CTLs) are thought to play an essential role in clearance of the infection[145,205]. Unfortunately, CTL responses could not be measured in PBMC of the seals due to the absence of a system to generate immortalized antigen presenting cells. Furthermore, the induction of CTL responses would be most efficiently studied following immunization with live attenuated virus vaccines. It is however generally recommended not to use this type of vaccines in free-ranging animals, or animals which are to be released.

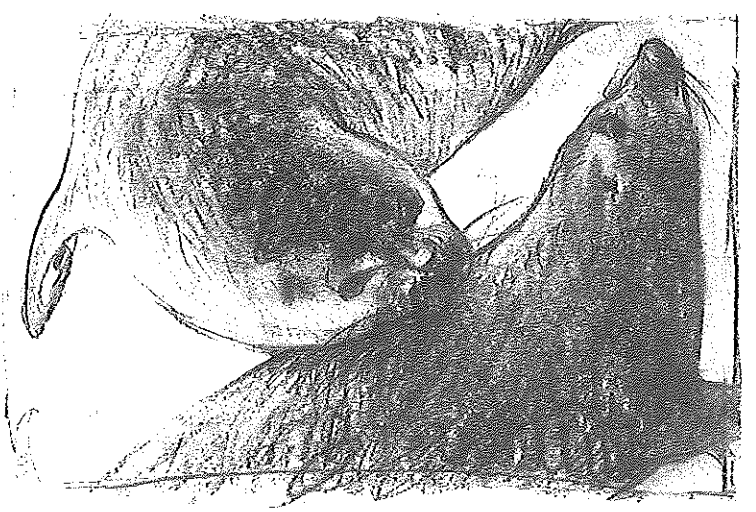
Before the outbreak of PDV among European harbour seals, this population was immunologically naive to morbilliviruses[157], which was probably also true for the other affected marine mammal populations. Therefore, immune responses at the start of the epizootics were largely dependent on the innate immune system (e.g. NK cells) and T lymphocyte responses. Since we have shown that both NK cells and T lymphocytes were functionally impaired in seals fed Baltic herring, it may be concluded that immunotoxic environmental contaminants may have rendered seals inhabiting certain areas more susceptible to morbillivirus infections.

Acknowledgements

The authors wish to thank Paul Thompson, Harry Ross, Henk Loggen, Jos Westendorp, Bert Weitering, Dick Jut, Djien Liem, Elze de Ruiter, Paul Mulder, Jaap Scholtens, Aline de Koeyer and all collaborators of the SRRC and the IBN-DLO for their contributions to these studies. R.L. de Swart and P.S. Ross were partially funded by the Nederlandse Aardolie Maatschappij (NAM) and a Natural Sciences and Engineering Research Council of Canada award, respectively.

Chapter 6

Contaminant-related suppression of delayed-type hypersensitivity responses in harbour seals fed herring from the Baltic Sea



Peter S. Ross, Rik L. de Swart, Peter J.H. Reijnders, Henk van Loveren, Joseph G. Vos and Albert D.M.E. Osterhaus

Environmental Health Perspectives 103: 162 - 167 (1995)

Abstract

Recent mass mortalities among several marine mammal populations have led to speculation about increased susceptibility to viral infections as a result of contaminant-induced immunosuppression. In a 2½ year study, we fed herring from either the relatively uncontaminated Atlantic Ocean or the contaminated Baltic Sea to two groups of captive harbour seals and monitored immune function in the seals. Seals fed the contaminated fish were less able to mount a specific immunological response to ovalbumin, as measured by *in vivo* delayed-type hypersensitivity (DTH) reactions and antibody responses. The skin reaction to this protein antigen was characterized by the appearance of mononuclear cells which peaked at 24 hours after intradermal administration, characteristic of DTH reactions in other animals studied. These DTH responses correlated well with *in vitro* tests of T lymphocyte function, implicating this cell type in the reaction. Aryl-hydrocarbon (*Ah*) receptor-dependent toxic equivalent (TEQ) profiles in blubber biopsies taken from the seals implicated PCBs rather than dioxins or furans in the observed immunosuppression. Marine mammal populations currently inhabiting polluted coastal environments in Europe and North America may therefore have an increased susceptibility to infections, and pollution may have played a role in recent virus-induced mass mortalities.

Introduction

The immunosuppressive potential of organochlorine chemicals has been well established in studies of laboratory animals[225], but little is known of the effects of environmentally-occurring mixtures on immune function in free-ranging animals. Because organochlorine chemicals bioaccumulate in many wildlife species occupying high trophic levels, these animals may serve as early warning indicators for problems of ecosystem health. Classes of chemicals that are of particular concern include the ubiquitous and highly immunotoxic 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related dioxins, furans and polychlorinated biphenyls (PCBs)[225]. Fish-eating animals including gulls, cormorants[74], seals[1], carnivorous whales[129,139], and certain groups of humans[95,194] can be exposed to high levels of such contaminants, and may therefore be expected to be the first to exhibit symptoms of toxicological stress. There is accumulating evidence that these organochlorines have adverse biological impacts in free-ranging animals, including skeletal malformations in gulls, terns, cormorants[74] and seals[12,136], and reproductive impairment in seals[85,163].

Mass mortalities among harbour (*Phoca vitulina*) and grey (*Halichoerus grypus*) seals in Europe in 1988[57,153,216], Baikal seals (*Phoca sibirica*) in 1987-88[78,155], bottlenose dolphins (*Tursiops truncatus*) in the Gulf of Mexico in 1987-88[113], and striped dolphins (*Stenella coeruleoalba*) in the Meditteranean Sea in 1990-91[208], led to speculation that environmental pollution was impairing the immunocompetence of these marine mammal populations, and had rendered them more susceptible to virus infection. The 1988 phocine distemper virus (PDV) epizootic among European harbour seals was particularly devastating, resulting in the deaths of approximately 20,000 seals[57,154]. While no studies could directly link pollution to the PDV epizootic, the speed at which the infection passed through the population and the high

mortality rate has fueled a continuing debate. Since the PDV epizootic, studies have provided additional clues: harbour seals surviving the epizootic had lower organochlorine burdens than seals that died[83]; PDV or a very similar virus had infected Canadian harbour seals prior to the European disaster, with no apparent mortality[87,177]; and harbour seals living in less contaminated areas of Britain had apparently lower mortality rates during the PDV epizootic than those from polluted areas[188]. In addition, we recently demonstrated that lymphocytes isolated from harbour seals fed herring from the contaminated Baltic Sea were functionally impaired compared to those isolated from seals fed herring from the relatively unpolluted Atlantic Ocean, as measured by *in vitro* T cell mitogen stimulation tests and natural killer cell activity[53,174]. Here, we extend these *in vitro* results by evaluating the *in vivo* immune response of these harbour seals, as measured by delayed-type hypersensitivity (DTH) and antibody responses to ovalbumin.

Materials and Methods

Captive harbour seals

Two groups of 11 healthy young harbour seals (*Phoca vitulina*) were housed at the Seal Rehabilitation and Research Centre in Pieterburen, The Netherlands, as described in detail elsewhere[53]. They had been captured as recently weaned pups from the relatively uncontaminated northeastern coast of Scotland, and all seals were fed relatively uncontaminated herring from the Atlantic Ocean for an acclimation period of one year. The 22 seals were matched for body weight and sex and subsequently divided at random between two feeding groups. At the start of the feeding experiment in October 1991, the control group continued to receive Atlantic Ocean herring and the treatment group received herring originating from the relatively contaminated Baltic Sea. Both groups received weekly vitamin supplements to compensate for losses during storage of the fish at -20°C. Estimated daily intakes of potentially immunotoxic compounds analyzed in the herring were three to ten times higher in the Baltic group of seals, as compared to the Atlantic group, and are summarized elsewhere[53]. The average daily intakes of *Ah* receptor-defined organochlorine contaminants in the Baltic Sea group were 288 ng TEQ per seal, compared to 29 ng TEQ per seal in the Atlantic group[53]. Similarities in the nutritional quality of the two diets, clinical chemistry profiles and weight gain of the animals, suggested that other than the differences in intake of environmental contaminants, the two groups of seals were comparable[52,53]. All animals were handled in accordance with institutional guidelines in the Netherlands, and their care was supervised by a Veterinary Consultant and the Veterinary Advisory Committee of the Seal Rehabilitation and Research Centre in Pieterburen.

Skin test

After approximately two years on the respective diets (week 100), seals of both groups were tested for DTH reactivity to ovalbumin. In this prescreen, aimed at ensuring that the seals were immunologically naive to the test antigen, we prepared a sterile solution of 250 µg/ml ovalbumin (Grade V, Sigma Chemicals, St. Louis, MO, USA) in physiological saline solution. Following cleansing of the skin with Betadine (Mundipharma, Switzerland), seals were injected with 100 µl of the ovalbumin solution (25 µg per injection) intradermally in the flipper webbing between two toes. We marked the site by placing a drop of waterproof paint

2 cm above the injection. We measured the thickness of the skin before and 48 hours after the injection using a Mitutoyo digital micrometer (Mitutoyo Corp., Tokyo, Japan).

Following the prescreen, all seals were immunized intramuscularly in the gluteal region (week 105) with 100 µg ovalbumin and 800 µg dimethyldioctadecyl-ammonium bromide (DDA; Eastman Kodak, Rochester, NY, USA) as adjuvant in 2 ml physiological saline solution.

We began the DTH recall skin test nine days later (week 106) using the same ovalbumin stock, concentration, and intradermal route of administration as the prescreen. In addition, a control injection of physiological saline was administered to assess the aspecific inflammation induced by the injection process alone. Sites were marked and skin thickness measured before and 24, 48, and 72 hours after injection.

We took a skin biopsy from three seals of each group at 72 hours after injection to assess the cellular infiltrate responsible for the observed swelling. Biopsies of the swelling were taken using a 6 mm biopsy plug (Codman and Shurtleff, Randolph, MA, USA) and scalpel after cleansing the area using Betadine and sterilizing surgical instruments in 95% isopropyl alcohol. Skin samples were immediately placed in 4% formaldehyde solution. Samples were later embedded in paraffin and 5 µm sections stained using haematoxylin and eosin (H&E), and mounted on glass microscope slides for evaluation. We assessed the cellular infiltrate by identifying and qualitatively ranking cell types observed using light microscopy.

Toxicological analyses of seal blubber

We took blubber biopsies from the study seals for toxicological analyses at week 104 of the experiment. After cleansing of the skin surface in the dorsal region approximately 10 cm lateral to the spinal column, a 1.5 cm incision was made. A 6 mm biopsy plug (Codman) was inserted into the incision and a sample of approximately 200 mg blubber was removed and frozen in glass vials at -20°C until analysis. Congener-specific analyses of planar (IUPAC numbers 77, 126, and 169) and mono- (IUPAC numbers 118, 156, and 189) and di-ortho (IUPAC number 180) PCBs were undertaken as described elsewhere[23,211]. We measured seven dioxin(2,3,7,8-TCDD, 1,2,3,7,8-PeCDD, 1,2,3,4,7,8-HxCDD, 1,2,3,6,7,8-HxCDD, 1,2,3,7,8,9-HxCDD, 1,2,3,4,6,7,8-HpCDD, and OCDD) and 10 furan (2,3,7,8-TCDF, 1,2,3,7,8-PeCDF, 2,3,4,7,8-PeCDF, 1,2,3,4,7,8-HxCDF, 1,2,3,6,7,8-HxCDF, 1,2,3,7,8,9-HxCDF, 2,3,4,6,7,8-HxCDF, 1,2,3,4,6,7,8-HpCDF, 1,2,3,4,7,8,9-HpCDF, and OCDF) congeners as described elsewhere[118]. 2,3,7,8-TCDD toxic equivalents (TEQs) were calculated on the basis of toxic equivalent factors (TEFs) designated for dioxins and furans[214] and for PCBs[4]. Concentrations of dioxin, furan, and planar PCB congeners measured around the detection limit suffered from variation due to an analytical source of error, which is not expected to appreciably alter total levels as presented here, or alter the relationship between the two groups of seals.

Detection of serum antibodies

Blood was sampled five weeks following the DTH test (week 111), and serum antibodies against ovalbumin were detected by an enzyme-linked immunosorbent assay (ELISA) as follows. ELISA plates (Costar, Cambridge, MA, USA) were coated with 1 µg/well ovalbumin in carbonate buffer (pH 9.6). Plates were washed in water-Tween (0.05%), and blocked at 37°C using ELISA buffer (phosphate buffered saline (PBS; 0.01 M) containing 0.3% milk powder, 5% NaCl, 0.1% Tween 20 (Merck, Munich, Germany), and 0.1% Triton

X 100 (Merck)). Plates were again washed, and sequential twofold dilutions of seal serum were made in ELISA buffer, starting at 1:1000. Plates were incubated at 37°C and washed. We used a conjugate preparation of protein A-horseradish peroxidase (Amersham Life Sciences, Little Chalfont, UK) at a 1:2500 dilution, and plates were developed using a solution 0.1% tetramethylbenzadine dimethylsulfoxide (10 mg/ml TMB in DMSO) and 0.001% H_2O_2. Antibody titers were expressed as the reciprocal of the dilution at 50% of the maximum optical density at 450 nm.

Correlation between DTH and in vitro test results

As part of the routine monitoring of immune function in the two groups of harbour seals, mitogen-induced proliferative responses of peripheral blood mononuclear cells (PBMC) were measured and reported elsewhere[53]. Here, we correlate results of these *in vitro* responses with the *in vivo* DTH responses in the same animals. Briefly, PBMC were isolated from heparinized blood and stimulated with the T cell mitogens concanavilin A (Con A, 5 µg/ml) and phytohaemagglutinin-M (PHA, 20 µg/ml), the T and B cell mitogen pokeweed mitogen (PWM, 2.5 µg/ml), or the B cell mitogen lipopolysaccharide from *Salmonella typhimurium* (LPS, 100 µg/ml). We measured proliferation after four (Con A, PHA and PWM) or five (LPS) days in culture as ^{3}H-thymidine incorporation, expressed in counts per minute (cpm). For each animal, proliferative responses were measured from seven blood samplings (weeks 67, 75, 80, 93, 104, 111, and 121) were averaged after subtraction of medium controls. These proliferation data were log-transformed before correlation to the 24-hour DTH values obtained at week 106 of the experiment.

Table 1: *Ah* receptor defined contaminant concentrations in blubber biopsies at week 104 from seals fed herring originating from the relatively uncontaminated Atlantic Ocean or from the contaminated Baltic Sea for a period of two years. Values represent the means of 11 seals per group ± SE

	ng TEQ kg^{-1} lipid	
	Atlantic group	Baltic group
PCB (mono and di-ortho)	63.7±6.01[1]	217.3±13.70
PCB (planar)	22.2±1.04	52.4±3.18
dioxins & furans (total)	4.1±0.20	17.2±3.98
TEQs (total)	90.0±6.36	286.1±16.87

[1] Values represent the means of 11 seals per group ± SE

Results

Toxicology

Baltic Sea seals had higher average *Ah* receptor-binding contaminant burdens than North Sea seals, with 3.4 times higher TEQ levels in blubber samples (table 1). Also apparent was the predominant contribution of the PCBs to these levels, representing 93 ± SE 0.6% of the total TEQ values, compared to only 7 ± SE 0.6% for the dioxins and furans.

Ovalbumin-specific DTH responses

In a prescreen of seals of both groups, there was no significant skin swelling 48 hours after intradermal injection with ovalbumin (paired *t* test; $p>0.05$; results not shown), indicating that the seals were immunologically naive to this antigen. Following the post-immunization recall skin test, both Atlantic and Baltic group seals responded significantly to the antigen (univariate repeated measures *F* test; $p<0.01$; figure 1), with a localized and palpable swelling. After intradermal injection, the seals fed the Baltic Sea herring had significantly lower responses than those fed the Atlantic herring (repeated measures analysis of variance with grouping factors; $p<0.01$), as the former had a mean swelling of 47% of that observed in the Atlantic group at the peak response time of 24 hours. In addition, an inverse relationship was found between DTH swelling and *Ah* receptor-binding contaminant levels (total TEQ) in blubber samples of the same seals ($r=-0.64$; $p<0.01$).

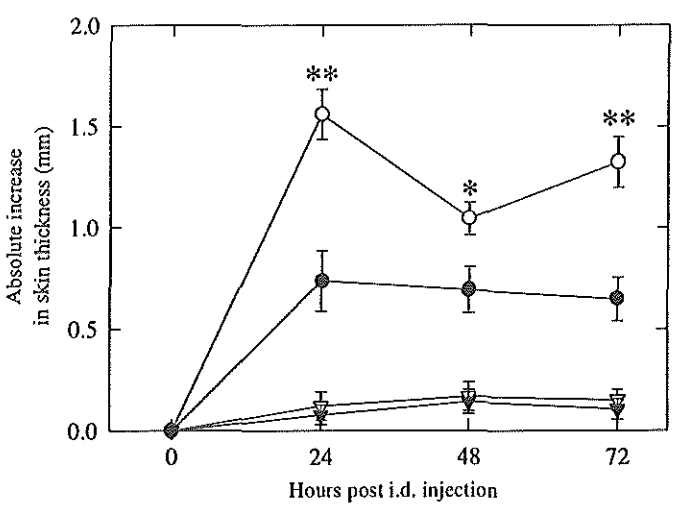
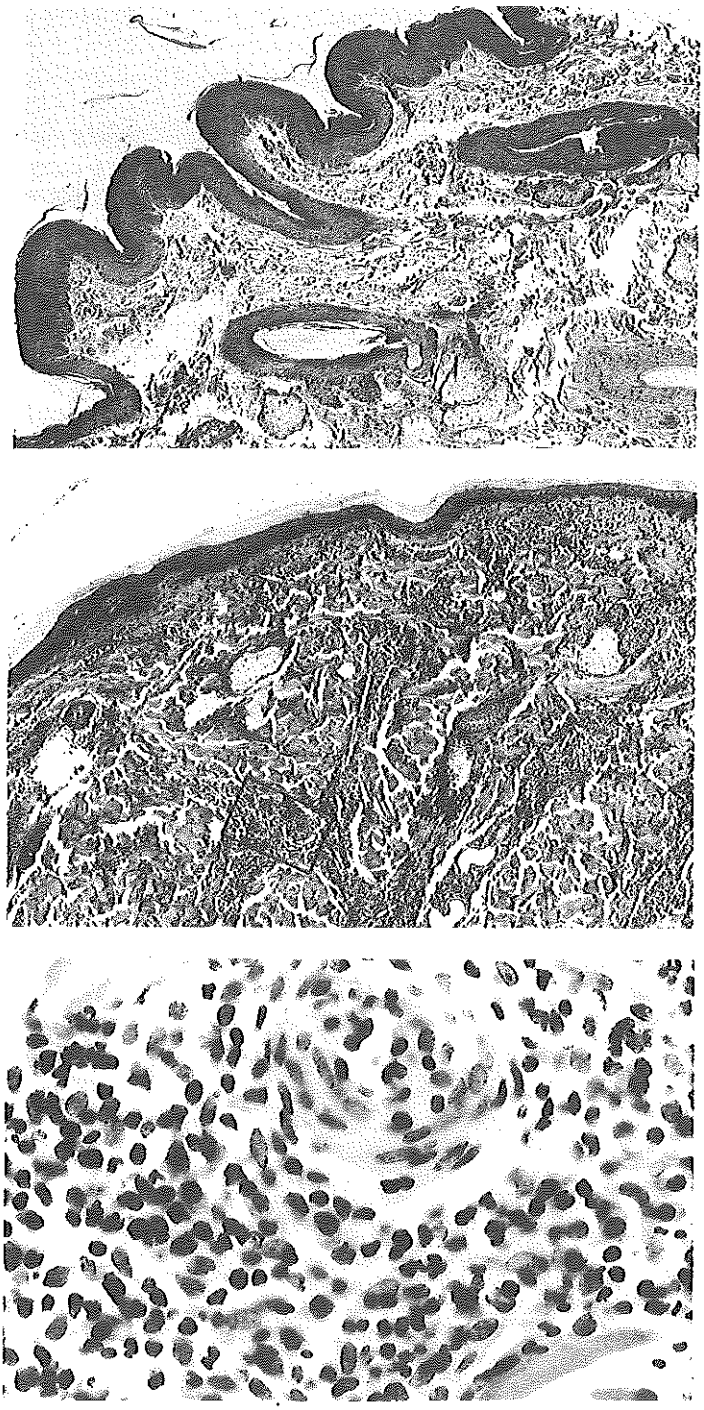


Figure 1: nine days after immunization with ovalbumin and the adjuvant DDA, harbour seals of both Atlantic (open triangles) and Baltic (closed triangles) groups exhibited a delayed type hypersensitivity (DTH) response to an intradermal skin challenge using ovalbumin. Seals fed the relatively contaminated Baltic Sea herring for a two-year period had a significantly lower response to the antigen (repeated measures ANOVA with grouping factors; $p<0.01$). The peak swelling took place 24 hours following injection. A control injection of 100 µl saline resulted in only a very small swelling for both Atlantic (open triangles) and Baltic (closed triangles). Data points represent the means of 11 seals ± SE. Significant differences between the two groups at each measured time point were analyzed by an independent *t* test (* $p<0.05$; ** $p<0.01$).



A

B

C

Figure 2: Biopsies of seal skin from (A) a control sample (40x H&E), (B) a positive skin test 72 hours after intradermal injection with the recall antigen ovalbumin (40x, H & E), and (C) an enlargement of an indicated section (see outlined square) of the same cut (200x H&E). The enlarged section reveals a perivascular infiltrate characterized by mononuclear cells, with the presence of some polymorphonucleargranulocytes. Biopsies of three seals from each group were examined.

In microscope preparations of skin biopsies taken from the ovalbumin reaction sites of 6 of the 22 seals 72 hours after injection, cellular infiltrates in the dermis were characterized as typical DTH reactions by the presence of perivascular mononuclear cells (likely lymphocytes), and a limited presence of polymorphonuclear cells (figure 2).

Ovalbumin-specific antibody responses

Before immunization and the recall skin test, seals of both groups had no detectable antibodies against ovalbumin, confirming the immunological naivety to this antigen. In a blood sampling four weeks subsequent to the immunization, seals of both groups had mounted antibody responses to ovalbumin, titers about 37% lower in the Baltic group than the Atlantic group (independent *t* test; $p<0.01$; figure 3).

Correlation between DTH and in vitro immune function test results

In correlation analyses between *in vitro* tests of immune function and the DTH response, the DTH response correlated best with lymphocyte stimulation by Con A ($r=0.62$; $p<0.01$) and PHA ($r=0.57$; $p<0.01$), and less with PWM ($r=0.35$; $p>0.05$) and LPS ($r=0.29$; $p>0.05$) (figure 4).

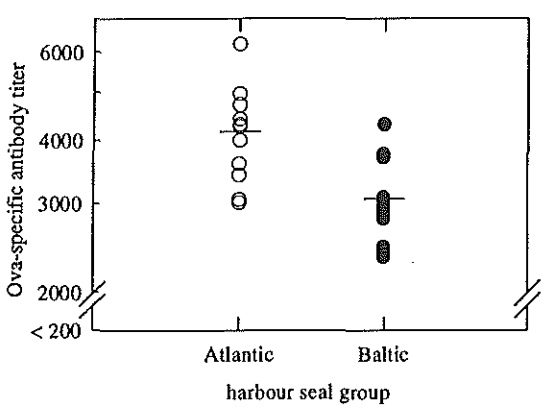


Figure 3: Serum antibody titers mounted against ovalbumin four weeks after immunization were significantly lower (independent *t* test; $p<0.01$) in the Baltic Sea group of seals (closed circles) as compared to the Atlantic group (open circles). Seals of both groups had no detectable antibodies to ovalbumin before immunization.

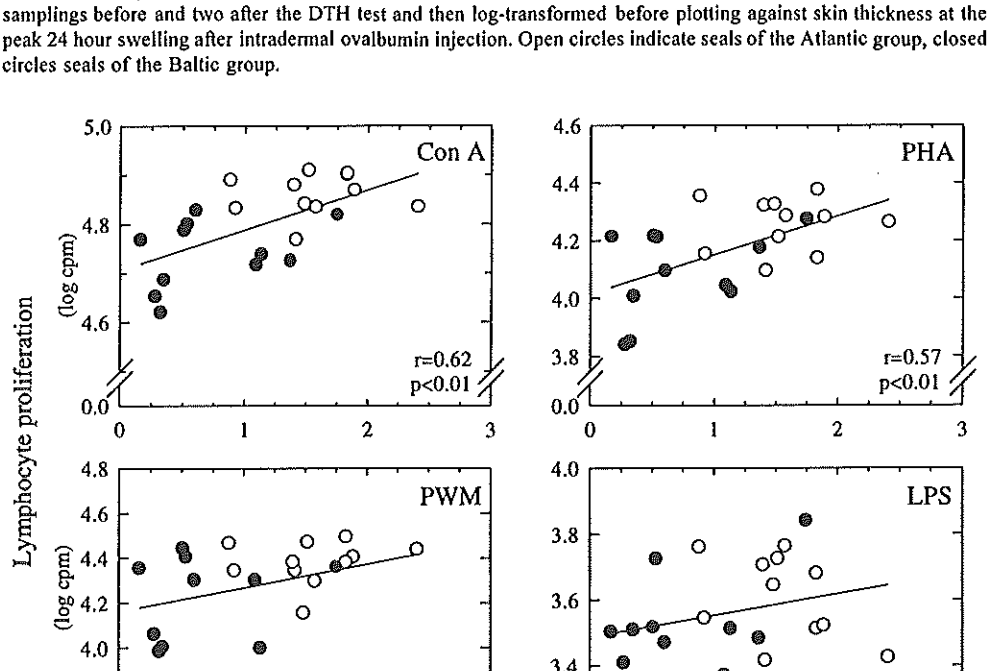
Discussion

Exposure to contaminants occurring at levels found in the Baltic Sea herring impaired the ability of captive harbour seals to mount a specific immune response to the T lymphocyte-dependent antigen ovalbumin with DDA as adjuvant. This was evidenced by impaired DTH and serum antibody responses. DDA was selected as an adjuvant because it is particularly effective in stimulating the induction of DTH responses[91]. The skin reaction to ovalbumin was characterized by a mononuclear infiltrate in the dermis, as observed in classical DTH responses in other species studied[238]. In addition, the correlation between the mean DTH response and the *in vitro* lymphocyte stimulation tests with the mitogens Con A and PHA, and not PWM and LPS, support the notion that T lymphocytes are involved in the mechanism of DTH swelling in our study seals. Con A and PHA specifically stimulate T lymphocytes *in*

vitro, whereas PWM stimulates T and B lymphocytes, and LPS stimulates B lymphocytes in many species[138], including the harbour seal[49]. These results not only strengthen our previous evidence of a contaminant-induced suppression of T lymphocyte function in the harbour seals[53], but also lend support to the use of *ex-vivo/in vitro* tests of immune function.

The DTH skin test represents the only practical *in vivo* test for cellular immunity. Moreover, it reflects a system-wide immune response, ranging from antigen processing and presentation after immunization, to the T helper cell response which coordinates a secondary response in the skin reaction. It is difficult to extrapolate from the immunological responses using ovalbumin as an antigen to a seal's ability to mount a specific immune response against a pathogen in the natural environment, though the DTH response does provide an overview of an animal's ability to mount a response to a foreign protein in a manner similar to which it would defend itself against infection by a viral agent.

Figure 4: Correlations between *in vitro* tests of immune function conducted previously and DTH responses. Good correlations existed between DTH and lymphocyte stimulation by the mitogens Con A ($r=0.62$, $p<0.01$) and PHA ($r=0.57$, $p<0.01$), but not PWM ($r=0.35$, not significant) and LPS ($r=0.29$, not significant), implicating T lymphocytes in the DTH response. Mean counts per minute were averaged for *in vitro* lymphocyte stimulation tests from five samplings before and two after the DTH test and then log-transformed before plotting against skin thickness at the peak 24 hour swelling after intradermal ovalbumin injection. Open circles indicate seals of the Atlantic group, closed circles seals of the Baltic group.



Impairment of DTH reactions following exposure to TCDD has been observed in Guinea pigs receiving 8 weekly doses of 0.04 μg/kg[241]; C57BL/6 mice receiving 4 weekly doses of 4 μg/kg[236] or a one time dose of 50 μg/kg[239]; and Fischer-344 rats exposed pre- and postnatally to four doses of 5 μg/kg or postnatally alone via nursing to three doses of 5 μg/kg[237]. Guinea pigs exposed to 50 μg/kg of a dietary PCB mixture (Clophen A60) had suppressed DTH responses to purified protein derivative and antibody responses to tetanus toxoid[242]. Vos and Moore[226] noted that rats must be exposed to TCDD during ontogenesis of the immune system for immunosuppression to take place, whereas the adult immune system is less sensitive to suppression of thymus-dependent immunity. Although the results of several studies of human exposure to organochlorines have been difficult to interpret, the accidental exposure of people in Taiwan to rice oil contaminated with PCBs (most likely contaminated with dioxins and furans) led to a significant impairment of delayed-type hypersensitivity responses[235]. Impairment in B cell responses has been observed in adult animals, with lower antibody responses to various antigens reported following exposure to PCBs[225] and dioxins[241]. Because few studies have examined the effects of environmentally occurring mixtures of anthropogenic contaminants on immune function in mammals, it is difficult to relate results of other studies to those reported here.

Although our results suggest an impairment in the function of the T cell mediated immune system of the Baltic group of seals, we cannot conclude that the T lymphocyte or its precursor are the targets of immunotoxic action by the contaminants. Possible effects of the contaminants at the antigen presentation level or a multidirected action (e.g. at both T and B lymphocytes) are conceivable. However, the concurrent and similar results in the DTH and the antibody responses in the Baltic Sea group as compared to the Atlantic group rather point to a common site of action. This is consistent with the findings of Lundberg *et al.*[239], among others, who observed reduced DTH responses, antibody responses and specific lymphocyte stimulation to ovalbumin in mice and normal function of antigen presenting cells. In addition, the thymus is a sensitive target for TCDD-induced immunosuppression, leading to impaired T lymphocyte responses[225].

Many different classes of potentially immunosuppressive contaminants bioaccumulate in the Baltic Sea ecosystem, making it impossible to identify any one contaminant as responsible for the impaired immune responses in our study. Evidence for the mediation of immunotoxicity via the *Ah* receptor in animals and the high affinity of 2,3,7,8-TCDD and its dioxin and PCB analogs for this cytosolic receptor[180] suggest a cumulative effect of the many contaminants found in the Baltic Sea herring. The observed impairment of DTH and antibody responses suggests that the mixture of contaminants in the Baltic Sea herring has immunosuppressive properties. Assuming that the observed effects are mediated by *Ah* receptor-binding contaminants, the blubber profile of TEQ values suggests that the PCBs are largely responsible for these effects, as opposed to the dioxins and furans. However, we cannot rule out an immunotoxic contribution from non-*Ah* receptor binding classes of chemicals.

Our findings with captive seals have direct relevance, because three seal species (ringed, harbour and grey) currently inhabit the Baltic Sea. Furthermore, the Baltic Sea herring which led to the impairment of immune function in our study seals was destined for human consumption, raising concerns about the potential for adverse immunological effects in certain human consumer groups. Current levels of contaminants in the marine food chain

along the industrialized coastlines of North America and Europe may be affecting the immunocompetence of marine mammals, and may predispose populations in these areas to an increased incidence and severity of disease. We speculate that anthropogenic contaminants, in particular PCBs, played a role in the 1988 PDV epizootic in Europe and other recent mass mortalities of marine mammals caused by virus infections.

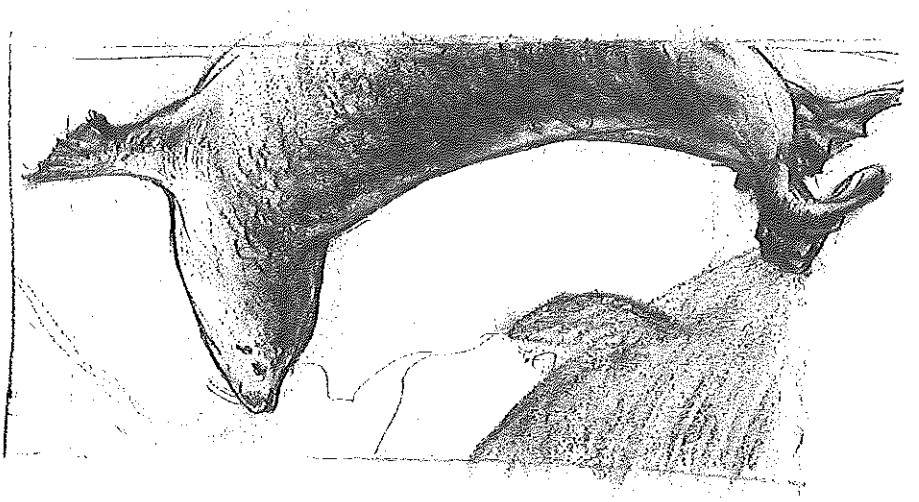
Efforts to detect possible immunosuppression in free-ranging populations of marine mammals is fraught with difficulties, due to the complexity of the mammalian immune system and the difficulty in obtaining reliable samples. However, field studies have demonstrated that it is possible to obtain useful immunological information from free-ranging seals[175,176], and correlative approaches similar to those used in wildlife toxicology[240] may provide the best direction for research in the future.

Acknowledgements

The assistance of many individuals is gratefully acknowledged: Lies Vedder and all the staff of the Seal Rehabilitation and Research Centre; Paul Thompson and Harry Ross for assistance in establishing the captive group of seals; Jan Groen with the ELISA; Hans van Dijk for the gift of DDA; Wim de Jong for assistance with histopathology; Jan Dormans with photographic layout; and Djien Liem and Elze de Ruiter for toxicological analyses. Peter S. Ross was partially funded by a Natural Sciences and Engineering Research Council of Canada award. Rik de Swart was partially funded by a grant from the Nederlandse Aardolie Maatschappij.

Chapter 7

Haematology and clinical chemistry values of harbour seals (Phoca vitulina) fed environmentally contaminated herring remain within normal ranges



Rik L. de Swart, Peter S. Ross, Lies J. Vedder, Fons B.T.H. Boink, Peter J.H. Reijnders, Paul G.H. Mulder and Albert D.M.E. Osterhaus

Submitted for publication

Abstract

Twenty-two young harbour seals (*Phoca vitulina*) were fed herring from either the relatively unpolluted Atlantic Ocean or the heavily polluted Baltic Sea as part of a 2½ year immunotoxicological study. Blood samples taken at regular intervals were analyzed for routine haematology and clinical chemistry. Minimal differences between the two groups were observed in these parameters over the course of the experiment. Of the 20 clinical chemistry parameters analyzed, slight differences were found in serum levels of urea, creatinine, magnesium and cholesterol. In haematology profiles, red blood cell counts and haematocrit values were higher in seals fed Baltic herring, but these differences diminished over time. Neutrophil counts were also higher in this group of seals, especially during the second half of the feeding study. Factors affecting haematological and clinical chemistry parameters within feeding goups included gender, age and season. The data collected demonstrate an insensitivity of clinical chemistry parameters to the effects of chronic exposure to environmental contaminants accumulated through the food chain, but suggest the induction of clear alterations in differential white blood cell counts. Finally, the data present a comprehensive set of normal ranges for healthy harbour seals.

Introduction

In 1988, an outbreak of a highly infectious disease among European harbour seals (*Phoca vitulina*) caused the deaths of approximately 20,000 animals[157]. On the basis of serological studies, virus isolation and characterization, and experimental infection of dogs and seals, it was concluded that the primary cause of this epizootic was infection with a previously unrecognized morbillivirus, phocid distemper virus-1 (PDV-1)[8,42,125,153,220]. However, the contribution of other factors to the severity of this outbreak, such as an environmental contaminant-related immunosuppression, could not be excluded[158]. This hypothesis became even more plausible when in subsequent years other morbilliviruses caused heavy losses among marine mammal populations inhabiting contaminated coastal waters[217].

In order to test the hypothesis that environmental pollutants accumulated through the food chain adversely affect immune function in marine mammals, we conducted a semifield prospective study using two groups of captive harbour seals. One group received herring from the relatively uncontaminated Atlantic Ocean, while the other group was fed on herring from the heavily polluted Baltic Sea. Estimated daily intakes of dioxin-like organochlorines were 288 ng toxic equivalents (TEQ)/day for the seals fed Baltic herring and 29 ng TEQ/day for the seals fed Atlantic herring[53]. Analyses of total TEQ levels in blubber biopsies of the seals taken after about two years on the respective diets, showed 3.4 times higher levels in seals feeding on Baltic herring (means±SE: 62±4 and 209±12 ng/kg on lipid base, independent t-test: $p<0.01$)[173]. Previously, we reported an impairment of immune function in the seals feeding on polluted fish, as measured *ex vivo/in vitro* by impaired natural killer (NK) cell activity and T cell responses[53] and *in vivo* by impaired delayed type hypersensitivity (DTH) responses and antigen-specific antibody production[173].

Haematology and clinical chemistry parameters were measured during this feeding

study for use as possible indicators of (immuno)toxic stress as well as indicators of general health. An important aspect of immunotoxicological studies is to ascertain that possible effects measured in specific immunological assays are caused by a direct influence of the chemical(s) under investigation, and not the result of indirect causes including nutritional status, impaired protein synthesis or stress. A full set of routine diagnostic parameters was therefore evaluated in order to control for such potential indirect effects. Since these parameters were measured repeatedly in a relatively large group of clinically healthy pinnipeds, the data collected not only shed light on the effects of chronic exposure to environmental contaminants accumulated through the food chain, but also present a useful set of normal ranges for captive young harbour seals.

Materials and methods

Seals and diets

22 harbour seals, caught as weaned pups from the north-east coast of Scotland, were fed herring from the Atlantic Ocean for an adaptation period of one year. The seals were matched for weight and gender and divided into two groups (seven females and four males in each group). The diet of one of the groups was changed to herring from the heavily polluted Baltic Sea in September 1991 (week 0), when the seals were approximately 15 months old. The animals were housed at the Seal Rehabilitation and Research Centre in Pieterburen, in two similar basins with approximately 40 m^3 water and haul-out platforms of approximately 24 m^2. The animals were fed in groups, and both received weekly vitamin supplements to compensate for losses during storage of the fish at -25°C. Vitamins supplemented were (per animal): vitamin A (12500 IE), B1 (50 mg), B2 (2 mg), B3 (80 mg), B5 (2.5 mg), B6 (25 mg), C (250 mg) and D3 (2500 IE).

During the experiment, three different batches of herring were fed to both groups. Batch 1 of the Atlantic herring was fed to both groups during the adaptation period, and to the Atlantic group until week 16; batch 2 was fed from week 17 to week 66; and batch 3 from week 67 onwards. Batch 1 of the Baltic herring was fed from week 0 to week 50; batch 2 from week 51 to week 80; and batch 3 until the end of the study. The mean composition of the Baltic and Atlantic herring diets, respectively, was: water (75% / 69%), fat (7.1% / 12.3%), protein (17.1% / 16.4%) and minerals (1.7% / 2.3%). The seals in the Baltic group were fed more than the seals in the Atlantic group to compensate for the lower fat percentage in the Baltic herring (average daily fish-intake 5.6 and 3.7 kg/seal, respectively). This led to similar daily fat intakes in the two groups (0.40 and 0.45 kg/seal, respectively), but higher daily protein intakes in the Baltic group seals (0.96 and 0.61 kg/seal, respectively).

During captivity the seals were under veterinary supervision, and were observed by animal care technicians at least four times per day. This study was undertaken in accordance with Dutch law, and was approved by the Animal Care Committees of the institutes involved. These rules are in accordance with the guidelines of the Canadian Council on Animal Care.

Blood sampling

Blood samples were taken at 21 weeks before, and 7, 16, 22, 28, 34, 42, 51, 58, 67, 75, 80, 93, 104, 111 and 121 weeks following the start of the feeding study. Blood was obtained from the epidural vein using an 18G3½ (1.2 x 90 mm) spinal needle and a

Vacutainer tube holder with luer lock adapter (Becton-Dickinson). A total volume of approximately 75 ml was collected for measurement of immunological, toxicological, haematological and clinical chemistry parameters. Samples were taken between 9.00 and 13.00 hrs., following a 15-day fasting period, with the exception of the sampling at weeks 42 and 93, when the seals were fed shortly before blood sampling. Samples were kept in the dark at 4°C until analyses, which were carried out on the same day.

Haematology and clinical chemistry analyses

Haematological analyses were carried out on blood with EDTA as anti-coagulant using an automated haematology analyzer (Sysmex E-5000), which uses automatic windowing to differentiate between different cell subsets. Differentiation of leukocyte subsets was carried out by counting 100 leucocytes in Giemsa stained blood smears. Clinical chemistry values were analyzed in serum on a selective discrete clinical chemistry analyzer (Hitachi 717). Clinical chemistry values measured at week 34 were excluded from statistical analyses. Results from this measurement deviated strongly from normal values, and an analytical error was suspected.

Statistical analysis

Longitudinal data were analyzed using a repeated measures ANOVA model (BMDP module 5V), with sex and diet as between subject grouping factors and time as within factor. The method of restricted maximum likelihood was used to estimate parameters of the coefficients of the model. For the covariance matrix of the residuals a first order autoregressive structure was assumed. Differences between the two groups were considered significant if p-values for both the influence of diet and the interaction of diet and time (diet x time) were below 0.01.

Calculation of normal ranges

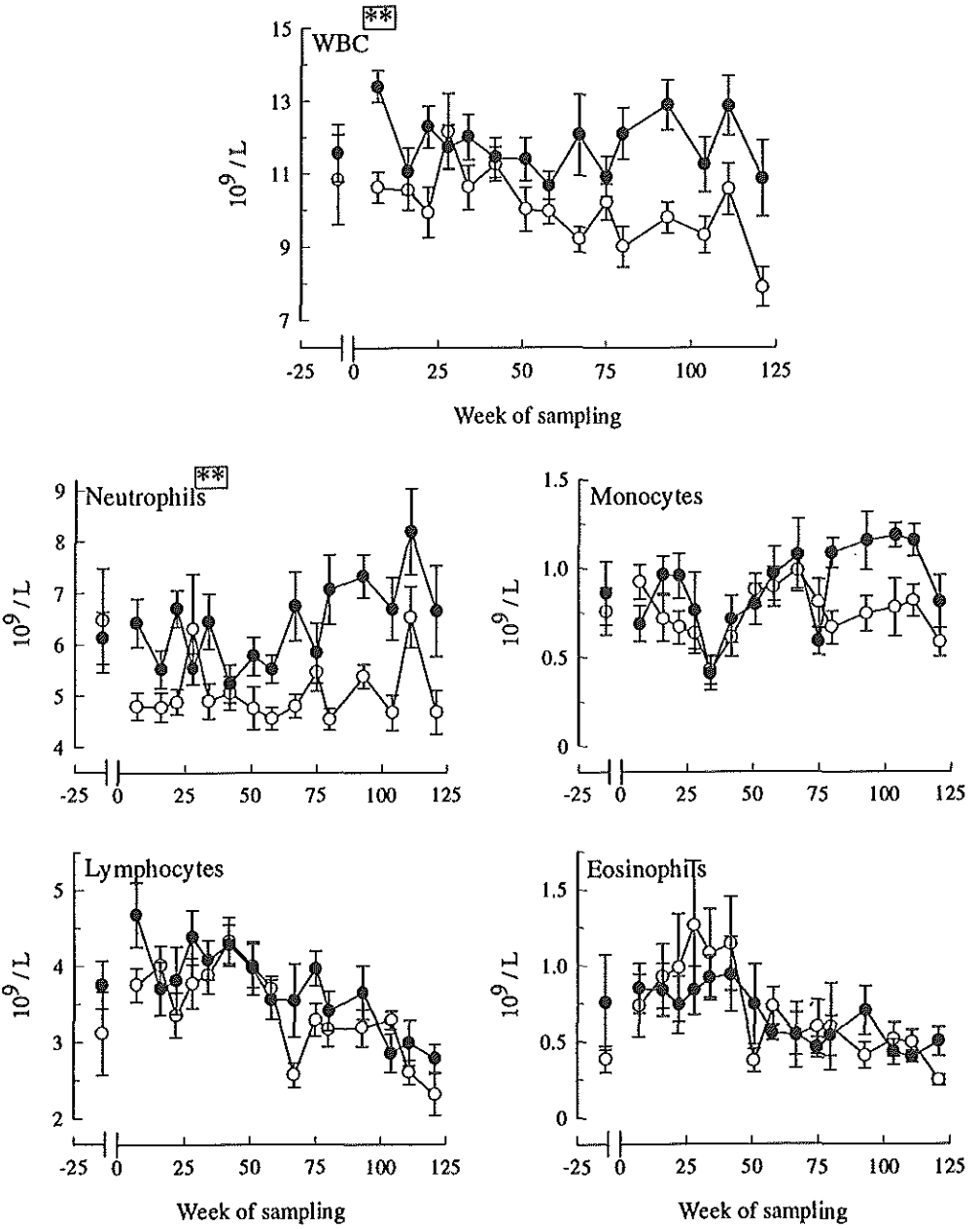
Normal ranges were calculated as the intervals between the 2.5% and 97.5% centiles (means±1.96 x standard deviation) of data obtained from all 22 seals at all time points, provided no significant differences were found between the two feeding groups. Where significant differences were found, only data from the 11 seals of the Atlantic group were used. The standard deviation (SD) of a single measurement was calculated as the square root of the sum of the between-animal and within-animal variance.

Results

Haematology

Longitudinal measurements of leukocyte numbers in both groups of seals are shown in figure 1. White blood cell (WBC) counts were significantly higher in seals fed Baltic herring. This was particularly striking in the second half of the experiment. The higher WBC counts resulted mainly from elevated numbers of neutrophils in these animals, since no significant differences were found in the numbers of lymphocytes, monocytes or eosinophils. However, coinciding with the higher neutrophil counts, monocyte counts measured during the last five samplings were also increased in Baltic group seals. This did not lead to a statistically significant difference between the two groups over time. High numbers of

Figure 1: Longitudinal measurements of white blood cell (WBC)-, neutrophil-, lymphocyte- monocyte- and eosinophil-counts in harbour seals fed Atlantic (open circles) or Baltic (closed circles) herring. Data are presented as means±SE measured 21 weeks before and 7-121 weeks following the start of the feeding experiment. Significant differences (ANOVA repeated measures, $p < 0.01$) between the two groups are indicated by asterisks.



eosinophils often coincided with the presence of heartworm larvae (*Dipetalonema spirocauda*) in peripheral blood mononuclear cell (PBMC) samples, isolated from heparinized blood as previously described[49]. These microfilaria were observed amidst PBMC isolated from seals of both groups. These parasitic infections were not treated and the animals did not show any signs of clinical illness.

Longitudinal measurements of red blood cell (RBC) parameters are shown in figure 2. Red blood cell (RBC) counts and haematocrit (Hct) values were significantly higher in seals fed Baltic Sea herring. No significant differences were found in mean corpuscular volume (MCV), haemoglobin (Hb), mean corpuscular haemoglobin (MCH) or mean corpuscular haemoglobin concentrations (MCHC).

Table 1: Normal ranges for haematology (1A) and clinical chemistry (1B) values of harbour seals. Normal ranges calculated from this study are the intervals between the 2.5% and 97.5% centiles of longitudinal data of all 22 harbour seals (14 females and 8 males, age 15-42 months) fed Atlantic or Baltic herring, unless otherwise specified. Normal ranges reported by Roletto (1993)[169] are the intervals between the 2.5% and 97.5% centiles of values obtained from clinically healthy pups at a rehabilitation centre. Normal ranges reported by Bossart and Dierauf (1990)[26] were obtained from adult harbour seals housed in fresh water.

table 1A

Parameter	Unit	This study	Roletto 1993[169]	Bossart and Dierauf 1990[26]
WBC*	10^9/L	6.0 - 14.2	7.5 - 18.9	7.6 - 19.4
Neutrophils*	%	33 - 68	45 - 81	46 - 85
	10^9/L	2.2 - 7.9	-	-
Lymphocytes§	%	16 - 50	10 - 42	9 - 45
	10^9/L	1.5 - 5.7	-	-
Monocytes	%	0 - 14	0 - 15	1 - 17
	10^9/L	0 - 1.7	-	-
Eosinophils§	%	0 - 16	0 - 9	0 - 9
	10^9/L	0 - 2.0	-	-
thrombocytes	10^9/L	150 - 800	-	-
RBC*¶	10^{12}/L	3.9 - 5.7	3.9 - 5.5	4.0 - 5.6
Hct*¶	%	35 - 63	39 - 62	40 - 66
Hb§	g/L	135 - 240	143 - 229	144 - 240
MCH§	pg	30.9 - 45.7	34.5 - 43.9	33 - 44
MCHC§	g/L	336 - 396	348 - 388	340 - 380
MCV§	fL	88 - 121	93 - 121	92 - 121

table 1b

Parameter	Unit	This study	Roletto 1993[169]	Bossart and Dierauf 1990[26]
Creatinine*	µmol/L	75 - 111	27 - 97	35 - 124
Urea*	mmol/L	8.0 - 14.8	2.9 - 12.7	4.2 - 16.1
AP	U/L	20 - 84		30 - 303
LD‡	U/L	285 - 1071	292 - 1330	321 - 1250
GGT	U/L	4.9 - 14.8	-	-
ASAT‡	U/L	5 - 91	45 - 221	70 - 241
ALAT‡	U/L	6 - 90	-	36 - 69
Total bilirubin	µmol/L	2.0 - 6.6	0.1 - 6.8	1.7 - 8.6
Cholesterol*	mmol/L	5.4 - 8.3	-	5.5 - 7.8
Triglycerides	mmol/L	0.2 - 1.0	0 - 2.9	0.25 - 4.1
Glucose	mmol/L	6.9 - 9.8	-	2.3 - 9.1
Total protein	g/L	67 - 84	57 - 89	61 - 97
Na^+‡	mmol/L	147 - 158	144 - 156	145 - 156
K^+‡	mmol/L	2.8 - 4.6	3.9 - 5.9	4.0 - 6.0
Cl^-	mmol/L	101 - 112	99 - 111	97 - 111
Ca	mmol/L	2.3 - 2.7	2.2 - 2.7	2.2 - 2.7
Mg*‡	mmol/L	0.68 - 0.87	-	-
Phosphorus‡	mmol/L	0.9 - 2.2	-	1.4 - 2.6
Total CO_2	mmol/L	23 - 32	22 - 34	24 - 26
Osmolality‡	mosm/kg	295 - 325	-	-

* significant difference between Atlantic and Baltic group seals: results of Atlantic group seals used for calculation of normal ranges; ‡ significant differences found between males and females; § parameter apparently influenced by age (see figures); ¶ parameter apparently influenced by season (see figures)

Clinical chemistry

Longitudinal measurements of serum biochemistry parameters in both groups of seals are shown in figures 3 and 4. Serum levels of urea and magnesium were significantly higher in seals fed Baltic herring, while levels of creatinine and cholesterol were significantly lower in these animals. No significant differences were found in levels of total protein, lactate dehydrogenase (LD), aspartate aminotransferase (ASAT), alanine aminotransferase (ALAT),

triglycerides, sodium, potassium, chlorine, calcium, phosphorus and bicarbonate or in osmolality. In addition, no significant differences were found in levels of alkaline phosphatase (AP), gamma glutamyl transpeptidase (GGT), total bilirubin, or glucose (not shown). Cholesterol and triglyceride levels measured at weeks 42 and 93 were not included in the statistical analysis, since the animals had been fed shortly before blood sampling, and serum levels of these blood constituents were elevated at this time (see figure 3). Significant gender-related differences were found in levels of LD, ASAT, ALAT, sodium, potassium, phosphorus and osmolality (higher in males) and levels of magnesium (lower in males).

Normal ranges

Normal ranges calculated from results of this study, including clinical chemistry parameters not displayed in the figures, are listed together with previously reported normal ranges for captive harbour seals by Roletto (1993)[169] and Bossart and Dierauf (1990)[26] in table 1. Normal ranges of parameters for which significant gender-related differences were found are not shown separately for males and females, since the numbers of animals from which these ranges would be calculated would become relatively small. Mean haematology and clinical chemistry values of seals in both groups remained within the previously reported normal ranges, with the exception of eosinophil counts in one of the animals in the Atlantic group.

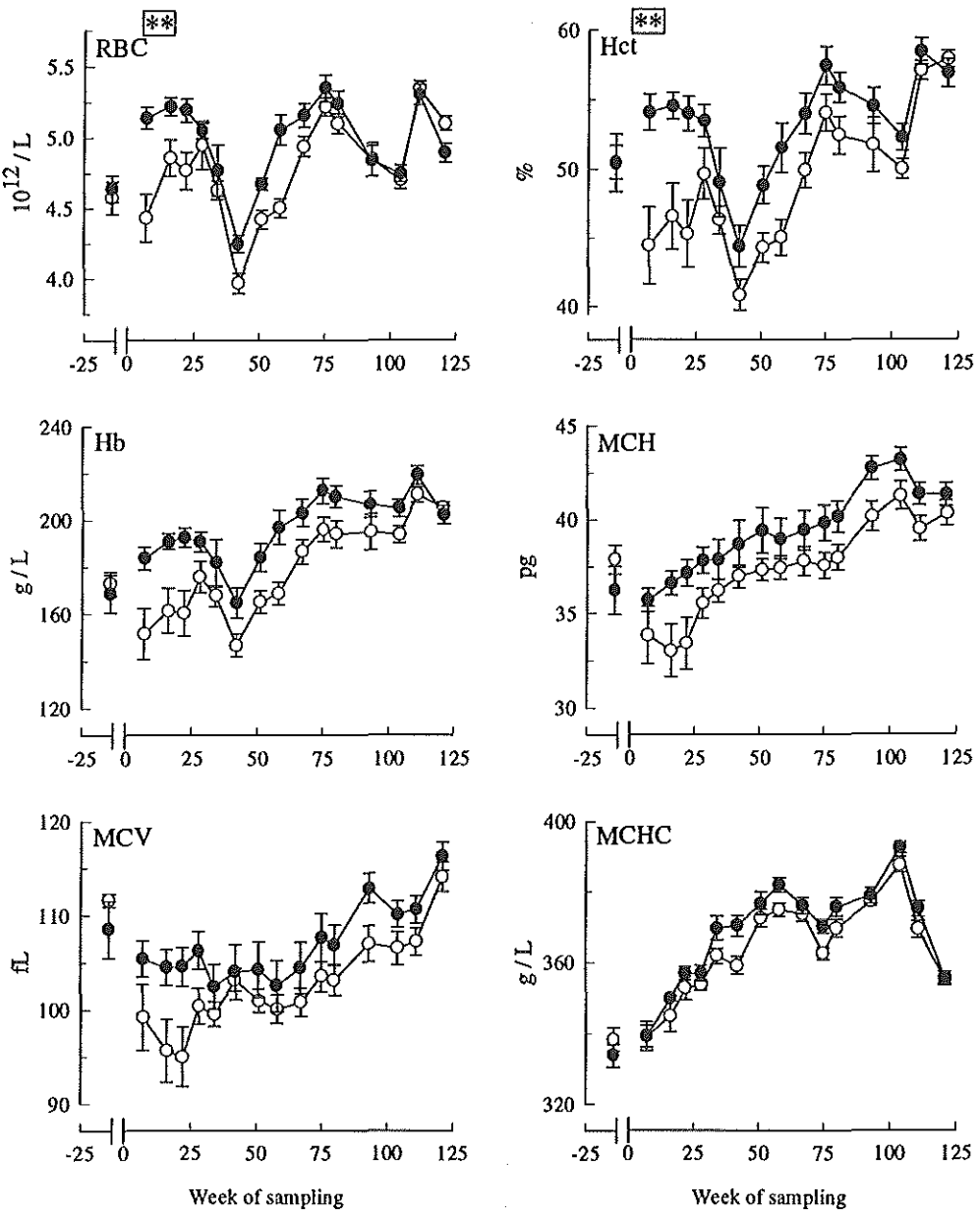
Discussion

Although we previously found impaired immunological responses in the seals fed Baltic herring[53,173], mean values of most haematology and clinical chemistry parameters remained within previously reported normal ranges for captive harbour seals[26,66,169]. This suggests that both the Atlantic and the Baltic herring diets were of adequate nutritional value for the captive seals, and that the observed impaired immunological responses did not result from an indirect effect related to nutrition.

The most prominent difference observed in the parameters measured between the two groups was the increase in neutrophil counts in the Baltic groups seals (figure 1). Differential WBC counts can serve as indicators of problems related to infection and immunity, and are routinely included in screening programs for immunotoxicity of chemicals[213]. Previously, we reported significantly higher percentages of neutrophils in peripheral blood leucocytes of seals fed Baltic herring measured over a shorter period (up to week 93)[53]. We then speculated that the observed impairment of immune function in these animals, as measured *ex vivo/in vitro* by NK cell activity and T cell proliferation, had led to an increase in subclinical bacterial infections. However, it is equally plausible that an effect at the myeloid stem cell level is responsible for this observation. Studies using laboratory animals have demonstrated that lack of T cell activity (e.g. in the congenital athymic nude rat) can be physiologically compensated for by an increased activity of myeloid stem cells, leading in turn to a higher production of both monocytes and granulocytes[224]. The increase in monocyte counts during the last five blood samples may support the latter hypothesis.

The significantly higher RBC counts and Hct values in seals fed Baltic herring (figure 2) could not be related to iron deficiency, since MCHC values were not significantly different. The chronic contaminant exposure is not likely to be the cause of these differences

Figure 2: Longitudinal measurements of red blood cell (RBC) counts, haemoglobin levels (Hb), mean corpuscular volume (MCV), haematocrit values (Hct), mean corpuscular haemoglobin (MCH) and mean corpuscular haemoglobin concentrations (MCHC) in harbour seals fed on Atlantic (open circles) or Baltic (closed circles) herring. Data are presented as means±SE measured 21 weeks before and 7 - 121 weeks following the start of the feeding experiment. Significant differences (ANOVA repeated measures, $p < 0.01$) between the two groups are indicated by asterisks.



either, since they were most pronounced during the first half of the experiment and decreased towards the end. Mean corpuscular volume values also appeared to be higher in Baltic group seals during the first half year, perhaps indicating a shift in red cell age distribution. At the beginning of the experimental period the animals in the Baltic group initially refused to eat when their diet was changed from Atlantic to Baltic herring, leading to a sudden drop in body weights[53]. Although the weights returned to those of the control group four to five weeks later, it is possible that this fasting period led to a shorter life-span of their red cells, which was compensated for by an increased production of erythrocytes.

The differences in cholesterol, creatinine, urea and magnesium levels between the two groups of seals were relatively small. Estimated daily fat intake was similar in both dietary groups, and no differences were found in serum triglyceride levels. The observed differences in cholesterol values could be related to possible differences in fatty acid composition between the two diets. Creatinine is formed in muscles during the metabolism of creatine and phosphocreatine, and enters circulation only for transportation to the kidneys. Since daily production of creatinine is relatively constant and serum levels are unaffected by diet[26], levels are generally used as indicators of kidney function. Since there was no indication of renal problems in the seals of our study groups, the lower creatinine values in the Baltic group of seals may reflect a slightly lower muscle mass in these animals. Serum urea levels are known to be elevated by high protein diets[26]. However, although estimated daily protein intakes of the seals in the Baltic group were 50% higher than those of seals in the Atlantic group, urea levels were only slightly higher in these animals. The largest difference was found seven weeks following the start of the experiment, and was probably related to the high fish intake in the Baltic group in this period related to the fasting period that they had gone through immediately following their change of diet. The higher serum magnesium levels in the seals fed Baltic herring probably result directly from differences in magnesium intake.

The observed differences in haematology and serum chemistry values differ from those reported previously by Reijnders (1988)[164]. In a captive feeding study in which two groups of harbour seals were fed fish with different levels of contaminants he found elevated levels of lymphocyte- and basophil-counts and levels of ALAT, ASAT, AP and GGT in the seals fed on contaminated fish. Reduced levels of total bilirubin, creatinine, uric acid, calcium, magnesium, albumin and gamma-globulin were found in these animals. However, these results are difficult to compare with the present data, since Reijnders studied adult female harbour seals fed on two different fish species (herring and flatfish), and data were obtained from a single blood sampling.

Apart from differences between the two dietary groups, within group differences in haematology and clinical chemistry parameters were observed. Apparently age-related changes were observed in lymphocyte and eosinophil counts, which decreased with age, and in Hb, MCH, MCHC and MCV values, which increased with age (see figures 2 and 3). A decrease in lymphocyte counts with increasing age has also been observed in other species like dogs, cattle and sheep[99]. Haemoglobin, MCH, MCHC and MCV values also tend to be influenced by age in these species, although observations within species are often inconsistent[99]. Red blood cell counts and Hct values showed dips around weeks 42 and 104 (July 1992 and September 1993, respectively), indicating a seasonal trend (figure 2). Seasonable influences on erythrocyte parameters are also commonly found in other mammals[99,166], and may be related to temperature-dependent changes in behaviour or water balance. Since the seals spent

Figure 3: Longitudinal measurements of creatinine-, total protein-, aspartate aminotransferase (ASAT)-, cholesterol-, urea-, lactic dehydrogenase (LD)-, alanine aminotransferase (ALAT)-, and triglyceride levels in harbour seals fed Atlantic (open circles) or Baltic (closed circles) herring. Data are presented as means±SE measured 21 weeks before and 7 - 121 weeks following the start of the feeding experiment. Significant differences (ANOVA repeated measures, $p < 0.01$) between the two groups are indicated by asterisks. Cholesterol and triglyceride levels measured at weeks 42 and 93 were not included in the statistical analysis, since the animals had been fed shortly before blood sampling, and serum levels were elevated at this time.

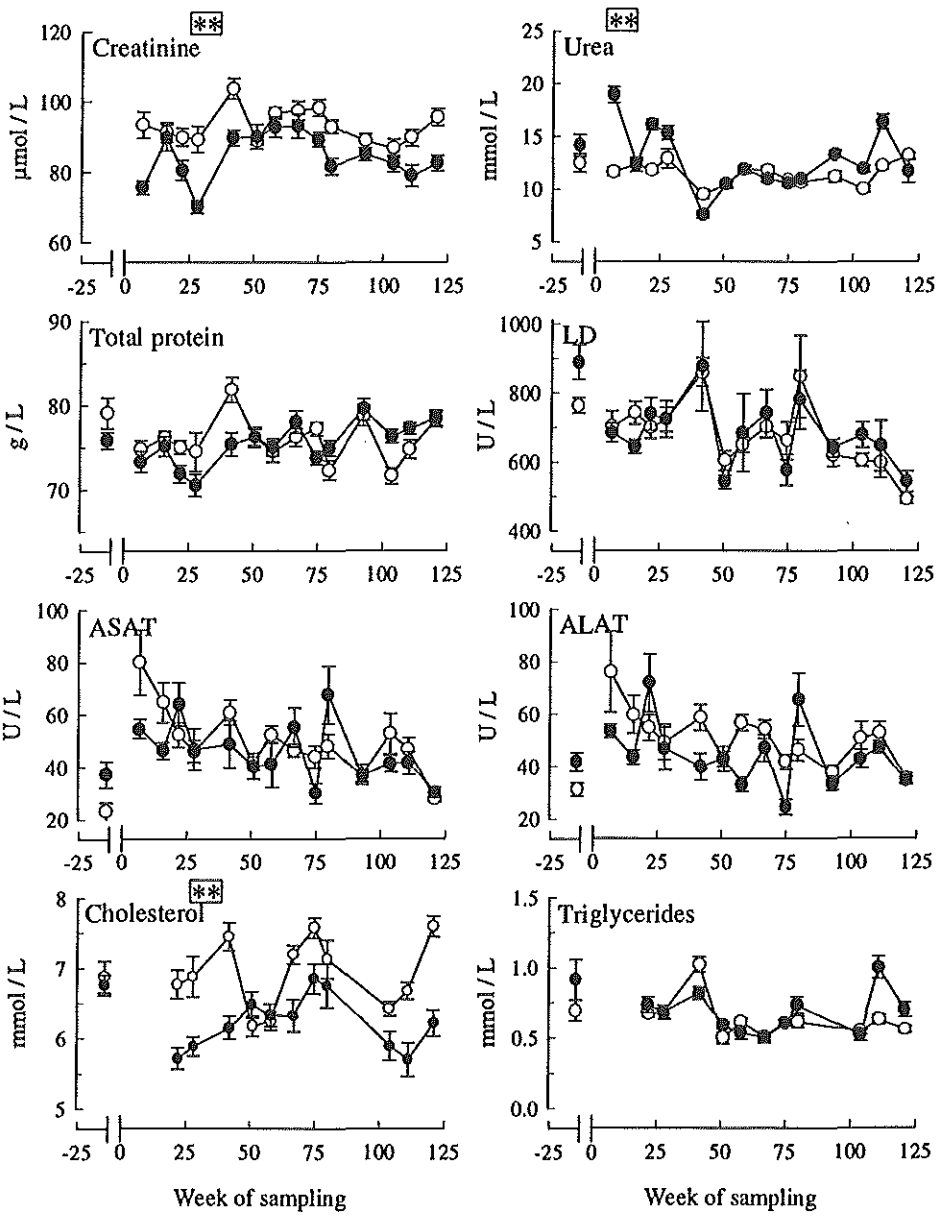
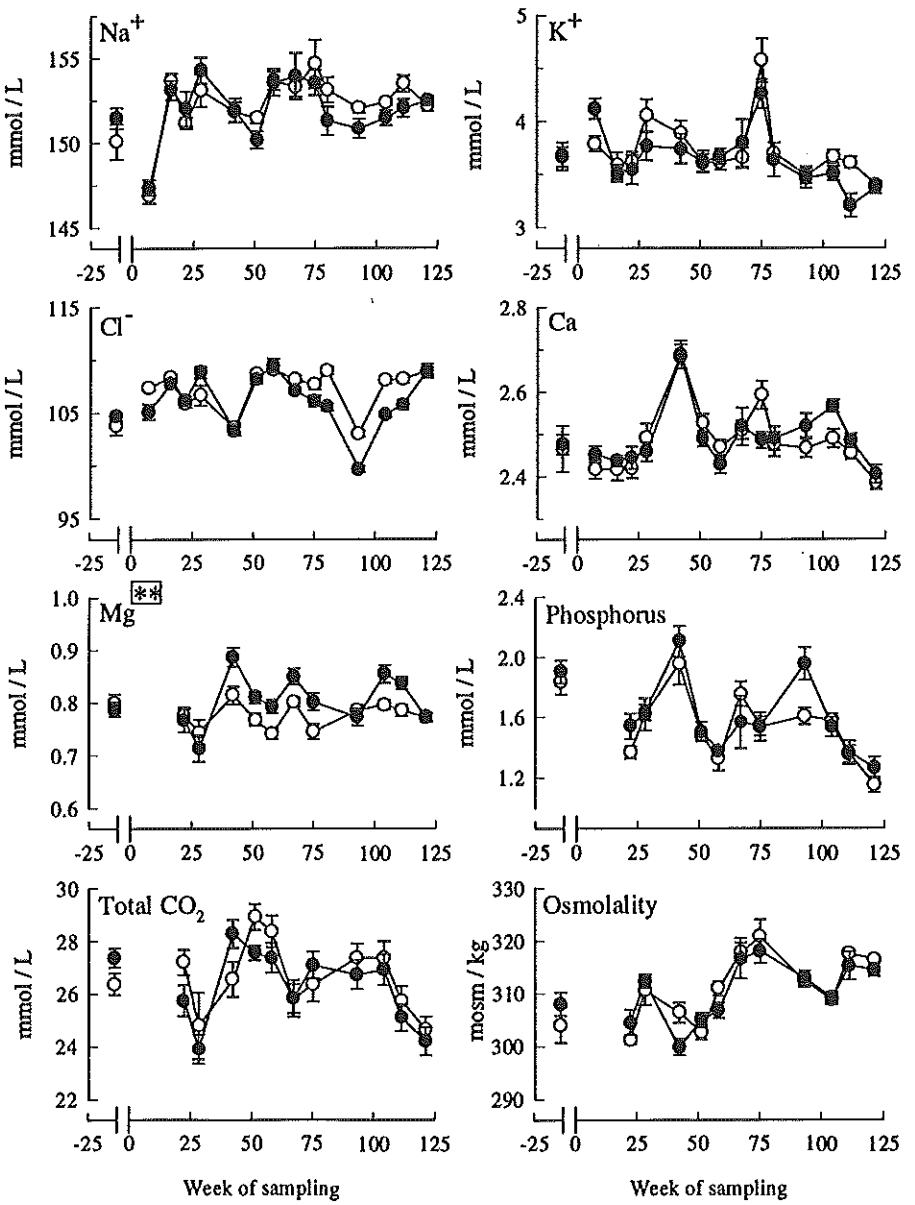


Figure 4: Longitudinal measurements of sodium (Na^+), potassium (K^+), chlorine (Cl^-), calcium (Ca^{2+}), magnesium (Mg^{2+}), phosphorus, total carbon dioxide (Total CO_2) levels, and osmolality in serum of harbour seals fed Atlantic (open circles) or Baltic (closed circles) herring. Data are presented as means±SE measured 21 weeks before and 7 - 121 weeks following the start of the feeding experiment. Significant differences (ANOVA repeated measures, $p < 0.01$) between the two groups are indicated by asterisks.



most of their time hauled out in summer, the decreased RBC parameters might reflect a reduced oxygen requirement. Statistical analysis also revealed gender-related differences in several of the clinical chemistry parameters measured. Similar differences have been reported in other species[6,166], although from these data no discernable patterns were detected. In studies of healthy free-ranging harbour seal mothers and their pups, factors including age, fasting and reproductive status apparently influenced several haematological and clinical chemistry parameters[171,175].

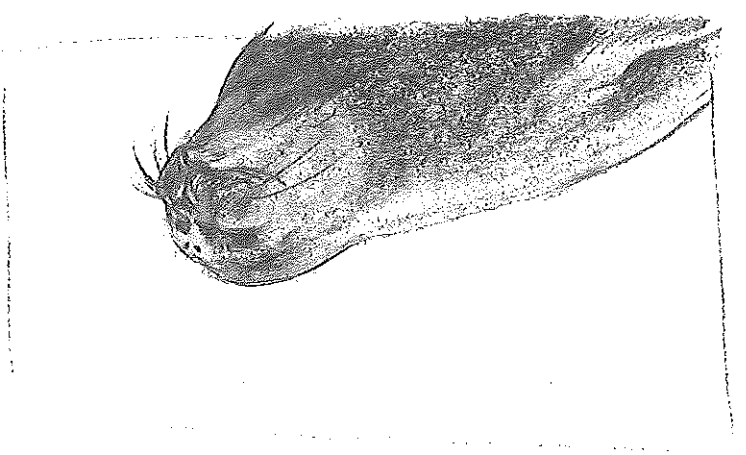
The importance of the data obtained during this study is not restricted to their value for the immunotoxicological feeding experiment, but is also related to their utility as a set of normal ranges and longitudinal patterns of haematology and clinical chemistry values in healthy captive harbour seals. These values will be of particular use for the interpretation of haematology and clinical chemistry values measured in seals suspected of acute or chronic disease.

Acknowledgements

We thank Paul Thompson, Harry Ross, Jaap Scholtens, the Martini Hospital in Groningen and all collaborators of the SRRC and the DLO-IBN for their contribution to these studies, and Dr. J.G. Vos for critical reading of the manuscript. Rik de Swart and Peter Ross were partially funded by a grant from the Nederlandse Aardolie Maatschappij (NAM) and a Natural Sciences and Engineering Research Council of Canada award, respectively.

Chapter 8

Short-term fasting does not aggravate immunosuppression in harbour seals (Phoca vitulina) with high body burdens of organochlorines



Rik L. de Swart, Peter S. Ross, Helga H. Timmerman, Willy C. Hijman, Elze de Ruiter, A.K. Djien Liem, Abraham Brouwer, Henk van Loveren, Peter J.H. Reijnders, Joseph G. Vos and Albert D.M.E. Osterhaus

Submitted for publication

Abstract

It has been postulated that the health of marine mammals inhabiting contaminated waters may be at risk during normally occurring fasting periods, when persistent chemicals mobilized from their lipid reserves could induce acute immunotoxic effects. In order to test this hypothesis, two groups of 11 harbour seals (*Phoca vitulina*) with different body burdens of organochlorines were subjected to an experimental 15-day fasting period. Prior to this experiment, the seals had been fed herring either from the polluted Baltic Sea or from the relatively uncontaminated Atlantic Ocean for a period of 2½ years. This had resulted in two to eight times higher blubber and blood levels of organochlorines in the former group. We previously reported a contaminant-related impairment of immune function in this group of seals, as evidenced by suppressed natural killer (NK) cell activity and specific T lymphocyte responses. During the fasting experiment, the seals of both groups lost an average 11.1 kg, representing 16.5% of their body weights. Blood levels of the most persistent organochlorines showed an approximate twofold increase. Since approximately half of their lipid stores were metabolized, this may reflect a redistribution of unaltered body burdens over the remaining body lipids. However, levels of aryl hydrocarbon receptor-binding organochlorines remained largely unaffected. In line with this observation, few differences were observed between the two dietary groups. Numbers of circulating lymphocytes dropped to about 65% of the initial values and NK cell activity showed a slight increase in both groups. Mitogen- and antigen-induced lymphoproliferative responses of the Baltic group of seals remained within normal ranges. These results suggest that relatively short-term fasting periods do not present an additional immunotoxicological risk to seals with high body burdens of organochlorines.

Introduction

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[43]. The lengths of these periods of partial or total food deprivation may vary from one or two weeks up to more than three months, during which the animals may lose more than 40% of their body mass[27,44,45]. True seals are physiologically adapted to long fasting periods. Metabolization of lipids from their blubber supplies most of their energy- and water requirements, thus limiting protein oxidation[27,34,143,151,161]. Serum levels of ketone bodies, which serve as energy source for the central nervous system during fasting periods, never reach levels which could affect the acid-base balance leading to metabolic acidosis or ketosis, as is seen in non-adapted mammalian species during prolonged fasting[34].

Although seals are adapted to fasting, the risk posed by the release of anthropogenic chemicals from their lipid reserves during these episodes may be of concern, since marine mammals inhabiting polluted marine areas carry high body burdens of lipophilic environmental contaminants[96,199]. Chronic contaminant exposure has been associated with a number of adverse biological effects in marine mammals, including skeletal deformations[12,136], reproductive toxicity[85,86,163], hormonal alterations[28,192], and immunotoxicity[51,53,173]. Recent morbillivirus related mass mortalities among marine mammals inhabiting polluted coastal

waters[48,217] led to speculation about a possible contribution of environmental contaminant-induced immunosuppression to the severity and extent of these epizootics[158,187]. Epidemiological studies showed high levels of potentially immunotoxic contaminants in animals affected by the morbillivirus outbreaks[3,83], although a causal involvement of pollution in the epizootics could not conclusively be demonstrated[114,149].

We carried out an immunotoxicological study in which captive young harbour seals (*Phoca vitulina*) were fed fish contaminated through the food chain of two different marine regions. During a 2½ year period, two groups of eleven seals were fed herring from the heavily polluted Baltic Sea or the relatively uncontaminated Atlantic Ocean, respectively. The long-term intake of contaminated herring led to significantly higher burdens of immunotoxic xenobiotics in the blubber and blood of the seals of the Baltic group[173]. We previously reported an impairment of immune function in this group of seals, as evidenced by suppressed natural killer (NK) cell activity and specific T lymphocyte mediated responses[51,53,173].

In order to test the hypothesis that mobilization of contaminants during fasting periods might pose an additional immunotoxic risk, we subjected the two groups of seals to a 15-day fasting. Here we report the results of functional immunological assays carried out before, during and after this fasting experiment. The data are evaluated in the light of the levels of organochlorines measured in the fish, seal blubber and in blood samples taken before and during the fasting period.

Materials and Methods

Animals

22 harbour seals were caught as weaned pups from the north-east coast of Scotland, and fed relatively unpolluted herring for an adaptation period of one year. The seals were matched for weight and gender and divided over two groups, which were fed herring from the heavily polluted Baltic Sea or from the relatively uncontaminated Atlantic Ocean for a period of 126 weeks (this period will further be referred to as "feeding study"). From week 126 onward (end of February 1994), the seals were subjected to an experimental fasting period of 15 days (further referred to as "fasting experiment"). The two-week duration of the fasting experiment was chosen since free-ranging harbour seals are known to fast between 15 and 20 days under natural circumstances[27], and an experimental fasting of similar length was previously carried out with harbour seals without adverse health effects[128]. The seals that had been fed Atlantic herring are further referred to as "Atlantic group", the seals that had been fed Baltic herring as "Baltic group". Following the fasting experiment, both groups were fed Atlantic herring ad libitum for a period of six months, after which they were released in the North Sea. The seals (seven females and four males in both groups) were housed at the Seal Rehabilitation and Research Centre in Pieterburen. At the beginning of the feeding study (week 0) the animals were approximately 15 months old, and at the moment of their release approximately 4 years. During captivity the seals were under veterinary supervision. This study was approved by the Animal Care Committee of the National Institute of Public Health and Environmental Protection in The Netherlands.

Diets

Composition and vitamin supplementation of herring diets have been described

previously[52]. Baltic herring was caught approximately 100 km off the southwest coast of Finland, while Atlantic herring was caught a few hundred km north of the British isles. Estimated daily intakes of potentially immunotoxic xenobiotics including polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs), and dibenzofurans (PCDFs), hexachlorobenzene (HCB), and the pesticides β-hexachloro-cyclohexane (β-HCH), dieldrin and DDT, were three to more than ten times higher in the Baltic group[53].

Chemical analyses

Chemical analyses were carried out on herring, seal blubber and seal blood. 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 frozen at -20°C. Blubber biopsies (±200 mg) were taken from all the seals at week 104 of the experiment as previously described[173], and frozen at -20°C. Blood samples were taken six weeks before the fasting experiment ("pre", week 121), and at days 8 and 15 after the start of the fasting experiment. Blood was obtained from the epidural vein using an 18G3½ (1.2 x 90 mm) spinal needle connected to a Vacutainer tube holder with a luer lock adapter (Becton-Dickinson). For each animal, a total volume of approximately 15 ml was collected into non-siliconized tubes without additives. Blood samples of the seals of both dietary groups taken on one day were pooled and stored at -20°C.

Lipid content of Baltic herring was lower than that of Atlantic herring: 7.1% and 12.1%, respectively[53]. Extractable lipid percentages (±SE) of the blubber biopsies were 74±4% and 83±4% for biopsies obtained from the seals of the Atlantic or Baltic group, respectively (not significantly different by Student's *t* test). Lipid percentages of the blood samples were 0.23% and 0.22% (pre), 0.25% and 0.23% (day 8) and 0.26% and 0.28% (day 15) for pooled blood samples obtained from the seals of the Atlantic or Baltic group, respectively.

Organochlorine concentrations were determined on the basis of extractable lipid. Total and congener-specific levels of PCBs and DDTs, as well as dieldrin, HCB and β-HCH were measured as previously described[23]. Congener-specific analysis of PCDDs, PCDFs and non-ortho chlorine substituted (coplanar) PCBs was carried out as previously described[118,211]. 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin (TCDD) toxic equivalents (TEQ) were calculated using toxic equivalency factors (TEF) as described[4,214], on the basis of levels of the 17 2,3,7,8-chlorine substituted PCDDs and PCDFs, and non-ortho chlorine substituted PCBs 77 (3,3',4,4'-TCB), 126 (3,3',4,4',5-PeCB) and 169 (3,3',4,4',5,5'-HxCB), mono-ortho PCBs 118 (2,3',4,4',5'-PeCB), 156 (2,3,3',4,4',5-HxCB) and 189 (2,3,3',4,4',5,5'-HpCB), and di-ortho PCB 180 (2,2',3,4,4',5,5'-HpCB). Since mono- and di-ortho chlorine substituted PCBs were the major contributors to the total TEQ levels[173], and PCBs 105, 114, 123, 157, 167 and 170 could not be analyzed, this value probably represents an underestimation of the true TEQ levels. Levels of organotin compounds, including triphenyltin, tributyltin and cyhexatin, in herring and seal blood were measured in extractable lipid after pentylation using gas chromatography methods. Levels of di-nitrophenol compounds in herring and seal blood were measured in extractable lipid using HPLC methods.

Estimation of cumulative intakes and body burdens of organochlorines

Cumulative organochlorine intakes were estimated by multiplying contaminant levels in herring (in μg/kg lipid) with estimated daily lipid intakes (in kg/day), and multiplying this

with the total duration of the feeding study (882 days). Lipid intakes were estimated at 0.45 and 0.4 kg/day for the Atlantic and Baltic groups, respectively, based on estimated daily herring intakes and lipid percentages in herring[52].

Mean body burdens were estimated on the basis of the mean contaminant levels in the blubber (table 1), assuming that more than 90% of lipophilic persistent contaminants are stored in the blubber layer[96]. Mean blubber lipid mass per seal was estimated at 15 kg (approximately 25% of mean body weight).

Haematology and clinical chemistry

Haematology and serum chemistry parameters were measured in blood samples taken five weeks before ("pre", week 121 of the feeding study), six weeks following ("post") and at days 8 and 15 of the fasting experiment. Haematological parameters were measured in blood with EDTA as anti-coagulant on an automated haematology analyzer (Sysmex E-5000), which uses automatic windowing to discriminate between cell subsets. Clinical chemistry parameters were analyzed in serum using a selective discrete clinical chemistry analyzer (Hitachi 717). Haematology and clinical chemistry data of the seals during the feeding study are reported elsewhere[52].

Immunizations

At day three of the fasting experiment, seals received a booster immunization with tetanus toxoid (TT) adsorbed to aluminium phosphate (intramuscularly). All seals had been immunized with the same antigen 93 and 76 weeks earlier (weeks 33 and 50 of the feeding study, respectively[51]).

Table 1A: Organochlorine contaminants in herring, seal blubber and seal blood before the start of the fasting experiment: non *Ah* receptor-binding compounds.

	------herring[1]------		-----seal blubber[2]-----		--seal blood[3]--	
	Atlantic	Baltic	Atlantic	Baltic	Atlantic	Baltic
sum PCBs	875±158[4]	4398±715	6884±493	16488±1023	7109	15062
sum DDTs	152±23	2155±807	3050±420	12690±1920	788	2779
p,p'-DDT	31±3	272±35	306±55	2448±368	89	552
HCB	21±4	88±6	12±2	19±4	30	86
β-HCH	<10	87±23	<130	<130	<1	12

Legend to Table 1: For a period of 128 weeks, two groups of harbour seals were fed herring from the heavily polluted Baltic Sea or the relatively uncontaminated Atlantic Ocean. At week 104, blubber samples were taken from all seals for chemical analyses. At week 121 blood samples were taken and mixed for each dietary group. Table 1A shows levels of non-*Ah* receptor-binding organochlorines in herring, seal blubber and seal blood, while table 1B shows levels of *Ah* receptor-binding organochlorines. [1]means±SE of three batches of herring; [2]means±SE of 11 seals; [3]concentration in pooled blood sample of 11 seals; [4]levels shown are in μg kg^{-1}; [5]levels shown are in ng kg^{-1} lipid unless otherwise specified; [6]N.D. not determined due to analytical error.

Table 1B: Organochlorine contaminants in herring, seal blubber and seal blood before the start of the fasting experiment: *Ah* receptor-binding compounds.

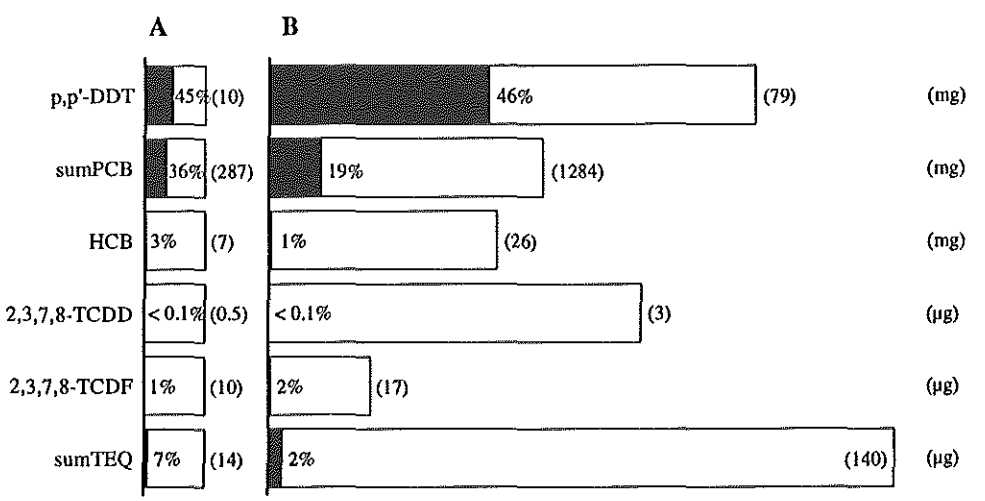
	------herring[1]------		---seal blubber[2]---		---seal blood[3]---	
	Atlantic	Baltic	Atlantic	Baltic	Atlantic	Baltic
2,3,7,8-TCDD	1.6±0.2[5]	10.9±2.6	<1	<1	2.4	2.1
1,2,3,7,8-PeCDD	4.6±0.3	35.9±7.7	<1	<1	<0.1	3.8
1,2,3,4,7,8-HxCDD	0.8±0.1	4.2±0.9	<1	<1	<0.1	0.8
1,2,3,6,7,8-HxCDD	2.8±0.2	26.0±4.9	5.0±0.3	15.9±2	3.5	9.1
1,2,3,7,8,9-HxCDD	0.8±0.1	3.4±0.7	<1	<1	<0.1	<0.1
1,2,3,4,6,7,8-HpCDD	3.5±1.3	8.4±2.8	3.3±0.8	7.8±1.8	81.0	130.8
OCDD	17±10	58.9±29	8.0±2.2	23.9±5.9	419.3	608.7
2,3,7,8-TCDF	31±2	58.0±4.7	5.0±0.2	20.9±2.2	3.7	2.9
1,2,3,7,8-PeCDF	5±0.3	44.0±9.1	<1	<1	<0.1	0.5
2,3,4,7,8-PeCDF	18±2	252±54	<1	<1	1.8	7.1
1,2,3,4,7,8-HxCDF	1.3±0.1	12.0±2.7	1.9±0.2	5.0±2.2	<0.1	1.5
1,2,3,6,7,8-HxCDF	1.4±0.1	16.8±4.1	1.6±0.2	5.4±2.3	<0.1	1.8
1,2,3,7,8,9-HxCDF	<0.1	0.6±0.2	<1	<1	<0.1	<0.1
2,3,4,6,7,8-HxCDF	1.8±0.1	15.9±4	<1	<1	0.9	1.1
1,2,3,4,6,7,8-HpCDF	0.9±0.1	4.5±1.5	1.2±0.4	5.7±1.8	<0.1	1.9
1,2,3,4,7,8,9-HpCDF	0.1±0.05	0.4±0.1	<1	<1	<0.1	<0.1
OCDF	0.4±0.2	1.2±0.4	4.5±0.7	10.6±2.4	1.9	<0.1
PCB077	538±22	1444±272	48±5	64±5	51	64
PCB126	202±29	1569±463	219±10	513±31	105	238
PCB169	40±5	371±89	30±2	100±6	N.D.[4]	37
PCB118 (μg kg^{-1})	29±3	223±20	78±4	219±10	71	184
PCB156 (μg kg^{-1})	2±1	23±2	49±7	219±14	<1	20
PCB189 (μg kg^{-1})	<1	<1	4±2	9±1	<1	<1
PCB180 (μg kg^{-1})	23±7	112±7	256±19	770±58	286	822
total TEQ	42±4	426±83	62±4	209±12	26	72

Six weeks before the fasting experiment the seals received a primary immunization with keyhole limpet haemocyanin (KLH, 200 µg) adjuvanted with dimethyl-dioctadecacyl-ammoniumbromide (DDA, 800 µg) (intramuscularly). At day 6 of the fasting experiment the animals received a booster immunization with the same antigen/adjuvant combination.

ELISAs

Serum antibody responses to the antigens TT and KLH were measured using an enzyme linked immunosorbent assay (ELISA) as previously described[49]. Briefly, plates were coated with the respective antigens and blocked with bovine serum albumin. After incubation with serial dilutions of seal plasma (in duplicate per sample), bound IgG antibodies were detected using horseradish peroxidase labeled protein A. Results are shown as 50% titers (sample dilution at which extinction at 450 nm is reduced to 50% of the maximal signal).

Figure 1: Estimated mean body burdens of organochlorines (gray bars), as percentage of estimated cumulative intakes of these chemicals (open bars) of two groups of seals (n=2 x 11) following a diet of Atlantic (A) or Baltic (B) herring for a period of two years. Cumulative intakes of the respective contaminants were estimated on the basis of contaminant levels in herring (table 1) and estimated daily lipid intakes, and are shown between brackets. Bar lengths of the cumulative intakes of the Baltic group are shown relative to the cumulative intakes of the Atlantic group. Lipid intakes were estimated at 0.45 and 0.4 kg day^{-1} for the Atlantic and Baltic groups, respectively, based on estimated daily herring intakes and lipid percentages in herring[32]. Mean body burdens were estimated on the basis of the mean contaminant levels in the blubber (table 1), since most lipophilic persistent contaminants are largely stored in the blubber layer[96]. Mean blubber lipid mass per seal was estimated at 15 kg (approximately 25% of mean body weight).



Immunological assays

Immunological parameters were measured following previously described methods in blood samples taken five weeks before ("pre"), six weeks following ("post") or at days 8 and 15 of the fasting experiment. Briefly, peripheral blood mononuclear cells (PBMC) were isolated from heparinized blood samples by density gradient centrifugation[49]. Cells were counted, adjusted to concentration, and assays were carried out in a double-blind manner. NK cell activity was measured in a chromium-release assay using YAC-1 tumour cells as target[53,174]. Lymphoproliferative responses were measured after stimulation of PBMC with optimal concentrations of the mitogens concanavalin A (Con A, 5 µg/ml), pokeweed mitogen (PWM, 2.5 µg/ml), phytohaemagglutinin-M (PHA, 20 µg/ml) or lipopolysaccharide from *Salmonella typhymurium* (LPS, 100 µg/ml), or the antigens TT (20 LF/ml) or KLH (20 µg/ml)[49,51,53].

Hormonal assays

Serum cortisol levels were determined in a competitive fluorescence assay using europium-labeled cortisol (Pharmacia). Levels of total thyroxin (TT4) and tri-iodothyronine (TT3) were determined by a method based on enhanced luminescence as previously described[141].

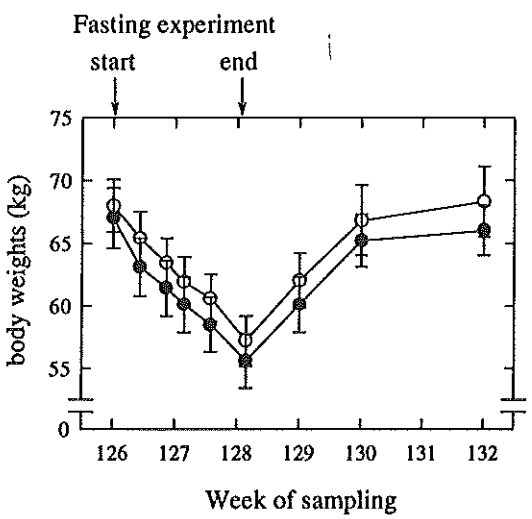


Figure 2: Body weights (means±SE) of harbour seals of the Atlantic group (open symbols, n=11) or the Baltic group (closed symbols, n=11) during a 15-day fasting period (week 126-128) and the subsequent recovery period.

Statistical analysis

Differences between the two dietary groups over the course of the fasting experiment were analyzed by two way ANOVA on natural log-transformed data, with time and diet as factors. Significant differences obtained with this test are shown as one ($p<0.05$) or two ($p<0.01$) boxed asterisks. When significant differences between the two groups were found in this test, differences on the individual sampling data were tested by Student's *t* test on log-transformed data: results shown by one ($p<0.05$) or two ($p<0.01$) asterisks without box.

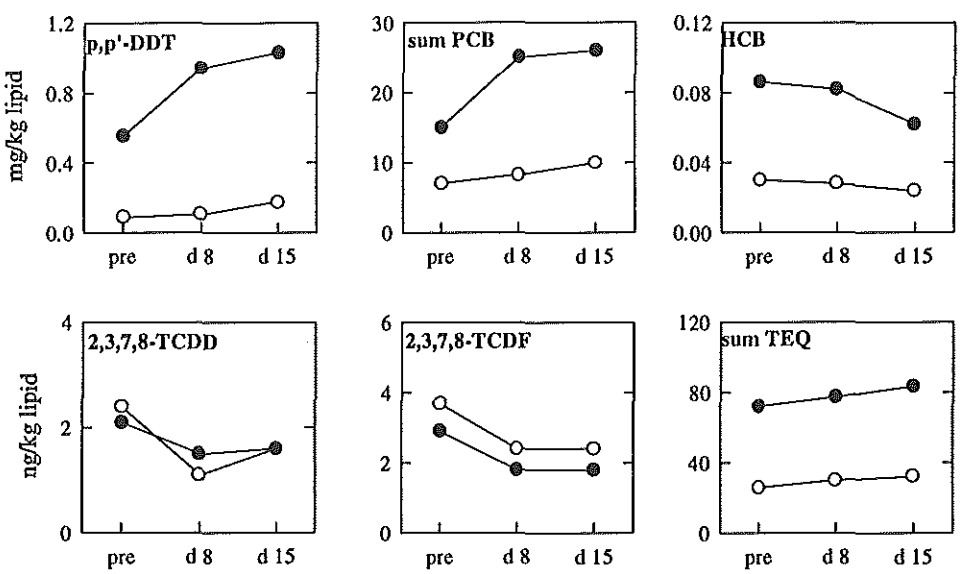
Results

Feeding study: accumulation of immunotoxic xenobiotics

Levels of non-aryl hydrocarbon (*Ah*) receptor-binding organochlorines in herring and in the blubber and blood of the seals are shown in table 1A. Sum PCB and sum DDT levels were strongly biomagnified from herring to seal blubber. Dieldrin levels were only biomagnified from herring to seal blood, but blubber levels remained relatively low. No biomagnification was observed for levels of HCB and ß-HCH. Levels of organotin compounds and di-nitrophenol compounds in herring and seal blood were below the detection limits (25 µg/kg and 10 µg/kg lipid, respectively).

As shown in table 1B, levels of *Ah* receptor-binding organochlorines (sum TEQ) in Baltic herring were approximately ten times higher than in the Atlantic herring. Total TEQ levels in the blubber of seals after two years on a diet of Baltic herring were approximately 3½ times higher than in the Atlantic group, but were lower than the levels measured in the Baltic herring. Total TEQ levels in blood samples of the Baltic group were even lower, although levels in the Baltic group were still a factor 2½ higher than levels in the Atlantic group.

Figure 3: Levels of organochlorines (on the basis of extractable lipid) in pooled blood samples from seals of the Atlantic group (open symbols) or Baltic group (closed symbols), six weeks before ("pre"), and at days 8 and 15 of the fasting experiment.



As shown in figure 1, estimated body burdens of p,p'-DDT and sum PCB (dominated by the persistent non *Ah* receptor-binding congeners) were in the same order of magnitude as the estimated cumulative intakes, reflecting an efficient uptake and storage of these persistent contaminants. Estimated body burdens of HCB, 2,3,7,8-TCDD, 2,3,7,8-TCDF and sum TEQ were less than 10% of the estimated cumulative intakes of these compounds, suggesting a partial metabolization and excretion. In general, the Baltic group of seals retained a smaller percentage of the estimated cumulative contaminant intakes in their blubber (figure 1 and data not shown).

Fasting experiment: mobilization of organochlorines

During the 15-day fasting experiment from week 126 onward, the seals lost 11.1±0.4 kg, representing 16.5±0.5% of their body weight (mean±SE). Although the body weights of the animals of the Baltic group were slightly lower, at the start of the fasting experiment, the parallel course of the lines in figure 2 indicates that the weight loss was similar in both dietary groups. Throughout the fasting experiment study animals remained active and displayed a normal behaviour.

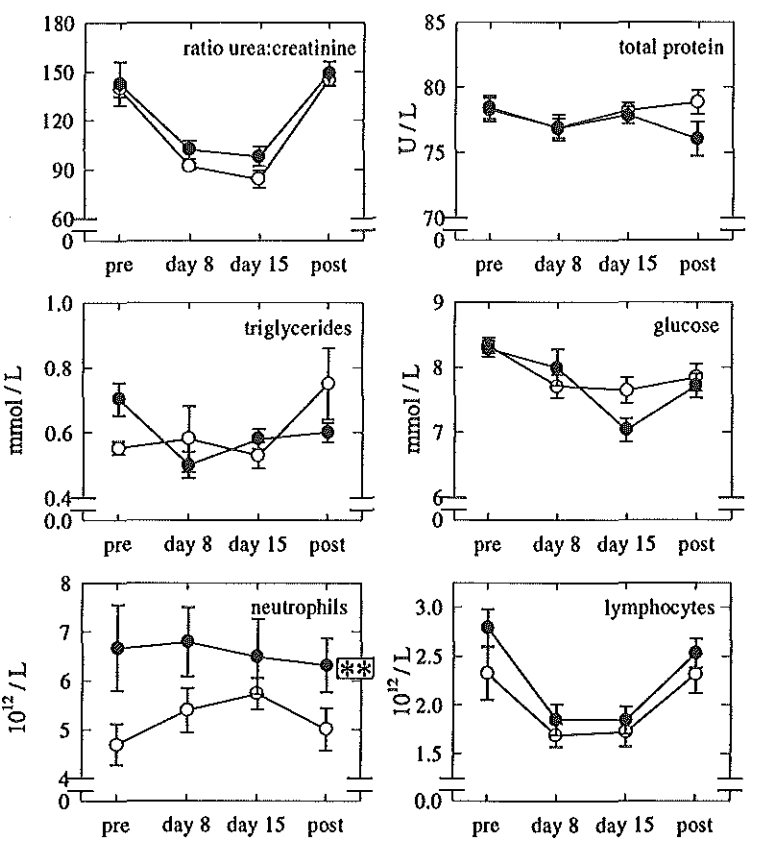


Figure 4: Haematology and clinical chemistry parameters (means±SE) of seals of the Atlantic group (open symbols, n=11) or Baltic group (closed symbols, n=11), six weeks before ("pre"), five weeks following ("post") and at days 8 and 15 of the fasting experiment.

In order to get an indication of the amount of organochlorines mobilized into the bloodstream during this period, chemical analyses were carried out on pooled blood samples drawn on days 8 and 15. As shown in figure 3, blood levels of p,p'-DDT and sum PCBs showed an approximate twofold increase during the fasting experiment, while levels of HCB, 2,3,7,8-TCDD and 2,3,7,8-TCDF showed a decrease. Blood concentrations of *Ah* receptor-binding organochlorines (in ng TEQ/kg lipid) remained largely unaffected. Levels of most 2,3,7,8-chlorine substituted dioxins and dibenzofurans stayed extremely low, ranging from below the detection limit (0.1 ng/kg lipid) to 15 ng/kg lipid.

Fasting experiment: clinical chemistry and haematology

In general, haematology and clinical chemistry parameters of the seals in both groups were similarly influenced by the fasting experiment. During fasting, the ratio of serum urea to creatinine showed a decline of 30 to 40% in both groups of seals, while the fasting experiment did not strongly influence serum levels of total protein, triglycerides or glucose (figure 4). In differential white blood cell counts, reduced numbers of circulating lymphocytes were observed in both groups of seals. Levels of neutrophils remained significantly higher (two way ANOVA, $p<0.01$) in seals of the Baltic group[52,53], but were not influenced by fasting (figure 4).

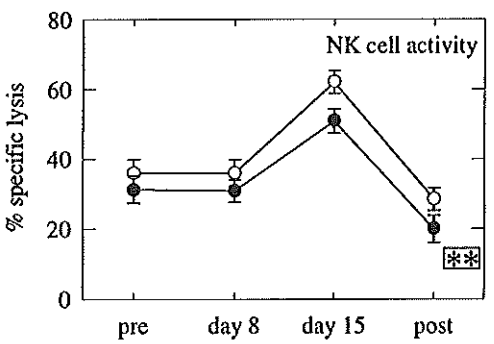


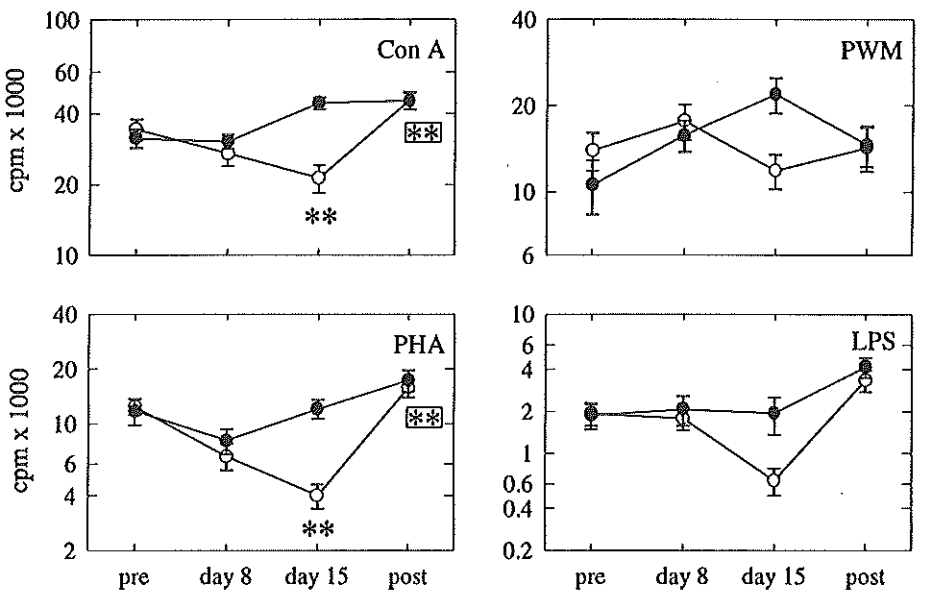
Figure 5: Natural killer (NK) cell activity (means±SE) of seals of the Atlantic group (open symbols) or the Baltic group (closed symbols), six weeks before ("pre"), five weeks following ("post") and at days 8 and 15 of the fasting experiment. NK cell activity was measured in a chromium release assay with seal PBMC as effector cells and ^{51}Cr-labeled YAC-1 tumour cells as target cells, using an effec-tor:target ratio of 50:1. Asterisks in boxes indicate a significant difference between the two dietary groups over the course of the fasting experiment (two-way ANOVA on log-transformed data, $p<0.01$).

Fasting experiment: immunological responses

NK cell responses of PBMC isolated from seals of both dietary groups were similarly influenced by the fasting experiment. The pre-existing difference between the two dietary groups (two way ANOVA, $p<0.01$) remained constant during and six weeks following the fasting experiment, while both groups showed an increased NK cell activity following the 15-day fasting experiment (figure 5).

No significant differences were found in the overall PWM- or LPS-induced lymphoproliferative responses between the two groups of seals during the fasting experiment. However, Con A- and PHA-induced proliferative responses were significantly lower in the Atlantic group (figure 6, two way ANOVA $p<0.01$), resulting from significantly lower responses in the seals of this group at day 15 (Student's *t* test, $p<0.01$). Following booster immunizations with TT and KLH on days 3 and 6 of the fasting experiment, respectively,

Figure 6: Mitogen-induced proliferative responses of PBMC obtained from seals of the Atlantic group (open symbols) or the Baltic group (closed symbols), six weeks before ("pre"), five weeks following ("post") and at days 8 and 15 of the fasting experiment. Seal PBMC were stimulated with the T cell mitogens Con A or PHA, the B cell mitogen LPS, or the T and B cell mitogen PWM. Proliferative responses were quantified by measuring the incorporation of ^{3}H-labeled thymidine, and expressed in cpm (means±SE), after subtraction of background responses. Asterisks in boxes indicate a significant difference between the two dietary groups over the course of the experiment (two-way ANOVA on log-transformed data, $p<0.01$). Asterisks without boxes indicate a significant difference between the two groups at one sampling point (Student's *t* test, $p<0.01$).



the overall antigen-specific humoral and lymphoproliferative responses were not significantly different between the two groups (figure 7). It is interesting to note that all lymphoproliferative responses of the Atlantic group of seals tended to be lower than those of the Baltic group at day 15 of the fasting experiment (figures 6,7).

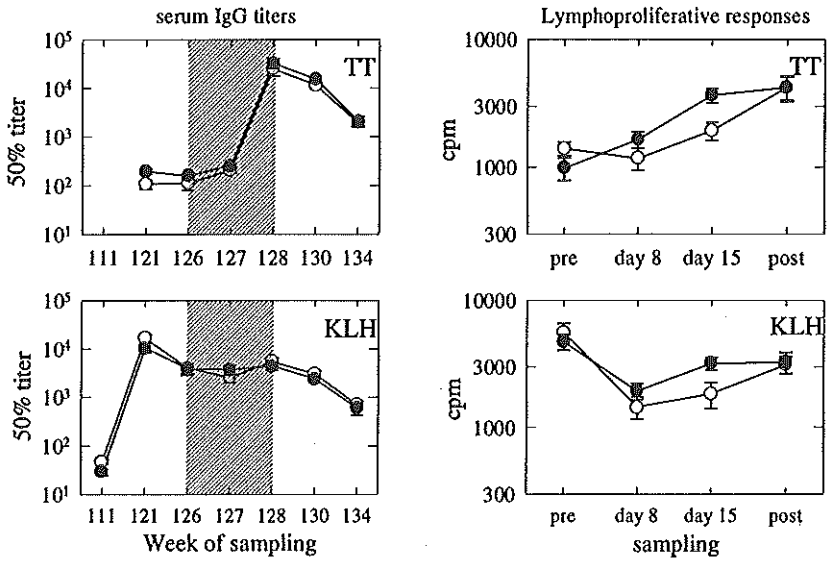
Fasting experiment: hormonal responses

During the fasting experiment, cortisol levels increased in both groups of seals (figure 8). Levels of the thyroid hormones TT4 and TT3 showed an increase in the seals of the Atlantic group on day 8 of the fasting experiment, but less so in the seals of the Baltic group (figure 8). Serum levels of TT3 were significantly higher in the Atlantic group over the course of the fasting experiment (two way ANOVA, $p<0.05$), and the difference was most pronounced at day 8 (Student's *t* test, $p<0.05$).

Discussion

After having shown that seals fed fish contaminated through the food chain had impaired immune functions[51,53,173], the present studies focused on the bioaccumulation of

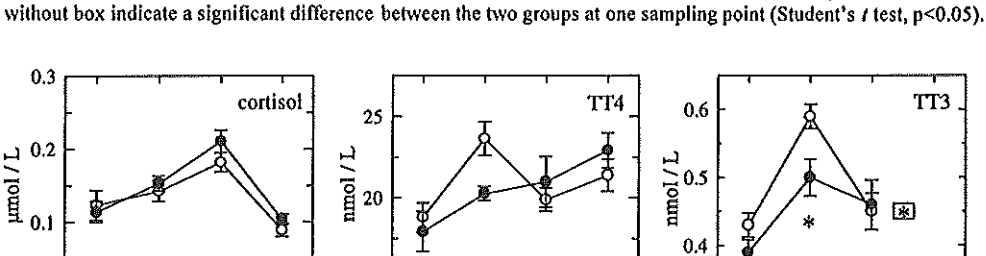
Figure 7: Antigen-specific humoral and cellular immune responses of seals of the Atlantic group (open symbols) or the Baltic group (closed symbols). Serum IgG titers (means±SE) following booster immunizations with tetanus toxoid at day 3 of the fasting experiment (TT, upper left) or keyhole limpet haemocyanin at day 6 of the fasting experiment (KLH, lower left) were measured in a direct ELISA using protein A as a conjugate. In the left plots the fasting period is indicated by gray shading. Antigen-specific proliferative responses of PBMC (means±SE) were measured six weeks before ("pre"), five weeks following ("post") and at days 8 and 15 of the fasting experiment (TT-specific responses: upper right, KLH-specific responses: lower right).



potentially immunotoxic chemicals in the seals, and showed that the observed immunosuppression was not aggravated by a two-week fasting.

Organochlorine levels in the herring were in line with previously published data on Baltic and Atlantic herring[55,82,150,170]. Levels of DDTs and PCBs were strongly biomagnified from herring to seal blood and blubber, although the levels in the Baltic group were slightly below the range reported for harbour seals inhabiting the Baltic Sea[20,121,149]. This may be explained by the relatively low level perinatal contaminant exposure in our study seals. PCB and DDT levels measured in seals of the Atlantic group were within the range previously reported for seals inhabiting the north-east coast of the United Kingdom[20,83]. When estimated body burdens of the persistent organochlorine p,p'-DDT were compared with the estimated cumulative intakes during a two-year period in both groups, almost half of the estimated intake could be accounted for, suggesting that the kinetics essentially resembled those of a "closed system" (figure 1). In agreement with previous observations[11,13,55], blubber levels of polychlorinated dioxins and dibenzofurans were relatively low, especially when compared to the estimated cumulative intakes (less than 0.1%, figure 1). This confirms that seals must have an efficient mechanism of either excreting or metabolizing these contaminants[11]. In seals of the Atlantic group, generally higher percentages of the estimated cumulative intakes accumulated in the blubber. This may reflect the induction of enzyme systems (e.g. cytochrome p450) in the Baltic group, resulting in a more efficient metabolization of organochlorines[23].

Figure 8: Serum levels of cortisol and levels of the thyroid hormones TT4 and TT3 (means±SE) of seals of the Atlantic group (open symbols) or the Baltic group (closed symbols) at days 0 ("pre"), 8 and 15 and five weeks following ("post") the fasting experiment. Asterisks in boxes indicate a significant difference between the two dietary groups over the course of the fasting experiment (two-way ANOVA on log-transformed data, $p<0.05$). Asterisks without box indicate a significant difference between the two groups at one sampling point (Student's *t* test, $p<0.05$).



Development of body weights during fasting and subsequent refeeding was similar as previously described for harbour seals[128]. During the fasting, blood levels of p,p'-DDT showed an approximate two-fold increase, suggesting that less than 1% of the total burden of this compound that was originally present in the lipids metabolized during the fasting, was present in the blood. This implies that this contaminant was either not mobilized along with the metabolized lipids, or left the blood soon after mobilization, e.g. back to the blubber[3,96]. However, Boon *et al.*[22] suggested that lipids in blubber, blood and peripheral organs essentially represent a single compartment. Assuming that p,p'-DDT cannot be metabolized by liver enzymes, the total burden of this compound should then be redistributed over the remaining body lipids. Since approximately half of the original lipid reserves were estimated to be metabolized during the fasting period, this would indeed lead to a two-fold increase in the p,p'-DDT concentrations in lipids. In starved marine mammals, blubber levels of persistent organochlorines are often extremely high, indeed suggesting a concentration effect during the metabolization of blubber lipids[1,3,20]. The lack of increasing blood levels observed for certain compounds during fasting may be explained by detoxification in the liver and subsequent excretion[3]. While the total body burden of non-metabolizable compounds like p,p'-DDT remains the same, body burdens of metabolizable compounds like the *Ah* receptor-binding organochlorines may decrease during fasting. The data shown in figure 3 suggest a half-life for 2,3,7,8-TCDD of less than one week, which is shorter than the estimated half-life of this compound in most other species [210].

Blood levels of *Ah* receptor-binding organochlorines did not increase during fasting, which is not inconsistent with our finding that only limited differences were found in functional immunological parameters measured during fasting. Numbers of circulating lymphocytes dropped to 30-40% of the initial values in both groups. Since the levels were back to normal six weeks after fasting, we speculate that this phenomenon is related to the stress of fasting. Acute stress has previously been shown to affect white blood cell counts in bottlenose dolphins[76] and other mammals[99]. The decreased serum urea:creatinine ratios in the absence of changes in serum levels of proteins, triglycerides and glucose likely reflect an adaptive response as previously observed in fasting seals[34,45].

The increased NK cell responses in both groups may also be related to fasting stress, or may alternatively have been due to the alterations in leucocyte profiles in the blood during this period: the decreased numbers of lymphocytes may have led to higher proportions of NK cells in PBMC. NK cell activity continued to be significantly lower in seals of the Baltic group as compared to the Atlantic group[53,174], whereas mitogen- and antigen-induced lymphoproliferative responses of these animals remained normal during fasting. Con A- and PHA-induced lymphoproliferative responses of seals of the Atlantic group were unexpectedly and unexplained reduced at day 15.

Serum cortisol levels showed a gradual increase in both groups during fasting, which was somewhat more pronounced in the Baltic group of seals (figure 8). Increased cortisol levels have previously been reported in harbour seals during the moulting period, which is associated with decreased food intake[168]. However, post-weaning fasting periods generally induce no or only limited changes in serum cortisol levels[144]. The serum levels of both TT3 and TT4 increased to a larger extent in the Atlantic group than in the Baltic group. Increases in thyroid hormone levels have previously been observed in moulting harp seals[67,100] and in the seals of this study during the moulting period (manuscript in preparation). The increase in thyroid hormone levels may be necessary for the metabolic switch to the use of blubber lipids as the principle source of energy. However, other reports have not indicated any changes in TT3 or TT4 levels during fasting in harp seals[143]. Previously, chronic exposure to organochlorines has also been associated with reduced thyroid hormone levels in harbour seals[28]. Irrespective of the physiological role of the increase in thyroid hormone levels during the fasting experiment, the seals of the Baltic group seemed to be less capable of this adaptive response.

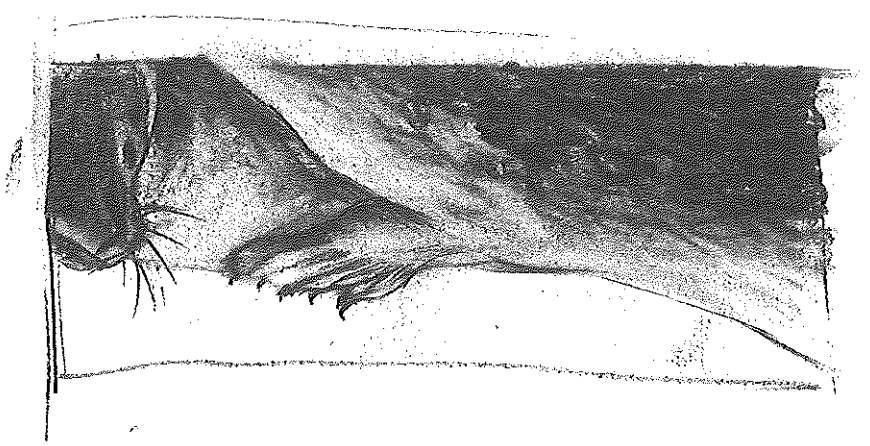
Taken together, the results of our experiments indicate that although chronic exposure to environmental contaminants accumulated through the food chain leads to impaired immune function in harbour seals, relatively short-term fasting does not seem to pose a major additional immunotoxic threat to these animals. Blood levels of persistent organochlorine chemicals showed an approximate twofold increase, but levels of *Ah* receptor-binding chemicals remained largely unaffected, which was in line with the fact that functional immunological assays did not provide evidence for an aggravated immunosuppression in the Baltic group. The chronic contaminant exposure influenced the endocrine response to fasting stress, as suggested by a lesser increase in serum thyroid hormone levels in the Baltic group. This suggests that marine mammals with high levels of accumulated environmental contaminants may be less able to cope with stressful situations than animals inhabiting less contaminated areas.

Acknowledgements

The authors wish to thank Paul Thompson, Harry Ross, Ineke Lutke-Schipholdt, Henk Loggen, Jos Westendorp, Martin Olling, Henk Derks, Jan Boon and all collaborators of the SRRC and the DLO-IFN for their contributions to these studies. R.L. de Swart and P.S. Ross were partially funded by the Nederlandse Aardolie Maatschappij (NAM) and a Natural Sciences and Engineering Research Council of Canada award, respectively.

Chapter 9

Summarizing discussion. Impaired immunity in harbour seals (Phoca vitulina) exposed to bioaccumulated environmental contaminants: review of a long-term study



Rik L. de Swart, Peter S. Ross, Joseph G. Vos and Albert D.M.E. Osterhaus

Submitted for publication

Abstract

Mass mortalities among seals and dolphins inhabiting contaminated marine regions have led to speculation about a possible involvement of environmental pollution-induced immunosuppression. In order 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 eleven 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 2½ year period. The seals fed contaminated Baltic herring developed significantly higher body burdens of potentially immunotoxic organochlorines, and displayed impaired immune responses as evidenced by suppression of natural killer cell activity and specific T cell responses. During a two week fasting experiment carried out 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, while relatively short-term fasting periods, which are normal for seals, do not seem to pose an additional risk. The seals of this study were not perinatally exposed to high levels of environmental chemicals, and body burdens of organochlorines measured towards the end of the study were lower than those generally observed in free-ranging seals inhabiting many contaminated regions. It may therefore be expected that environmental contaminants adversely affect immune function of free-ranging seals at least as seriously as observed in these studies.

Introduction

In recent years, serious disease outbreaks among seals and dolphins were attributed to infection with established or newly recognized morbilliviruses (for review see 48). 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 approximately 20,000 harbour seals (*Phoca vitulina*) in northwestern Europe[153]. A similar epizootic among Baikal seals (*Phoca sibirica*) in Siberia in 1987 was later attributed to infection with canine distemper virus (CDV)[78,155]. A morbillivirus 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[105,219]. A similar virus, dolphin morbillivirus (DMV), was the primary cause of a mass mortality among striped dolphins (*Stenella coeruleoalba*) in the Mediterranean from 1990-1992[59,207]. These morbillivirus related mass mortalities amongst aquatic mammals led to speculation about a possible involvement of environmental pollution-induced immunosuppression in the severity and extent of these outbreaks[158].

In the early seventies, laboratory animal studies had demonstrated that the mammalian immune system can be a sensitive target for environmentally occurring toxic chemicals[225,226]. This led to the identification of a number of groups of different chemicals with immunotoxic properties. Of these, the polyhalogenated aromatic hydrocarbons (PHAH), including polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs), seemed the most likely candidates for causing immunotoxic effects in the marine environment[228]. The receptor-mediated mechanism elucidated in laboratory animal studies has suggested an additive toxicity of different PHAH congeners, while the most immunotoxic congener, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), was shown to cause immune alterations at levels below 1 μg/kg body weight in certain species[93]. Top predators inhabiting contaminated marine regions carry high body burdens of lipophilic PHAH[121,199], and estimated yearly intakes of these compounds expressed in TCDD toxic equivalents (TEQ) often exceed the 1 μg/kg body weight level[53]. The association of bioaccumulated organochlorine contaminants in marine mammals with adverse biological effects including reproductive failure, endocrinological alterations, developmental irregularities and increased tumour incidence, has been the subject of a considerable number of reports over the last decades[46,96].

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. As a result of the consumption of environmentally contaminated herring, seals 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[53].

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[213]. Results of these studies are then extrapolated to estimate the potential immunotoxicity of these compounds in humans[227]. Additional information on immunotoxic effects for humans may be obtained from events of accidental exposure[93]. 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.

In order 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 realized that extensive information on the immune system and its components as available for rodents and humans is largely lacking in free-ranging animals.

First, mitogen- and antigen-induced proliferative B and T cell responses were established for peripheral blood mononuclear cells (PBMC) isolated from harbour seals. It was shown that concanavalin A (Con A) and pokeweed mitogen (PWM) induced strong

proliferative responses, while phytohaemagglutinin (PHA) and lipopolysaccharide (LPS) induced comparatively low responses[49]. Proliferation of mitogen stimulated PBMC in response to recombinant human interleukin 2 (rhIL-2) and *ex vivo/in vitro* antibody (Ab) production by PBMC were measured to discriminate between T and B cell responses. Con A and PHA were shown to stimulate phocine T cells, PWM stimulated both T and B cells while LPS predominantly stimulated phocine B cells[49]. Antigen-specific immune responses were measured after immunization of seals with rabies virus antigen (RV), tetanus toxoid (TT), keyhole limpet haemocyanin (KLH) or ovalbumin (Ova). Specific proliferative responses, antibodies and antibody forming cells were demonstrated in PBMC cultures of immunized animals[49,51]. Responses measured *ex vivo/in vitro* proved to correlate well with specific serum antibody production *in vivo*. In addition, measurement of one-way mixed lymphocyte (MLR) responses of PBMC was carried out using the harbour seal lymphosarcoma cell line PV1.P1 (ATCC CRL 6526) as irradiated stimulatory cells, following methods routinely applied for humans and rodents[51]. Apart from 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 measured correlated well with results of *ex vivo/in vitro* tests of T lymphocyte function, implicating this cell type in the reaction[173]. Natural killer cells are leucocytes which play an important role in the first line of defence against virus infections. The natural cytotoxic activity of harbour seal PBMC was characterized, and found IL-2 responsive, sensitive to antibody anti-Asialo GM1, and higher against a virus-infected target cell line, like NK cells described for other mammalian species[174].

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 would appear to be the collection of information from free-ranging animals. Although many attempts in this direction have been made, results have proved inconclusive in most cases since experimental design was flawed by unavoidable and uncontrollable factors[3,83,114]. 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.* 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[84]. No differences were found in mortality rates or other virological parameters between PCB-exposed and unexposed animals. The interpretation of the results was seriously confounded by the small sample size and lesser PCB body burdens than those of free-ranging animals inhabiting contaminated areas.

In our experiments, we fed fish originating from two regions with different contamination levels to two groups of young harbour seals caught in a relatively unpolluted area, both diets being originally destined for human consumption. This 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 realized that even in this experimental setup, the duration of exposure was more limited than under natural circumstances, since perinatal exposure was not feasible.

An immunotoxicological feeding study in seals under semifield conditions

Toxicological aspects

During a 2½ 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[53]. The animals had been caught as weaned pups off the relatively uncontaminated northeast coast of Scotland, and were fed on relatively uncontaminated herring for an adaptation period of one year prior to this feeding study. Chemical residue analyses were carried out 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[53]. Persistent compounds such as p,p'-DDT and total PCBs were biomagnified from herring to seal blood and -blubber, while lipid-based levels of aryl hydrocarbon (*Ah*) receptor binding organochlorines (sum TEQ) in seals were not higher than in the fish they were fed[50]. The latter finding confirms that seals have the capacity to metabolize or excrete these compounds[13,55]. 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), while estimated body burdens of sum TEQ totalled 7% and 2.2% of the estimated cumulative intakes by seals fed Atlantic or Baltic herring, respectively (chapter 8, figure 3). However, blubber and blood levels of these chemicals were still a factor three higher in the seals fed Baltic herring (chapter 8, table 1). Generally higher percentages of the total contaminant intakes accumulated in the Atlantic group as compared to the Baltic group, which may have reflected liver enzyme induction and subsequently more efficient biotransformation in the Baltic group[50]. Total PCB levels in the blubber of seals fed Baltic herring were between 15 and 20 mg/kg body weight (chapter 8, table 1), which is relatively low compared to seals inhabiting polluted waters like the Baltic Sea or the Dutch Wadden Sea[70,121].

Immunological aspects

During the course of the 2½ year feeding study, a total of 17 blood samples were taken at regular intervals from the seals of both groups 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[174]. 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 following stimulation with the T cell mitogens Con A and PHA and the T/B cell mitogen PWM were also significantly reduced as 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 two years after the onset of the experiment[51]. MLR-induced PBMC proliferation, reflecting a non-specific immunological response involving a complex sequence

of events involving antigen processing and -presentation, were significantly lower in seals fed Baltic herring[51]. Mean MLR responses from the second half of the experiment were also inversely correlated with total TEQ levels in blubber biopsies. Upon immunization with RV (before the start of the feeding study) and TT (half-way the feeding study), specific proliferative responses of PBMC from the Baltic group were also significantly reduced. Again, these impaired cellular responses were most pronounced towards the end of the experiment, with rabies virus antigen-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 hours after intradermal administration, was significantly lower in the Baltic group. These DTH responses correlated with *ex vivo/in vitro* Con A- and PHA-induced lymphoproliferative responses, but not to PWM- or LPS-induced responses, implicating T cells as effectors.

In contrast with 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[51]. In line with this observation, *ex vivo/in vitro* mitogen-induced total Ab production proved to be unaffected in the Baltic group. Furthermore, primary antigen-specific serum antibody responses to immunization with RV, TT and poliovirus antigen were also not lower in the Baltic group. Serum Ab 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 dimethyldioctadecyl-ammonium bromide (DDA) in this study, which has a major effect on the induction of T helper cell responses. Consequently, the difference observed in these serum Ab titers may be predominantly related to an impaired T cell response in the Baltic group[51].

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 in order to control for such potential indirect effects in our experiments. Haematology and clinical chemistry parameters were monitored longitudinally, as possible indicators of immunotoxic stress, as well as indicators of general health state[52]. 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 haematology profiles. The most striking finding was an increase in neutrophil counts in the Baltic group, which became more pronounced towards the end of the experiment[52]. A summary of the immunological effects observed in the seals fed Baltic herring is shown in table 1.

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 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. In order to test this

Table 1: Summary of differences in immunological parameters between the two groups of seals

parameter	assay	effect	chapter
NK cell	^{51}Cr release assay	↓[1]	3 and 4
T lymphocyte	mitogen-induced proliferation	↓	3 and 5
	antigen-induced proliferation	↓	5
	mixed lymphocyte reaction	↓	5
	delayed type hypersensitivity skin test	↓	6
B lymphocyte	mitogen-induced proliferation	-[2]	3 and 5
	Specific serum antibody responses	-/↓	5 and 6
	Ex vivo/in vitro immunoglobulin production	-	5
overall	Lymphocyte counts in peripheral blood	-	3 and 7
	Neutrophil counts in peripheral blood	↑[3]	3 and 7

[1]significantly lower responses in the seals fed Baltic herring, as compared to the seals fed Atlantic herring; [2]no significant differences over time between the two groups of seals; [3]significantly higher responses in the seals fed Baltic herring, as compared to the seals fed Atlantic herring

hypothesis, the seals of our study were subjected to an experimental two-week fasting directly after the end of the feeding study. The animals of both groups lost an average 11.1 kg, representing 16.5% of their body weights. Metabolization of blubber lipids led to an approximate twofold increase in blood levels of persistent organochlorine compounds, but did not influence blood levels of *Ah* receptor binding organochlorines (chapter 8, figure 3). This is not inconsistent with the observation that few differences in immunological parameters were observed between the Baltic and Atlantic groups. 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 normal ranges. Unexpectedly and unexplained, lymphoproliferative responses of the Atlantic group were reduced after the 15-day fasting. Taken together, our results suggest that relatively short term fasting does not pose a major additional immunotoxic threat to seals with relatively high organochlorine body burdens. Since thyroid hormone responses upon fasting stress were lower in the Baltic group, it may be speculated that marine mammals with high body burdens of environmental chemicals are less capable to cope with stressful situations than animals inhabiting relatively uncontaminated areas.

Conclusions

The experiments described here have underlined the utility of a semifield approach to immunotoxicological studies of complex mixtures of environmentally accumulated contaminants. Although major efforts are needed to adapt functional immunological assays to use in the species under investigation, once established they allow the monitoring of changes in immune function over time in relation to contaminant mixtures as they occur in the natural environment. The numbers of assays used and immune parameters studied in an integrated manner may justify the extrapolation of experimental data to 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 largely reinforced one another, adding 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. For ethical reasons and due to legal restrictions, it was not possible to challenge the seals with PDV or another infectious agent in order 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 case fatality rates.

References

1. Addison RF. Organochlorines and marine mammal reproduction. *Can J Fish Aquat Sci* 46: 360-368 (1989).
2. Aguilar A and Borrell A. Hetrogeneous distribution of organochlorine contaminants in the blubber of Baleen whales: implications for sampling procedures. *Mar Environ Res* 31: 275-286 (1991).
3. Aguilar A and Borrell A. Abnormally high polychlorinated biphenyl levels in striped dolphins (*Stenella coeruleoalba*) affected by the 1990-1992 Mediterranean epizootic. *Sci Total Environ* 154: 237-247 (1994).
4. Ahlborg UG, Becking GC, Birnbaum LS, Brouwer A, Derks HJGM, Feeley M, Golor G, Hanberg A, Larsen JC, Liem AKD, Safe SH, Schlatter C, Waern F, Younes M and Yrjanheikki E. Toxic equivalency factors for dioxin-like PCBs. *Chemosphere* 28: 1049-1067 (1994).
5. Anderson J, Sjöberg O and Möller G. Induction of immunoglobulin and antibody synthesis in vitro by lipopolysaccharides. *Eur J Immunol* 2: 349-353 (1972).
6. Audigé L. Serum biochemical values of rusa deer (*Cervus timorensis russa*) in New Caledonia. *Aust Vet J* 69: 268-271 (1992).
7. Banerjee BD. Effects of sub-chronic DDT exposure on humoral and cell-mediated immune responses in albino rats. *Bull Environ Contam Toxicol* 39: 827-834 (1987).
8. Barrett T, Crowther J, Osterhaus ADME, Subbarao SM, Groen J, Haas L, Mamaev LV, Titenko AM, Visser IKG and Bostock CJ. Molecular and serological studies on the recent seal virus epizootics in Europe and Siberia. *Sci Total Environ* 115: 117-132 (1992).
9. Barrett T, Visser IKG, Mamaev LV, Goatley L, Van Bressem MF and Osterhaus ADME. Dolphin and porpoise morbilliviruses are genetically distinct from phocine distemper virus. *Virology* 193: 1010-1012 (1993).
10. Bengtson JL, Boveng P, Franzen U, Have P, Heide-Jörgensen M-P and Härkönen T. Antibodies to canine distemper virus in antarctic seals. *Mar Mamm Sci* 7: 85-87 (1991).
11. Bergek S, Bergqvist P-A, Hjelt M, Olsson M, Rappe C, Roos A and Zook D. Concentrations of PCDDs and PCDFs in seals from Swedish waters. *Ambio* 21: 553-556 (1992).
12. Bergman A, Olsson M and Reiland S. Skull-bone lesions in the baltic grey seal (*Halichoerus grypus*). *Ambio* 21: 517-519 (1992).
13. Bignert A, Olsson M, Bergqvist P-A, Bergek S, Rappe C, De Wit C and Jansson B. Polychlorinated dibenzo-p-dioxins (PCDD) and dibenzo-furans (PCDF) in seal blubber. *Chemosphere* 19: 551-556 (1989).
14. Biron CA. Cytokines in the generation of immune responses to, and resolution of, virus infection. *Curr Opin Immunol* 6: 530-538 (1994).
15. Biron CA, Byron KS and Sullivan JL. Severe herpesvirus infections in an adult without natural killer cells. *New England J Med* 320: 1731-1735 (1989).
16. Bitman J, Cecil HC, Harris SJ and Fries GF. DDT induces a decrease in eggshell calcium. *Nature* 224: 44-46 (1969).
17. Black FL. Epidemiology of Paramyxoviridae. In: *The paramyxoviruses*, Kingsbury DW (ed), Plenum Press, New York, pp. 509-536 (1991).
18. Black FL. Why did they die? *Science* 258: 1739-1740 (1992).
19. Blixenkrone-Møller M. Biological properties of phocine distemper virus and canine distemper virus. *APMIS* 101: 5-51 (1993).
20. Blomkvist G, Roos A, Jensen S, Bignert A and Olsson M. Concentrations of sDDT and PCB in seals from Swedish and Scottish waters. *Ambio* 21: 539-545 (1992).
21. Boctor FN, Charmy RA and Cooper EL. Seasonal differences in the rhythmicity of human male and female lymphocyte blastogenic responses. *Immunol Invest* 18: 775-784 (1989).
22. Boon JP, Oostingh I, Van der Meer J and Hillebrand MTJ. A model for the bio-accumulation of chlorobiphenyl congeners in marine mammals. *Eur J Pharmacol-Environ Toxicol* 270: 237-251 (1994).
23. Boon JP, Reijnders PJH, Dols J, Wensvoort P and Hillebrand MTJ. The kinetics of individual

polychlorinated biphenyl congeners in female harbour seals (*Phoca vitulina*), with evidence for structure-related metabolism. *Aquat Toxicol* 10: 307-324 (1987).

24. Boos J and Wheelock EF. Correlation of survival from MCMV infection with spleen cell responsiveness to concanavilin A. *Proc Soc Exp Biol Med* 149: 443-446 (1971).
25. Borrow P and Oldstone MBA. Measles virus-mononuclear cell interactions. In: *Measles virus*, Ter Meulen V and Billeter MA (eds), Springer-Verlag, Berlin, pp. 85-100 (1995).
26. Bossart GD and Dierauf LA. Marine mammal clinical laboratory medicine. In: *CRC Handbook of marine mammal medicine: health, disease and rehabilitation*, Dierauf LA (ed), CRC Press, Boca Raton, pp. 1-52 (1990).
27. Bowen WD, Oftedal OT and Boness DJ. Mass and energy transfer during lactation in a small phocid, the harbor seal (*Phoca vitulina*). *Physiol Zool* 65: 844-866 (1992).
28. Brouwer A, Reijnders PJH and Koeman JH. Polychlorinated biphenyl (PCB)- contaminated fish induces vitamin A and thyroid hormone deficiency in the common seal (*Phoca vitulina*). *Aquat Toxicol* 15: 99-106 (1989).
29. Brouwer A and Van den Berg KJ. Binding of a metabolite of 3,4,3',4'- tetrachlorobiphenyl to transthyretin reduces serum vitamin A transport by inhibiting the formation of the protein complex carrying both retinol and thyroxin. *Toxicol Appl Pharmacol* 85: 301-312 (1986).
30. Bukowski JF and Welsh RM. Inability of interferon to protect virus-infected cells against lysis by natural killer (NK) cells correlates with NK cell-mediated antiviral effects in vivo. *J Immunol* 135: 3537-3541 (1985).
31. Campos M, Rossi CR, Ohmann HB, Beskorwayne T, Rapin N and Babiuk LA. Characterization and activation requirements of bovine lymphocytes acquiring cytotoxic activity after interleukin-2 treatment. *Vet Immunol Immunopathol* 32: 205-223 (1992).
32. Carter SD, Hughes DE and Baker JR. Characterization and measurement of immuno-globulins in the grey seal (*Halichoerus grypus*). *J Comp Pathol* 102: 13-23 (1990).
33. Casali P and Trinchieri G. Natural killer cells in viral infection. In: *Concepts in viral pathogenesis*, Notkins AL and Oldstone MBA (eds), Springer-Verlag, New York, pp. 11-19 (1984).
34. Castellini MA and Rea LD. The biochemistry of natural fasting at its limits. *Experientia* 48: 575-582 (1992).
35. Cavagnolo RZ. The immunology of marine mammals. *Develop Comp Immunol* 3: 245-257 (1979).
36. Chambers WH, Vujanovic AB, DeLeo AB, Olszowy MW, Herberman RB and Hiserodt JC. Monoclonal antibody to a triggering structure expressed on rat natural killer cells and adherent lymphokine-activated killer cells. *J Exp Med* 169: 1373-1389 (1989).
37. Chandra RK and Kumari S. Nutrition and immunity: an overview. *J Nutr* 124: 1433S- 1435S (1994).
38. Chong YC, Duffus WPH and Hannant D. Natural killer cells in normal horses and specific pathogen-free foals infected with equine herpesvirus. *Vet Immunol Immunopathol* 33: 103-113 (1992).
39. Colgrove GS. Stimulation of lymphocytes from a dolphin (*Tursiops truncatus*) by phytomitogens. *Am J Vet Res* 39: 141-144 (1978).
40. Colmenares C and Lopez C. Enhanced lysis of herpes simplex virus type 1-infected mouse cell lines by NC and NK effectors. *J Immunol* 136: 3473-3480 (1986).
41. Cornacoff JB, Lauer LD, House RV, Tucker AN, Thurmond LM, Vos JG, Working PK and Dean JH. Evaluation of the immunotoxicity of β-hexachlorocyclohexane (β-HCH). *Fundam Appl Toxicol* 11: 293-299 (1988).
42. Cosby SL, McQuaid S, Duffy N, Lyons C, Rima BK, Allan GM, McCullough SJ, Kennedy S, Smyth JA, McNeilly F, Craig C and Örvell C. Characterization of a seal morbillivirus. *Nature* 336: 115-116 (1988).
43. Costa DP. Reproduction and foraging energetics of pinnipeds: implications for life history patterns. In: *The behaviour of pinnipeds*, Renouf D (ed), Chapman and Hall, London, pp. 301-344 (1995).
44. Costa DP, Le Boeuf BJ, Huntley AC and Ortiz CL. The energetics of lactation in the northern elephant seal, *Mirounga angustirostris*. *J Zool* 209: 21-33 (1986).
45. Costa DP and Ortiz CL. Blood chemistry homeostasis during prolonged fasting in the northern elephant seal. *Am J Physiol* 242: R591-R595 (1982).

46. De Guise S, Lagacé A and Béland P. Tumors in St Lawrence beluga whales (*Delphinapterus leucas*). *Vet Pathol* 31: 444-449 (1994).
47. De Jong WH, Steerenberg PA, Ursem PS, Osterhaus ADME, Vos JG and Ruitenberg EJ. The athymic nude rat: III. Natural cell-mediated cytotoxicity. *Clin Immunol Immunopathol* 17: 163-172 (1980).
48. De Swart RL, Harder TC, Ross PS, Vos HW and Osterhaus ADME. Morbilliviruses and morbillivirus diseases of marine mammals. *Infect Agent Dis*, in press.
49. De Swart RL, Kluten RMG, Huizing CJ, Vedder LJ, Reijnders PJH, Visser IKG, UytdeHaag FGCM and Osterhaus ADME. Mitogen and antigen induced B and T cell responses of peripheral blood mononuclear cells from the harbour seal (*Phoca vitulina*). *Vet Immunol Immunopathol* 37: 217-230 (1993).
50. De Swart RL, Ross PS, Timmerman HH, Hijman WC, De Ruiter E, Liem AKD, Brouwer A, Van Loveren H, Reijnders PJH, Vos JG and Osterhaus ADME. Short-term fasting does not aggravate immunosuppression in harbour seals (*Phoca vitulina*) with high body burdens of organochlorines. *submitted*
51. De Swart RL, Ross PS, Timmerman HH, Vos HW, Reijnders PJH, Vos JG and Osterhaus ADME. Impaired cellular immune response in harbour seals (*Phoca vitulina*) feeding on environmentally contaminated herring. *Clin Exp Immunol*, in press.
52. De Swart RL, Ross PS, Vedder LJ, Boink FBTJ, Reijnders PJH, Mulder PGH and Osterhaus ADME. Haematology and clinical chemistry values of harbour seals (*Phoca vitulina*) fed environmentally contaminated herring remain within normal ranges. *submitted*
53. De Swart RL, Ross PS, Vedder LJ, Timmerman HH, Heisterkamp SH, Van Loveren H, Vos JG, Reijnders PJH and Osterhaus ADME. Impairment of immune function in harbor seals (*Phoca vitulina*) feeding on fish from polluted waters. *Ambio* 23: 155-159 (1994).
54. De Vries P, UytdeHaag FGCM and Osterhaus ADME. Canine distemper virus (CDV) immune-stimulating complexes (Iscoms), but not measles virus iscoms, protect dogs against CDV infection. *J Gen Virol* 69: 2071-2083 (1988).
55. De Wit C, Jansson B, Bergek S, Hjelt M, Rappe C, Olsson M and Andersson O. Polychlorinated dibenzo-p-dioxin and polychlorinated dibenzofuran levels and patterns in fish and fish-eating wildlife in the Baltic Sea. *Chemosphere* 25: 185-188 (1992).
56. Dietz R, Ansen CT and Have P. Clue to seal epizootic? *Nature* 338: 627 (1989).
57. Dietz R, Heide-Jörgensen M-P and Härkönen T. Mass deaths of harbor seals (*Phoca vitulina*) in Europe. *Ambio* 18: 258-264 (1989).
58. Djeu JY, Heinbaugh JA, Holden HT and Herberman RB. Augmentation of mouse natural killer cell activity by interferon and interferon inducers. *J Immunol* 122: 175-181 (1979).
59. Domingo M, Ferrer L, Pumarola M, Marco A, Plana J, Kennedy S, McAlisky M and Rima BK. Morbillivirus in dolphins. *Nature* 348: 21 (1990).
60. Domingo M, Visa J, Pumarola M, Marco AJ, Ferrer L, Rabanal R and Kennedy S. Pathological and immunocytochemical studies of morbillivirus infection in striped dolphins (*Stenella coeruleoalba*). *Vet Pathol* 29: 1-10 (1992).
61. Domok I and Magrath DI. Guide to poliovirus isolation and serological techniques for polyomyelitis surveillance. *WHO Offset Publication* 46: (1979).
62. Driskell WJ, Bashor MM and Neese JW. Beta-carotene determined in serum by liquid chromatography with an internal standard. *Clin Chem* 29: 1042-1044 (1983).
63. Duignan PJ, Sadove S, Saliki JT and Geraci JR. Phocine distemper in harbor seals (*Phoca vitulina*) from Long Island, New-York. *J Wildl Dis* 29: 465-469 (1993).
64. Duignan PJ, Saliki JT, St.Aubin DJ, House JA and Geraci JR. Neutralizing antibodies to phocine distemper virus in Atlantic walruses (*Odobenus rosmarus rosmarus*) from Arctic Canada. *J Wildl Dis* 30: 90-94 (1994).
65. Eis D. Simplification in the etiology of recent seal deaths. *Ambio* 18: 144 (1989).
66. Engelhardt FR. Haematology and plasma chemistry of captive pinnipeds and cetaceans. *Aquat Mammals* 7: 11-20 (1979).
67. Engelhardt FR and Ferguson JM. Adaptive hormone changes in harp seals, Phoca groenlandica, and gray

seals, *Halichoerus grypus*, during the postnatal period. *Gen Comp Endocrinol* 40: 434-445 (1980).
68. Erdman DD, Anderson LJ, Adams DR, Stewart JA, Markowitz LE and Bellini WJ. Evaluation of monoclonal antibody-based capture enzyme immunoassays for detection of specific antibodies to measles virus. *J Clin Microbiol* 29: 1466-1471 (1991).
69. Esolen LM, Ward BJ, Moench TR and Griffin DE. Infection of monocytes during measles. *J Infect Dis* 168: 47-52 (1993).
70. Falandysz J, Yamashita N, Tanabe S, Tatsukawa R, Rucinska L and Skora K. Congener-specific data on polychlorinated biphenyls in tissues of common porpoise from Puck Bay, Baltic Sea. *Arch Environ Contam Toxicol* 26: 267-272 (1994).
71. Falkoff R. Maternal immunologic changes during pregnancy: a critical appraisal. *Clin Rev Allergy* 5: 287-300 (1987).
72. Finberg R and Benacerraf B. Induction, control and consequences of virus specific cytotoxic T cells. *Immunological Rev* 58: 157-180 (1981).
73. Fournier M, Chevalier G, Nadeau D, Trottier B and Krzystyniak K. Virus-pesticide interactions with murine cellular immunity after sublethal exposure to dieldrin and aminocarb. *J Toxicol Environ Health* 25: 103-118 (1988).
74. Fox GA, Collins B, Hayakawa E, Weseloh DV, Ludwig JP, Kubiak TJ and Erdman TC. Reproductive outcomes in colonial fish-eating birds: a biomarker for developmental toxicants in Great Lakes food chains: spatial variation in the occurrence and prevalence of bill defects in young double-crested cormorants in the Great Lakes, 1979 - 1987. *J Great Lakes Res* 17: 158-167 (1991).
75. Funseth E and Ilbäck NG. Effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on blood and spleen natural killer (NK) cell activity in the mouse. *Toxicol Lett* 60: 247-256 (1992).
76. Geraci JR and Medway W. Simulated field blood studies in the bottle-nosed dolphin *Tursiops truncatus*. 2. Effects of stress on some hematologic and plasma chemical parameters. *J Wildl Dis* 9: 29-33 (1973).
77. Gilmartin WG, Delong RL, Smith AW, Sweeney JC, De Lappe BW, Risebrough RW, Griner LA, Dailey MD and Peakall DB. Premature parturition in the california sea lion. *J Wildl Dis* 12: 104-115 (1976).
78. Grachev MA, Kumarev VP, Mamaev LV, Zorin VL, Baranova LV, Denikjna NN, Belikov SI, Petrov EA, Kolesnik VS, Kolesnik RS, Dorofeev VM, Beim AM, Kudelin VN, Nagieva FG and Sidorov VN. Distemper virus in Baikal seals. *Nature* 338: 209 (1989).
79. Greaves M, Janossy G and Doenhoff M. Selective triggering of human T and B lymphocytes in vitro by polyclonal mitogens. *J Exp Med* 140: 1-18 (1974).
80. Griffin DE. Immune responses during measles virus infection. In: *Measles virus*, Ter Meulen V and Billeter MA (eds), Springer-Verlag, Berlin, pp. 117-134 (1995).
81. Gupta RK, Relyveld EH, Lindblad EB, Bizzini B, Ben-Efraim S and Gupta CK. Adjuvants - a balance between toxicity and adjuvanticity. *Vaccine* 11: 293-306 (1993).
82. Haahti H and Perttilä M. Levels and trends of organochlorines in cod and herring in the northern Baltic. *Mar Pollut Bull* 19: 29-32 (1988).
83. Hall AJ, Law RJ, Harwood J, Ross HM, Kennedy S, Allchin CR, Campbell LA and Pomeroy PP. Organochlorine levels in common seals (*Phoca vitulina*) which were victims and survivors of the 1988 phocine distemper epizootic. *Sci Total Environ* 115: 145-162 (1992).
84. Harder TC, Willhaus T, Leibold W and Liess B. Investigations on course and outcome of phocine distemper virus infection in harbour seals (*Phoca vitulina*) exposed to polychlorinated biphenyls. *J Vet Med B* 39: 19-31 (1992).
85. Helle E, Olsson M and Jensen S. PCB levels correlated with pathological changes in seal uteri. *Ambio* 5: 261-263 (1976).
86. Helle E, Olsson M and Jensen S. DDT and PCB levels and reproduction in ringed seal from the Bothnian Bay. *Ambio* 5: 188-189 (1976).
87. Henderson G, Trudgett A, Lyons C and Ronald K. Demonstration of antibodies in archival sera from Canadian seals reactive with a European isolate of phocine distemper virus. *Sci Total Environ* 115: 93-98 (1992).
88. Henney CS, Kuribayashi K, Kern DE and Gillis S. Interleukin-2 augments natural killer cell activity.

Nature 291: 335-338 (1981).
89. Herberman RB, Reynolds CW and Ortaldo JR. Mechanism of cytotoxicity by natural killer (NK) cells. *Ann Rev Immunol* 4: 651-680 (1986).
90. Hickey JJ and Anderson DW. Chlorinated hydrocarbons and eggshell changes in raptorial and fish-eating birds. *Science* 162: 271-273 (1968).
91. Hilgers LAT and Snippe H. DDA as an immunological adjuvant. *Ann Inst Pasteur/Immunol* 143: 494-503 (1992).
92. Holsapple MP, Morris DL, Wood SC and Snyder NK. 2,3,7,8-tetrachloro-dibenzo-p-dioxin-induced changes in immunocompetence: possible mechanisms. *Annu Rev Pharmacol Toxicol* 31: 73-100 (1991).
93. Holsapple MP, Snyder NK, Wood SC and Morris DL. A review of 2,3,7,8,-tetrachlorodibenzo-p-dioxin-induced changes in immunocompetence: 1991 update. *Toxicology* 69: 219-255 (1991).
94. House RV, Lauer LD, Murray MJ, Thomas PT, Ehrlich JP, Burleson GR and Dean JH. Examination of immune parameters and host resistance mechanisms in B6C3F1 mice following adult exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *J Tox Env Health* 31: 203-215 (1990).
95. Humphrey HEB. The human population - an ultimate receptor for aquatic con-taminants. *Hydrobiologica* 149: 75-80 (1987).
96. Hutchinson JD and Simmonds MP. Organochlorine contamination in pinnipeds. *Rev Environ Contam Toxicol* 136: 123-168 (1994).
97. Ikeda S, Tominaga T and Nishimura C. Thy 1+ asialo GM1+ dendritic epidermal cells in skin defense mechanisms of vaccinia virus-infected mice. *Arch Virol* 117: 207-218 (1991).
98. Ilbäck NG, Sundberg J and Oskarsson A. Methyl mercury exposure via placenta and milk impairs natural killer (NK) cell function in newborn rats. *Toxicol Lett* 58: 149-158 (1991).
99. Jain NC. *Schalm's veterinary hematology*. Lea & Febiger, Philadelphia (1986).
100. John TM, Ronald K and George JC. Blood levels of thyroid hormones and certain metabolites in relation to moult in the harp seal (*Phoca groenlandica*). *Comp Biochem Physiol [A]* 88: 655-657 (1987).
101. Kannan K, Tanabe S, Borrell A, Aguilar A, Focardi S and Tatsukawa R. Isomer-specific analysis and toxic evaluation of polychlorinated biphenyls in striped dolphins affected by an epizootic in the western Mediterranean Sea. *Arch Environ Contam Toxicol* 25: 227-233 (1993).
102. Kasai M, Yoneda T, Habu S, Maruyama Y, Okumura K and Tokunaga T. In vivo effect of anti-asialo GM1 antibody on natural killer activity. *Nature* 291: 334-335 (1981).
103. Katz M. Clinical spectrum of measles. In: *Measles virus*, Ter Meulen V and Billeter MA (eds), Springer-Verlag, Berlin, pp. 1-12 (1995).
104. Keller LH, Lillehoj HS and Solnosky JM. JMV-1 stimulation of avian natural killer cell activity. *Avian Pathol* 21: 239-250 (1992).
105. Kennedy S, Smyth JA, McCullough SJ, Allan GM and McQuaid S. Viral distemper now found in porpoises. *Nature* 336: 21 (1988).
106. Khansari DN, Murgo AJ and Faith RE. Effects of stress on the immune system. *Immunol Today* 11: 170-175 (1990).
107. King DP, Hay AWM, Robinson I and Evans SW. The use of monoclonal antibodies specific for seal immunoglobulins in an enzyme-linked immunosorbent assay to detect canine distemper virus-specific immunoglobulin in seal plasma samples. *J Immunol Methods* 160: 163-171 (1993).
108. King DP, Lowe KA, Hay AWM and Evans SW. Identification, characterisation, and measurement of immunoglobulin concentrations in grey (*Haliocherus grypus*) and common (*Phoca vitulina*) seals. *Dev Comp Immunol* 18: 433-442 (1994).
109. King DP, Robinson I, Hay AWM and Evans SW. Identification and partial characterization of common seal (Phoca vitulina) and grey seal (*Halichoerus grypus*) interleukin-6-like activities. *Develop Comp Immunol*, in press.
110. Knapp DW, Leibnitz RR, DeNicola DB, Turek JJ, Teclaw R, Shafer L and Chan TCK. Measurement of NK activity in effector cells purified from canine peripheral lymphocytes. *Vet Immunol Immunopathol* 35: 239-251 (1993).
111. Koeman JH, Noever de Brauw MC and Vos RH. Chlorinated biphenyls in fish, mussels and birds from

the River Rhine and the Netherlands coastal area. *Nature* 221: 1126-1128 (1969).
112. Krakowka S, Higgins RJ and Koestner A. Canine distemper virus: review of structural and functional modulations in lymphoid tissues. *Am J Vet Res* 41: 284-292 (1980).
113. Kuehl DW, Haebler R and Potter C. Chemical residues in dolphins from the U.S. Atlantic coast including atlantic bottlenose obtained during the 1987/1988 mass mortality. *Chemosphere* 22: 1071-1084 (1991).
114. Kuiken T, Bennett PM, Allchin CR, Kirkwood JK, Baker JR, Lockyer CH, Walton MJ and Sheldrick MC. PCBs, cause of death and body condition in harbour porpoises (*Phocoena phocoena*) from British waters. *Aquat Toxicol* 28: 13-28 (1994).
115. Lahvis GP, Wells RS, Casper D and Via CS. In vitro lymphocyte response of bottlenose dolphins (*Tursiops truncatus*): mitogen induced proliferation. *Mar Environ Res* 35: 115-119 (1993).
116. Lavigne DM and Schmitz OJ. Global warming and increasing population densities: a prescription for seal plagues. *Mar Pollut Bull* 21: 280-284 (1990).
117. Lewis CE and McGee JOD. Natural killer cells in tumour biology. In: *The natural killer cell*, Lewis CE and McGee JOD (eds), IRL Press, Oxford, pp. 175-203 (1992).
118. Liem AKD, De Jong APJM, Marsman JA, Den Boer AC, Groenemijer GS, Den Hartog RS, De Korte GAL, Hoogerbrugge R, Kootstra PR and Van 't Klooster HA. A rapid clean-up procedure for the analysis of polychlorinated dibenzo-*p*-dioxins and dibenzofurans in milk samples. *Chemosphere* 20: 843-850 (1990).
119. Lipscomb TP, Schulman FY, Moffett D and Kennedy S. Morbilliviral disease in Atlantic bottlenose dolphins (*Tursiops truncatus*) from the 1987-1988 epizootic. *J Wildlife Dis* 30: 567-571 (1994).
120. Loose LD, Silkworth JB, Pittman KA, Benitz KF and Mueller W. Impaired host resistance to endotoxin and malaria in polychlorinated biphenyl- and hexachlorobenzene-treatedmice. *Infect Immun* 1978: 30-35 (1978).
121. Luckas B, Vetter W, Fischer P, Heidemann G and Plötz J. Characteristic chlorinated hydrocarbon patterns in the blubber of seals from different marine regions. *Chemosphere* 21: 13-19 (1990).
122. Luster MI, Blank JA and Dean JH. Molecular and cellular basis of chemically induced immunotoxicity. *Annu Rev Pharmacol Toxicol* 27: 23-49 (1987).
123. Luster MI and Rosenthal GJ. Chemical agents and the immune response. *Environ Health Perspect* 100: 219-236 (1993).
124. Maestroni GJM, Conti A and Pierpaoli W. Role of the pineal gland in immunity: II. Melatonin enhances the antibody response via an opiatergic mechanism. *Clin Exp Immunol* 68: 384-391 (1987).
125. Mahy BWJ, Barrett T, Evans S, Anderson EC and Bostock CJ. Characterization of a seal morbillivirus. *Nature* 336: 115-116 (1988).
126. Makinodan T, James SJ, Inamizu T and Chang MP. Immunologic basis for susceptibility to infection in the aged. *Gerontology* 30: 279-289 (1984).
127. Mamaev LV, Visser IKG, Harder TC, Belikov SI, Denikina N, Liess B, Osterhaus ADME and Barrett T. Characterization of morbilliviruses in Lake Baikal seals (*Phoca sibirica*). *J Gen Virol*, in press.
128. Markussen NH, Ryg M and Oritsland NA. Metabolic rate and body composition of harbour seals, *Phoca vitulina*, during starvation and refeeding. *Can J Zool* 70: 220-224 (1992).
129. Martineau D, Béland P, Desjardins C and Lagacé A. Levels of organochlorine chemicals in tissues of beluga whales (*Delphinapterus leucas*) from the St. Lawrence Estuary, Québec, Canada. *Arch Environ Contam Toxicol* 16: 137-147 (1987).
130. Martineau D, De Guise S, Fournier M, Shugart L, Girard C, Lagacé A and Béland P. Pathology and toxicology of beluga whales from the St-Lawrence estuary, Quebec, Canada - past, present and future. *Sci Total Environ* 154: 201-215 (1994).
131. McChesney MB and Oldstone MB. Virus-induced immunosuppression: infections with measles virus and human immunodeficiency virus. *Adv Immunology* 45: 335-380 (1989).
132. McCullough SJ, McNeilly F, Allan GM, Kennedy S, Smyth JA, Cosby SL, McQuaid S and Rima BK. Isolation and characterization of a porpoise morbillivirus. *Arch Virol* 118: 247-252 (1991).
133. Mead R and Curnow RN. *Statistical methods in agriculture and experimental biology*. Chapman and Hall, London, New York (1983).

134. Michie CA, McLean A, Alcock C and Beverley PCL. Lifespan of human lymphocyte subsets defined by CD 45 isoforms. *Nature* 360: 264-265 (1992).
135. Mims CA and White DO. *Viral Pathogenesis and Immunology*. Blackwell Scientific Publications, Oxford (1984).
136. Mortensen P, Bergman A, Bignert A, Hansen H-J, Härkönen T and Olsson M. Prevalence of skull lesions in harbor seals (*Phoca vitulina*) in Swedish and Danish museum collections: 1835 - 1988. *Ambio* 21: 520-524 (1992).
137. Mosier DE. Consequences of secondary or co-infections for immunity. *Curr Opin Immunol* 6: 539-544 (1994).
138. Möller G. Lymphocyte activation by mitogens. *Transplant Rev* 11: 1-267 (1972).
139. Muir DCG, Wagemann R, Grift NP, Norstrom RJ, Simon M and Lien J. Organochlorine chemical and heavy metal contaminants in white-beaked dolphins (Lagenorhynchus albirostris) and pilot whales (*Globicephala melaena*) from the coast of Newfoundland, Canada. *Arch Environ Contam Toxicol* 17: 613-629 (1989).
140. Mumford DM, Stockman GD, Barsales PB, Whitman T and Wilbur JR. Lymphocyte transformation studies of sea mammal blood. *Experientia* 31: 498-500 (1975).
141. Murk AJ, Bosveld ATC, van den Berg M and Brouwer A. Effects of polyhalogenated aromatic hydrocarbons (PHAHs) on biochemical parameters in chicks of the common tern (*Sterna hirundo*). *Aquat Toxicol* 30: 91-115 (1994).
142. Nash DR and Mach J-P. Immunoglobulin classes in aquatic mammals. *J Immunol* 107: 1424-1430 (1971).
143. Nordoy ES, Aakvaag A and Larsen TS. Metabolic adaptations to fasting in harp seal pups. *Physiol Zool* 66: 926-945 (1993).
144. Nordoy ES, Ingebretsen OC and Blix AS. Depressed metabolism and low protein catabolism in fasting grey seal pups. *Acta Physiol Scand* 139: 361-369 (1990).
145. Norrby E and Oxman MN. Measles virus. In: *Virology*, Fields BN and Knipe DM (eds), Raven Press, New York, pp. 1013-1044 (1990).
146. O'Brien SJ, Roelke ME, Marker L, Newman A, Winkler CA, Meltzer D, Colly L, Evermann JF, Bush M and Wildt DE. Genetic basis for species vulnerability in the cheetah. *Science* 227: 1428-1434 (1985).
147. O'Shea J and Ortaldo JR. The biology of natural killer cells: insight into the molecular basis of function. In: *The natural killer cell*, Lewis CE and McGee JOD (eds), IRL Press, Oxford, pp. 1-40 (1992).
148. Olsson M, Andersson O, Bergman A, Blomkvist G, Frank A and Rappe C. Contaminants and diseases in seals from Swedish waters. *Ambio* 21: 561-562 (1992).
149. Olsson M, Karlsson B and Ahnland E. Diseases and environmental contaminants in seals from the Baltic and the Swedish west coast. *Sci Total Environ* 154: 217-227 (1994).
150. Olsson M and Reutergardh L. DDT and PCB pollution trends in the Swedish aquatic environment. *Ambio* 15: 103-109 (1986).
151. Ortiz CL, Costa DP and Le Boeuf BJ. Water and energy flux in elephant seal pups fasting under natural conditions. *Physiol Zool* 51: 166-178 (1978).
152. Osterhaus ADME, De Swart RL, Vos HW, Verhoef J and Zarnke RL. Prevalence of canine distemper virus and phocine distemper virus specific serum antibodies in marine mammals from Alaska and russia, 1978 - 1994. *submitted*
153. Osterhaus ADME, Groen J, De Vries P, UytdeHaag FGCM, Klingeborn B and Zarnke R. Canine distemper virus in seals. *Nature* 335: 403-404 (1988).
154. Osterhaus ADME, Groen J, Spijkers HEM, Broeders HWJ, UytdeHaag FGCM, De Vries P, Teppema JS, Visser IKG, Van de Bildt MWG and Vedder EJ. Mass mortality in seals caused by a newly discovered morbillivirus. *Vet Microbiol* 23: 343-350 (1990).
155. Osterhaus ADME, Groen J, UytdeHaag FGCM, Visser IKG, Van de Bildt MWG, Bergman A and Klingeborn B. Distemper virus in Baikal seals. *Nature* 338: 209-210 (1989).
156. Osterhaus ADME, UytdeHaag FGCM, Visser IKG, Vedder EJ, Reijnders PJH, Kuiper J and Brugge HN. Seal vaccination success. *Nature* 337: 21 (1989).

157. Osterhaus ADME and Vedder EJ. Identification of virus causing recent seal deaths. *Nature* 335: 20 (1988).
158. Osterhaus ADME and Vedder EJ. No simplification in the etiology of recent seal deaths. *Ambio* 18: 297-298 (1989).
159. Peakall DB. Thyroid function, retinols, haem and regulatory enzymes. In: *Animal biomarkers as pollution indicators*, Depledge MH and Sanders B (eds), Chapman & Hall, London, pp. 108-154 (1993).
160. Rabin BS, Cohen S, Ganguli R, Lysle DT and Cunnick JE. Bidirectional interaction between the central nervous system and the immune system. *Crit Rev Immunol* 9: 279-312 (1989).
161. Rea LD and Costa DP. Changes in standard metabolism during long-term fasting in northern elephant seal pups (*Mirounga angustirostris*). *Physiol Zool* 65: 97-111 (1992).
162. Reijnders PJH. Organochlorine and heavy metal residues in harbour seals from the Wadden Sea and their possible effects on reproduction. *Neth J Sea Res* 14: 30-65 (1980).
163. Reijnders PJH. Reproductive failure in common seals feeding on fish from polluted coastal waters. *Nature* 324: 456-457 (1986).
164. Reijnders PJH. Ecotoxicological perspectives in marine mammalogy: Research principles and goals for a conservation policy. *Mar Mamm Sci* 4: 91-102 (1988).
165. Richerson JT and Misfeldt ML. Host environment as a modulating factor of swine natural killer cell activity. *Vet Immunol Immunopathol* 23: 309-319 (1989).
166. Rietkerk FE, Delima EC and Mubarak SM. The hematological profile of the mountain gazelle (*Gazella gazella*): variations with sex, age, capture method, season, and anesthesia. *J Wildl Dis* 30: 69-76 (1994).
167. Risebrough RW, Pieche P, Peakall DB, Herman SG and Kirven MN. Polychlorinated biphenyls in the global ecosystem. *Nature* 220: 1098-1102 (1968).
168. Riviere JE, Engelhardt FR and Solomon J. The relationship of thyroxine and cortisol to the moult of the harbor seal, *Phoca vitulina*. *Gen Comp Endocrinol* 31: 398-401 (1977).
169. Roletto J. Hematology and serum chemistry values for clinically healthy and sick pinnipeds. *J Zoo Wildlife Med* 24: 145-157 (1993).
170. Roots O and Aps R. Polychlorinated biphenyls and organochlorine pesticides in Baltic herring and sprat. *Toxicol Environ Chem* 37: 195-205 (1993).
171. Ross PS. Immunocompetence of free-ranging harbour seal (*Phoca vitulina*) mothers and their pups over the course of lactation. In: *MSc Thesis, Dalhousie University, Halifax, Nova Scotia, Canada*, pp. 1-75 (1990).
172. Ross PS. *Seals, pollution and disease: environmental contaminant induced immuno-suppression*. Utrecht University, Utrecht (1995).
173. Ross PS, De Swart RL, Reijnders PJH, Van Loveren H, Vos JG and Osterhaus ADME. Contaminant-related suppression of delayed-type hypersensitivity and antibody responses in harbor seals fed herring from the Baltic Sea. *Environ Health Perspect* 103: 162-167 (1995).
174. Ross PS, De Swart RL, Timmerman HH, Vedder LJ, Van Loveren H, Vos JG, Reijnders PJH and Osterhaus ADME. Suppression of natural killer cell activity in harbour seals (*Phoca vitulina*) fed Baltic Sea herring. *submitted*
175. Ross PS, De Swart RL, Visser IKG, Murk W, Bowen WD and Osterhaus ADME. Relative immunocompetence of the newborn harbour seal, Phoca vitulina. *Vet Immunol Immunopathol* 42: 331-348 (1994).
176. Ross PS, Pohajdak B, Bowen WD and Addison RF. Immune function in free-ranging harbor seal (*Phoca vitulina*) mothers and their pups during lactation. *J Wildl Dis* 29: 21-29 (1993).
177. Ross PS, Visser IKG, Broeders HWJ, Van de Bildt MWG, Bowen WD and Osterhaus ADME. Antibodies to phocine distemper virus in Canadian seals. *Vet Rec* 130: 514-516 (1992).
178. Saboori AM and Newcombe DS. Environmental chemicals with immunotoxic properties. In: *Clinical immunotoxicology*, Newcombe DS, Rose NR and Bloom JC (eds), Raven press, New York, pp. 365-400 (1992).
179. Safe S. Polychlorinated biphenyls (PCBs), Dibenzo-p-dioxins (PCDDs), Dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the development of toxic equivalency factors (TEFs). *CRC Crit Rev Toxicol* 21: 51-88 (1990).

180. Safe SH. Polychlorinated biphenyls (PCBs) - environmental impact, biochemical and toxic responses, and implications for risk assessment. *Crit Rev Toxicol* 24: 87-149 (1994).
181. Sarokin D and Schulkin J. The role of pollution in large-scale population disturbances. 1. Aquatic populations. *Environ Sci Technol* 26: 1476-1484 (1992).
182. Schumacher U and Welsch U. Histological, histochemical, and fine structural observations on the spleen of seals. *Am J Anat* 179: 356-368 (1987).
183. Schuurman HJ, Krajnc-Franken MAM, Kuper CF, Van Loveren H and Vos JG. The immune system. In: *Handbook of Toxicologic Pathology*, Haschek-Hock WM (ed), Academic Press, New York, pp. 421-487 (1991).
184. Sedgwick JD and Holt PG. A solid phase immunoenzymatic technique for the enumeration of specific antibody-secreting cells. *J Immunol Methods* 57: 301-309 (1983).
185. Shellam GR, Allan JE, Papadimitriou JM and Bancroft GJ. Increased susceptibility to cytomegalovirus infection in beige mutant mice. *Proc Natl Acad Sci USA* 78: 5104-5108 (1981).
186. Silkworth JB and Grabstein EM. Polychlorinated biphenyl immunotoxicity: dependence on isomer planarity and the *Ah* gene complex. *Toxicol Appl Pharmacol* 65: 109-115 (1982).
187. Simmonds MP and Johnston PA. Seals, sense, and science. *Mar Pollut Bull* 20: 580-584 (1989).
188. Simmonds MP, Johnston PA and French MC. Organochlorine and mercury contamination in United Kingdom seals. *Vet Rec* 132: 291-295 (1993).
189. Skaare JU, Markussen NH, Norheim G, Haugen S and Holt G. Levels of polychlorinated biphenyls, organochlorine pesticides, mercury, cadmium, copper, selenium, arsenic, and zinc in the harbour seal, *Phoca vitulina*, in Norwegian waters. *Environ Pollut* (1992).
190. Stein-Streilen J and Guffee J. *In vivo* treatment of mice and hamsters with antibodies to asialo GM1 increases morbidity and mortality to pulmonary influenza infection. *J Immunol* 4: 1435-1441 (1986).
191. Stuen S, Arnemo JM, Have P, Osterhaus ADME and Moustgaard A. Serological investigation of virus infections in harp seals (Phoca groenlandica) and hooded seals (*Cystophora cristata*). *Vet Rec* 134: 502-503 (1994).
192. Subramanian A, Tanabe S, Tatsukawa R, Saito S and Miyazaki N. Reduction in the testosterone levels by PCBs and DDE in Dall's porpoises of northwestern North Pacific. *Mar Pollut Bull* 18: 643-646 (1987).
193. Svensson BG, Hallberg T, Nilsson A, Schutz A and Hagmar L. Parameters of immunological competence in subjects with high consumption of fish contaminated with persistent organochlorine compounds. *Int Arch Occup Environ Health* 65: 351-358 (1994).
194. Svensson BG, Nilsson A, Hansson M, Rappe C, Akesson B and Skerfving S. Exposure to dioxins and dibenzofurans through the consumption of fish. *N Engl J Med* 324: 8-12 (1991).
195. Talcott PA, Koller LD and Exon JH. The effect of lead and polychlorinated biphenyl exposure on rat natural killer cell cytotoxicity. *Int J Immunopharmacol* 7: 255-261 (1985).
196. Tanabe S, Iwata H and Tatsukawa R. Global contamination by persistent organochlorines and their ecotoxicological impact on marine mammals. *Sci Total Environ* 154: 163-177 (1994).
197. Tanabe S, Kannan N, Subramanian A, Watanabe S and Tatsukawa R. Highly toxic coplanar PCBs: occurrence, source, persistency and toxic implications to wildlife and humans. *Environ Pollut* 47: 147-163 (1987).
198. Tanabe S, Loganathan BG, Subramanian A and Tatsukawa R. Organochlorine residues in short-finned pilot whale. Possible use as tracers of biological parameters. *Mar Pollut Bull* 18: 561-563 (1987).
199. Tanabe S, Mori T, Tatsukawa R and Miyazaki N. Global pollution of marine mammals by PCBs, DDTs and HCHs (BHCs). *Chemosphere* 12: 1269-1275 (1983).
200. Timonen T, Ortaldo JR and Herberman RB. Characteristics of human large granular lymphocytes and relationship to natural killer cells and K cells. *J Exp Med* 153: 569-582 (1981).
201. Trichieri G. Biology of natural killer cells. *Adv Immunology* 47: 187-376 (1991).
202. UytdeHaag FGCM, Loggen HG, Logtenberg T, Lichtveld RA, Van Steenis B, Van Asten JAAM and Osterhaus ADME. Human peripheral blood lymphocytes from recently vaccinated individuals produce both type-specific and intertypic crossreacting neutralizing antibody on in vitro stimulation with one type of poliovirus. *J Immunol* 135: 3094-3101 (1985).

203. Van Binnendijk RS. *T cell function in measles Analysis of T cell responses to measles virus at the clonal level: antigen processing and presentation.* Utrecht University, Utrecht (1992).
204. Van Binnendijk RS, Poelen MCM, De Vries P, UytdeHaag FGCM and Osterhaus ADME. A role for CD8+ class I MHC-restricted CTLs in recovery from measles: implications for the development of inactivated measles vaccines. In: *Vaccines 90. Modern approaches to new vaccines including prevention of AIDS,* Brown F, Chanock RM, Ginsberg HS and Lerner RA (eds), Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 299-303 (1990).
205. Van Binnendijk RS, Poelen MCM, Kuijpers KC, Osterhaus ADME and UytdeHaag FGCM. The predominance of CD8+ T cells after infection with measles virus suggests a role for CD8+ class I MHC-restricted cytotoxic T lymphocytes (CTL) in recovery from measles. *J Immunol* 144: 2394-2399 (1990).
206. Van Binnendijk RS, van der Heijden RWJ, Van Amerongen G, UytdeHaag FGCM and Osterhaus ADME. Viral replication and development of specific immunity in Macaques after infection with different measles virus strains. *J Infect Dis* 170: 443-448 (1994).
207. Van Bressem MF, Visser IKG, De Swart RL, Örvell C, Stanzani L, Androukaki E, Siakavara K and Osterhaus ADME. Dolphin morbillivirus infection in different parts of the Mediterranean Sea. *Arch Virol* 129: 235-242 (1993).
208. Van Bressem MF, Visser IKG, Van de Bildt MWG, Teppema JS, Raga JA and Osterhaus ADME. Morbillivrirus infection in Mediterraneanstriped dolphins (*Stenella coeruleoalba*). *Vet Rec* 129: 471-472 (1991).
209. Van den Berg M, Blank F, Heeremans C, Wagenaar H and Olie K. Presence of polychlorinated dibenzo-p-dioxins and polychlorinated dibenzofurans in fish-eating birds and fish from The Netherlands. *Arch Environ Contam Toxicol* 16: 149-158 (1987).
210. Van den Berg M, De Jongh J, Poiger H and Olson JR. The toxicokinetics and metabolism of polychlorinated dibenzo-p-dioxins (PCDDs) and dibenzofurans (PCDFs) and their relevance for toxicity. *Crit Rev Toxicol* 24: 1-74 (1994).
211. Van der Velde EG, Marsman JA, De Jong APJM, Hoogerbrugge R and Liem AKD. Analysis and occurrence of toxic planar PCBs, PCDDs and PCDFs in milk by use of carbosphere activated carbon. *Chemosphere* 28: 693-702 (1994).
212. Van Loveren H, Krajnc EI, Rombout PJA, Blommaert FA and Vos JG. Effect of ozone, hexachlorobenzene, and bis(tri-n-butyltin)oxide on natural killer activity in the rat lung. *Toxicol Appl Pharmacol* 102: 21-33 (1990).
213. Van Loveren H and Vos JG. Immunotoxicological considerations: a practical approach to immunotoxicity testing in the rat. In: *Advances in applied toxicology,* Dayan AD and Paine AJ (eds), Taylor & Francis Ltd, pp. 143-163 (1989).
214. Van Zorge JA, Van Wijnen JH, Theelen RMC, Olie K and van den Berg M. Assessment of the toxicity of mixtures of halogenated dibenzo-p-dioxins and dibenzofurans by use of toxicity equevalency factors (TEF). *Chemosphere* 19: 1881-1895 (1989).
215. Visser IKG, Kumarev VP, Örvell C, De Vries P, Broeders HWJ, Van de Bildt MWG, Groen J, Teppema JS, Burger MC, UytdeHaag FGCM and Osterhaus ADME. Comparison of two morbilliviruses isolated from seals during outbreaks of distemper in North West Europe and Siberia. *Arch Virol* 111: 149-164 (1990).
216. Visser IKG, Teppema JS and Osterhaus ADME. Virus infections of seals and other pinnipeds. *Rev Med Microbiol* 2: 105-114 (1991).
217. Visser IKG, Van Bressem MF, Barrett T and Osterhaus ADME. Morbillivirus infections in aquatic mammals. *Vet Res* 24: 169-178 (1993).
218. Visser IKG, Van Bressem MF, De Swart RL, Van de Bildt MWG, Vos HW, van der Heijden RWJ, Saliki JT, Örvell C, Kitching P, Kuiken T, Barrett T and Osterhaus ADME. Characterization of morbilliviruses isolated from dolphins and porpoises in Europe. *J Gen Virol* 74: 631-641 (1993).
219. Visser IKG, Van Bressem MF, Van de Bildt MWG, Groen J, Örvell C, Raga JA and Osterhaus ADME. Prevalence of morbilliviruses among pinniped species. *Rev Sci Tech Off Int Epiz* 12: 197-202 (1993).
220. Visser IKG, Van de Bildt MWG, Brugge HN, Reijnders PJH, Vedder EJ, De Vries P, Groen J,

Walvoort HC, UytdeHaag FGCM and Osterhaus ADME. Vaccination of harbour seals (*Phoca vitulina*) against phocid distemper virus with two different inactivated canine distemper virus (CDV) vaccines. *Vaccine* 7: 521-526 (1989).

221. Visser IKG, van der Heijden RWJ, Van de Bildt MWG, Kenter MJH, Örvell C and Osterhaus ADME. Fusion protein gene nucleotide sequence similarities, shared antigenic sites and phylogenetic analysis suggest that phocid distemper virus type 2 and canine distemper virus belong to the same virus entity. *J Gen Virol* 74: 1989-1994 (1993).
222. Visser IKG, Vedder EJ, Van de Bildt MWG, Örvell C, Barrett T and Osterhaus ADME. Canine distemper virus ISCOMs induce protection in harbour seals (Phoca vitulina) against phocid distemper but still allow subsequent infection with phocid distemper virus-1. *Vaccine* 10: 435-438 (1992).
223. Vos JG. Immunotoxicity of hexachlorobenzene.In: *Hexachlorobenzene:proceedings of an international symposium*, Morris CR and Cabral JRP (eds), IARC Scientific Publications No. 77, Lyon, pp. 347-356 (1986).
224. Vos JG, Kreeftenberg JG, Kruijt BC, Kruizinga W and Steerenberg PA. The athymic nude rat. II. Immunological characteristics. *Clin Immunol Immunopathol* 15: 229-237 (1980).
225. Vos JG and Luster MI. Immune alterations. In: *Halogenated biphenyls, terphenyls, naphtalenes, dibenzodioxins and related products*, Kimbrough RD and Jensen S (eds), Elsevier Science Publishers B.V., Amsterdam, pp. 295-322 (1989).
226. Vos JG and Moore JA. Suppression of cellular immunity in rats and mice by maternal treatment with 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Int Arch Allergy* 47: 777-794 (1974).
227. Vos JG, Van Loveren H and Schuurman HJ. Immunotoxicity of dioxin: immune function and host resistance in laboratory animals and humans. In: *Banbury Report 35: Biological basis for risk assessment of dioxins and related compounds*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 79-93 (1991).
228. Vos JG, Van Loveren H, Wester PW and Vethaak AD. The effects of environmental pollutants on the immune system. *Europ Environm Rev* 2: 2-7 (1988).
229. Vos JG, Van Loveren H, Wester PW and Vethaak AD. Toxic effects of environmental chemicals on the immune system. *Trends Pharmacol Sci* 10: 289-292 (1989).
230. Warren HS and Chedid LA. Future prospects for vaccine adjuvants. *Crit Rev Immunol* 8: 83-101 (1988).
231. Welsh RM, Brubaker JO, Vargas-Cortes M and O'Donnell CL. Natural killer (NK) cell response to virus infections in mice with severe combined immunodeficiency. *J Exp Med* 173: 1053-1063 (1991).
232. Welsh RM and Vargas-Cortes M. Natural killer cells in viral infection. In: *The natural killer cell*, Lewis CE and McGee JO'D (eds), Oxford University Press, Oxford, pp. 107-150 (1992).
233. Wong S, Fournier M, Coderre D, Banska W and Krzystyniak K. Environmental immunotoxicology. In: *Animal biomarkers as pollution indicators*, Depledge MH and Sanders B (eds), Chapman & Hall, London, pp. 167-189 (1993).
234. Yang YG, Lebrec H and Burleson GR. Effect of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) on pulmonary influenza virus titer and natural killer (NK) activity in rats. *Fundam Appl Toxicol* 23: 125-131 (1994).

added in proof:

235. Chang K-J, Hsieh K-H, Tang S-Y, Tung T-C and Lee T-P. Immunological evaluation of patients with polychlorinated biphenyl poisoning: evaluation of delayed-type hypersensitivity response and its relation to clinical studies. *J Tox Env Health* 9: 217-223 (1982).
236. Clark DA, Gauldie J, Szewczuk MR and Sweeney G. Enhanced suppressor cell activity as a mechanism of immunosuppression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Proc Soc Exp Biol Med* 168: 290-299 (1981).
237. Faith RE and Moore JA. Impairment of thymus-dependent immune functions by exposure of the developing immune system to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). *J Tox Env Health* 3: 451-464 (1977).

238. Hurtrel B, Maire M-A, Hurtrel M and LaGrange PH. Different time course patterns of local expression of delayed-type hypersensitity to sheep red blood cells in mice. *Cell Immunol* 142: 252-263 (1992).
239. Lundberg K, Dencker L and Gronvik KO. Effects of 2,3,7,8-tetrachlorodibenzo-*para*-dioxin (TCDD) on the specific immune response to ovalbumin in the mouse. *Chemosphere* 25: 111-114 (1992).
240. Van den Berg M, Craane BLHJ, Sinnige T, Van Mourik S, Dirksen S, Boudewijn T, Van der Gaag M, Lutke-Schipholdt IJ, Spenkelink B and Brouwer A. Biochemical and toxic effects of polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) in the cormorant (*Phalacrocorax carbo*) after in ovo exposure. *Environ Toxicol Chem* 13: 803-816 (1994).
241. Vos JG, Moore JA and Zinkl JG. Effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin on the immune system of laboratory animals. *Environ Health Perspect* 5: 149-162 (1973).
242. Vos JG and Van Driel-Grootenhuis L. PCB-induced suppression of the humoral and cell-mediated immunity in guinea pigs. *Sci Total Environ* 1: 289-302 (1972).

Nederlandse samenvatting

Sinds het einde van de zestiger jaren wordt zowel door wetenschappers als door het grote publiek de dreiging van milieuvervuiling voor het evenwicht van ecosystemen erkend. In die tijd werd duidelijk dat hoge concentraties van insecticiden (o.a. DDT) in roofvogels de oorzaak waren van verminderde reproductie, met als gevolg sterk teruglopen van de de populaties. Al snel bleek dat milieuvervuilende stoffen, met name organochloorverbindingen waaronder PCBs en dioxines, wereldwijd in alle lagen van de verschillende ecosystemen voorkwamen. Als gevolg van hun vetoplosbaarheid en slechte biologische afbreekbaarheid, worden deze stoffen efficiënt opgenomen door biologische organismen, waardoor ze zich ophopen in de voedselketen. Zeehonden en dolfijnen, die aan de top van de mariene voedselketen staan, zijn daarom vaak blootgesteld aan zeer grote hoeveelheden van deze stoffen, die worden opgeslagen in hun speklaag. In het verleden zijn de hoge gehaltes bij deze diersoorten al in verband gebracht met verschillende toxische effecten, waaronder met name verminderde reproductie en veranderingen in embryonale ontwikkeling en hormonale huishouding.

In 1988 werd de noordwest-Europese populatie van de gewone zeehond (*Phoca vitulina*) plotseling getroffen door een raadselachtige ziekte. In het voorjaar werd voor het eerst meer dan normale sterfte waargenomen in het Kattegat bij Denemarken, waarna de ziekte zich snel verspreidde richting Oostzee en Waddenzee. In de loop van de zomer werden ook dieren rondom de Britse eilanden ziek, en aan het eind van het jaar werd geschat dat ongeveer 20,000 dieren, iets minder dan de helft van de populatie, waren dood gegaan. Een uitgebreide internationale zoektocht naar de oorzaak van de ziekte leidde uiteindelijk tot de Nederlandse ontdekking van het "zeehondenziekte-virus", een morbillivirus nauw verwant aan het virus dat hondeziekte veroorzaakt, en tevens verwant aan het virus dat mazelen veroorzaakt bij de mens. Deze en andere virusinfecties onder zeehonden en dolfijnen aan het eind van de tachtiger en het begin van de negentiger jaren leidden tot speculaties over een mogelijke betrokkenheid van milieuvervuilende stoffen bij deze massale sterftes.

Uit laboratoriumonderzoek bij proefdieren was al eerder duidelijk geworden dat milieuvervuilende stoffen, inclusief de organochloorverbindingen, toxische effecten kunnen hebben op het afweersysteem van zoogdieren. Blootstelling aan deze stoffen kan het functioneren van onderdelen van het immuunsysteem verzwakken, en daardoor verhoogde gevoeligheid voor infectieziekten tot gevolg hebben. In de meeste gevallen werden hierbij echter de effecten van één enkele stof in relatief hoge, acute doseringen op het afweersysteem van proefdieren bestudeerd. Tot nu toe was echter weinig bekend van de effecten van chronische blootstelling aan de mengsels van in het milieu voorkomende stoffen op het functioneren van het afweersysteem. Weliswaar is de blootstelling aan de afzonderlijke stoffen dan gering, maar het vermoeden bestaat dat het mechanisme van toxiciteit van een aantal van de organochloorverbindingen gelijk is, waardoor hun werking cumulatief zou kunnen zijn.

Studies naar het functioneren van het afweersysteem van dieren die in relatief vervuilde gebieden leven zijn over het algemeen zeer moeilijk uit te voeren, en leveren zelden duidelijke resultaten op. Het afweersysteem van zoogdieren bestaat uit een complex netwerk van cellen en moleculen die in verschillende compartimenten van het lichaam voorkomen, en het functioneren van dit systeem in kwantitatieve zin is daarom moeilijk te bestuderen. In

tegenstelling tot sommige andere biologische effecten, zoals bijvoorbeeld reproductietoxiciteit (te meten door het aantal jongen per volwassen vrouwtje te vergelijken tussen populaties) of veranderingen in hormoonspiegels (te meten door concentraties van een hormoon in een bloedmonster te bepalen), is het functioneren van het afweersysteem niet te bepalen door het uitvoeren van één enkele test, maar moet een groot aantal tests worden uitgevoerd om een geïntegreerd beeld te verkrijgen van het functioneren van het systeem. Daar komt bij dat het verkrijgen van goede monsters van in het wild levende dieren, met name van zeezoogdieren, zeer moeilijk is, aangezien een groot aantal van deze tests moet worden uitgevoerd met verse bloedmonsters. Het bleek daarom onmogelijk om met behulp van veldstudies een betrokkenheid van milieuvervuilende stoffen bij de recente virusuitbraken onder zeezoogdieren aan te tonen of uit te sluiten.

Wij hebben daarom een andere aanpak gevolgd om de mogelijke effecten van milieuvervuilende stoffen op het afweersysteem van zeehonden te bestuderen. Tweeëntwintig jonge zeehonden werden gevangen aan de relatief onvervuilde noordoost kust van Schotland, waar nog een groot aantal zeehonden voorkomt. De dieren verbleven tijdens de experimenten op de Zeehondencrèche in Pieterburen. Na een aanpassingsperiode van ongeveer een jaar waarin alle dieren met dezelfde, schone, vis werden gevoerd, werden ze verdeeld over twee groepen. Eén van de groepen werd gedurende 2½ jaar gevoerd met haring uit de relatief onvervuilde Atlantische Oceaan, terwijl de andere groep gedurende dezelfde periode werd gevoerd met haring afkomstig uit de sterk vervuilde Oostzee. De vis van beide diëten was oorspronkelijk bestemd voor menselijke consumptie. Door de opzet van deze zogenaamde "semi-veldstudie" kon het functioneren van het afweersysteem van de dieren in beide groepen gedurende lange tijd worden vervolgd en vergeleken. Het grote voordeel van deze experimentele opzet is dat de situatie goed vergelijkbaar is met de situatie van in het wild levende dieren, maar dat nu monsters kunnen worden verkregen van dieren met zoveel mogelijk dezelfde genetische achtergrond, leeftijd, geslacht en voedselsamenstelling, en als enige verschil tussen de groepen de gehaltes in de haring aanwezige milieuvervuilende stoffen. Resultaten van deze studie worden beschreven in dit proefschrift, en in het proefschrift van Peter Ross (Universiteit Utrecht, 1995).

Bepalingen van gehaltes aan organochloorverbindingen in de haring van beide diëten toonden aan dat de dagelijkse inname van deze stoffen door de zeehonden van de "Oostzeegroep" drie tot tien keer hoger was dan de "Atlantische groep". Let wel: een echte controlegroep waarin dieren totaal geen vervuilende stoffen binnen krijgen is onmogelijk. Al is de Atlantische haring "relatief onvervuild", ook de dieren in deze groep krijgen, mede als gevolg van hun relatief grote vis consumptie (ongeveer 5 kg per dag), nog aanzienlijke hoeveelheden organochloorverbindingen binnen (zie hoofdstuk 3, tabel 1). Aan het einde van het experiment werden ook de hoeveelheden organochloorverbindingen gemeten die zich in de speklaag van de zeehonden in beide groepen opgehoopt hadden en die in hun bloed circuleerden. Hieruit bleek dat de gehaltes in de Oostzeegroep duidelijk hoger waren dan in de Atlantische groep (zie hoofdstuk 8, tabel 1). Hierbij moet wel opgemerkt worden dat gehaltes van deze stoffen in zeehonden die in de Oostzee leven over het algemeen nog hoger zijn, mede als gevolg van de langduriger blootstelling én blootstelling via de moedermelk.

Het belangrijkste doel van onze experimenten was het vergelijken van het functioneren van het immuunsysteem van de dieren in beide groepen. Aangezien relatief weinig informatie beschikbaar was over het immuunsysteem van zeehonden, moesten eerst een aantal functionele

tests ontwikkeld worden. Dit is onder andere beschreven in hoofdstuk 2. Met behulp van deze tests, en een aantal aanvullende tests die later ontwikkeld werden, werd gedurende de experimentele periode het functioneren van verschillende onderdelen van het immuunsysteem vergeleken. In hoofdstuk 3 is een overzicht gegeven van een aantal belangrijke immunologische parameters: responsen van de dieren in de Oostzeegroep zijn weergegeven in verhouding tot responsen van de dieren in de Atlantische groep (zie figuur 2). De belangrijkste verschillen tussen de twee groepen bleken te liggen in het verminderd functioneren van twee typen cellen van het immuunsysteem in de dieren van de Oostzeegroep: "natural killer" (NK) cellen en T-lymfocyten. Deze resultaten werden bevestigd in verschillende andere tests, zoals in meer detail beschreven in de hoofdstukken 4, 5 en 6. In hoofdstuk 7 zijn de resultaten van een aantal routinematige klinisch-chemische en haematologische tests beschreven, waaruit geen aanwijzingen voor een algeheel verminderde gezondheid van de dieren in de Oostzeegroep kwamen. Tijdens het experiment werden ook verder geen gezondheidsproblemen waargenomen bij de dieren in de Oostzeegroep. Onze conclusie is daarom dat chronische blootstelling aan milieuvervuilende stoffen aanwezig in de Oostzee haring, de oorzaak is geweest van een verminderd functioneren van de eerder genoemde NK cellen en T-lymfocyten. Aangezien deze beide celtypen ook een belangrijke rol spelen bij de afweer tegen virusinfecties, en gehaltes van immuuntoxische stoffen in zeehonden in het wild vaak hoger zijn dan in de dieren van de Oostzeegroep, is het waarschijnlijk dat milieuvervuiling een rol heeft gespeeld in de recente virusuitbraken onder zoogdieren, en mogelijk de gevolgen van de infektie heeft verergerd.

In het wild levende zeehonden ondergaan regelmatig vastenperiodes van enkele weken. Zo zal een moederzeehond tijdens het zogen van haar jong niet of vrijwel niet eten, terwijl zij wel vette moedermelk moet produceren. Tijdens deze periodes verbruiken de dieren de vetreserves die zij opgeslagen hebben in hun speklaag. Aangezien de milieuvervuilende stoffen die de dieren gedurende hun leven ophopen zich grotendeels in deze speklaag bevinden, is het denkbaar dat tijdens vastenperiodes deze stoffen vrijkomen in het bloed, waardoor de dieren dan extra blootgesteld zouden worden aan deze toxische stoffen. Om deze hypothese te testen, zijn de zeehonden van ons experiment aan het einde van de studie twee weken aan een vastenperiode onderworpen (zie hoofdstuk 8). Tijdens deze periode verbrandden de dieren ongeveer de helft van hun vetreserve, en bleven ze verder gezond en aktief. De concentraties van een aantal organochloorverbindingen in het bloed stegen licht, maar gehaltes van de meest immuuntoxische stoffen bleven ongeveer gelijk. Vergelijking van immunologische parameters lieten geen verergering zien van de eerder waargenomen verschillen in immuunfunctie tussen de twee groepen. De resultaten suggereren dan ook dat relatief kortdurende vastenperiodes geen extra bedreiging voor de dieren vormen.

Aan het einde van de vastenperiode zijn de dieren uit beide groepen nog gedurende ongeveer een half jaar gevoerd met Atlantische haring, waarna ze weer uitgezet zijn in het wild. Ze waren op dat moment ongeveer vier jaar oud en dus bijna volwassen, en kunnen dus in de toekomst hun rol spelen in de Europese populatie gewone zeehonden. Deze heeft zich na de virusepidemie in 1988 weer aanzienlijk hersteld: de aantallen zijn nu ongeveer even hoog of zelfs hoger dan vóór 1988. De resultaten van onze studies hebben echter aangetoond dat zeehonden die er uiterlijk volkomen gezond uitzien, toch een verminderde weerstand kunnen hebben als gevolg van blootstelling aan milieuvervuilende stoffen.

Dankwoord

Er bestaan maar weinig cliché's in de wereld die zo veel waarheid bevatten als "een proefschrift schrijven doe je nooit alleen". Ik wil dan ook iedereen die zijn of haar steentje heeft bijgedragen aan dit onderzoek ontzettend bedanken voor de medewerking die ik heb gekregen. Op het gevaar af mensen te vergeten, wil ik toch een aantal mensen in het bijzonder noemen.

Allereerst natuurlijk mijn beide promotores. Ab, jouw veelzijdigheid en tomeloze energie stellen me elke keer weer voor een raadsel. Zowel voor wat betreft het uitvoeren en rapporteren van wetenschappelijk werk als het leren omgaan met de randvoorwaarden heb ik veel van je geleerd. Daarnaast zullen met name de "tap-uitstapjes" naar Pieterburen en de "concerted action" in je achtertuin als ultieme eindsprint naar het proefschrift me lang bijblijven. Sjef, ook jouw bijdrage aan het onderzoek vanuit de immuuntoxicologische hoek heb ik zeer gewaardeerd, wat trouwens ook geldt voor Henk van Loveren. Verder ben ik veel dank verschuldigd aan Lenie 't Hart: zonder jouw onvoorwaardelijke steun was het project mogelijk nooit van de grond gekomen. Ik heb zeer veel waardering voor jouw inzet voor de zeehond in het algemeen en de Crèche in het bijzonder, en hoop dat ik daar een bijdrage aan heb kunnen leveren. Vooral je enthousiasme tijdens het uitzetten van de zeehonden van het experiment zal ik niet snel vergeten.

Wat betreft de praktische kant van het werk moge duidelijk zijn dat ik dit onderzoek niet in mijn eendje gedaan heb. Peter: het begin van onze samenwerking was wat moeizaam, maar ik denk dat Ab uiteindelijk toch gelijk heeft gekregen met zijn bewering dat 1 + 1 soms meer dan twee kan zijn. Juist vanuit onze verschillende invalshoeken op het onderzoek hebben we elkaar uiteindelijk goed weten aan te vullen. Met name de al eerder genoemde uitstapjes naar Pieterburen, en vooral de daarop volgende marathon-sessies in het lab waren vaak bijzondere dagen. Helga, ook jij daarbij zeer bedankt, zowel voor de enorme hoeveelheid pipetteerwerk die je hebt verzet als voor je bijdrage aan de sfeer eromheen. Verder natuurlijk mijn dank aan de studenten Carla, Ruud, Aline en werk(of was het nou dienst?)weigeraar Jaap.

Ilona, jou wil ik bedanken voor onze prettige samenwerking in "vroeger jaren". Ook van jou heb ik veel geleerd, en ik bewaar nog steeds prettige herinneringen aan onze "uitstapjes" naar Cambridge en Texas. Verder wil ik alle medewerkers van de voormalige afdeling IMB en de afdeling Pathologie van het RIVM bedanken. Met name wil ik de "smokers" Rob, Carel en Fons bedanken voor de soms spontaan opborrelende brainstorm-sessions tussendoor. Helma: jij ontzettend bedankt voor alle hulp die ik de laatste 1½ jaar gehad heb. Buiten IMB en PAT hebben nog vele anderen op het RIVM bijgedragen aan dit onderzoek: Henk Loggen en Jos Westendorp: eindelijk rust (als jullie mijn kop maar zagen wist je alweer hoe laat het was), Dick Jut en Bert Weitering bedankt voor jullie bijdrage aan hoofdstuk vijf, Fons Boink voor zijn hulp bij hoofdstuk 7, en natuurlijk Djien Liem en Willy Hijman bedankt voor jullie belangrijke bijdrage aan de toxicologische kant van het onderzoek. Dat laatste geldt ook voor Elze de Ruiter van het IBN-DLO. Verder wil ik Siem Heisterkamp (RIVM) en Paul Mulder (EUR) danken voor hun hulp bij statistische problemen. Alle mensen in Rotterdam: dank voor jullie steun tijdens de laatste loodjes. Van de mensen buiten deze instituten wil ik met name Bram Brouwer en Ineke Lutke-Schipholdt bedanken voor de

stimulerende discussies en de nuttige samenwerking.

Tot nu toe is nog slechts het gedeelte van het werk dat in het laboratorium plaats vond genoemd, maar het moge duidelijk zijn dat ook in Pieterburen menigeen zich in het zweet heeft gewerkt om dit allemaal mogelijk te maken. Allereerst wil ik Lies Vedder bedanken: nog voor het eigenlijke experiment begon heb jij me tijdens uitstapjes naar het toenmalige RIN op Texel de edele kunst van het bloed afnemen van zeehonden bijgebracht. Maar daarnaast hebben tientallen medewerkers en vrijwilligers van de Zeehondencrèche geholpen bij grote en kleinere experimenten. Op het gevaar af mensen op hun ziel te trappen, ga ik voor één keer proberen ze allemaal te noemen: Abel, Aldert (hoe is 't met je rug?), Anita (hoe hou je buizen warm als je handen koud zijn?), Balasz, Boukje (wordt er nou nog gezongen met Ab op dat feest of hoe zit dat?), Geert (mis je ons nou stiekem niet toch een beetje?), Geesje, Ida, Inge, Jitske, Manuel, Marc, Lisette (waar is die ☹☻♂dia nou gebleven?), Peter (toch maar goed dat we op een gegeven moment het net zijn gaan gebruiken om zeehonden te tillen...), Pieter, Renate, Ria, Roelof, Simone, Stefania, Sylvia (lekkere soep), Tom, Trijnette, Vincent, Yvonne en alle bouwvakkers en andere vrijwilligers: zeer bedankt! Verder natuurlijk ook de mensen van het IBN-DLO (cq. RIN) bedankt: Peter, Piet-Wim, Koos, Ditta en Sofie. Ik bewaar nog steeds goede herinneringen aan de trip naar Schotland!

Buiten het werk wil ik twee groepen mensen noemen die erg belangrijk voor mij zijn geweest. Daarvan is er natuurlijk maar één groep die zich "de groep" mag noemen: Ca, DJ, El, Jean, Mo, Punt en Vennie: het waren goeie avonden cq. weekenden, zowel tijdens studie als in de periode daarna. En natuurlijk Voorheen Voorheen (twee keer toch? of zijn we inmiddels al nog een keer Voorheen?) Het Zaaltejater. Ik hoop dat ik me met de in de loop van de jaren samen met jullie opgedane toneelervaring ook door het nu komende toneelstuk weet te slaan. Verder natuurlijk ook alle andere vrienden bedankt voor jullie steun!

En dan natuurlijk alle mensen bedankt die in de laatste fase hebben geholpen om deze spoedklus te klaren: de commissieleden voor hun welwillendheid het manuscript versneld te beoordelen, mijn paranimfen Helma en Janneke voor de bergen werk die zij nu al verzet hebben en ongetwijfeld nog gaan verzetten, Rob voor de figuren uit zijn spelletjescomputer-zonder-muis, Conny voor het screenen van het manuscript op kleine en grote fouten, en de vele anderen die bereid waren om snel nog even te helpen.

Tenslotte Hetty en mijn familie. Niet voor niets heb ik het proefschrift aan jullie opgedragen. Van huis uit ben ik altijd gestimuleerd en gesteund in studie en later in promotie. Ik weet het, ik heb vaak geroepen "promoveren? dat nooit!", maar ben uiteindelijk toch voor de bijl gegaan. En jij Hetty: door je nuchtere kijk op het leven in het algemeen en wetenschap in het bijzonder heb jij het voor mij mogelijk gemaakt om eventuele problemen op het werk thuis weer tot normale proporties terug te brengen. Ik hoop, nee ben er van overtuigd dat we straks, hopelijk met z'n drietjes, op dezelfde voet door kunnen gaan.

Curriculum Vitae

De auteur van dit proefschrift werd op 22 januari 1966 geboren te Doetinchem, waar hij in 1984 zijn VWO diploma behaalde. In aansluiting hierop studeerde hij biologie aan de Rijksuniversiteit Utrecht. Het doctoraalexamen werd behaald in 1990, met als eerste hoofdvak een kombinatie van moleculaire microbiologie en biochemie, en als tweede hoofdvak immunologie. In 1990 trad hij als wetenschappelijk medewerker in dienst van de Stichting Zeehondencrèche Pieterburen, met als opdracht het bewerken van de in dit proefschrift beschreven vraagstelling. Deze studies werden in eerste instantie uitgevoerd als gastmedewerker van het Laboratorium voor Immunobiologie van het Rijksinstituut voor Volksgezondheid en Milieuhygiëne te Bilthoven, en vanaf begin 1994 als gastmedewerker van de afdeling Virologie van de Erasmus Universiteit Rotterdam, in beide gevallen onder begeleiding van Prof. Dr. A.D.M.E. Osterhaus en in nauwe samenwerking met Peter Ross. De laatstgenoemde is eveneens als wetenschappelijk medewerker in dienst van de Stichting Zeehondencrèche Pieterburen, en beschrijft andere aspecten van deze studies in zijn proefschrift (Universiteit Utrecht, 1995).

List of publications

De Vrije T, De Swart RL, Dowhan W, Tommassen J and De Kruiff B. Phosphatidylglycerol is involved in protein translocation across Escherichia coli inner membranes. *Nature* 334: 173-175 (1988).

Osterhaus ADME, Visser IKG, De Swart RL, Van Bressem MF, Van de Bildt MWG, Örvell C, Barrett T and Raga JA. Morbillivirus threat to Mediterranean monk seals? *Vet Rec* 130: 141-142 (1992).

De Swart RL, Kluten RMG, Huizing CJ, Vedder LJ, Reijnders PJH, Visser IKG, UytdeHaag FGCM and Osterhaus ADME. Mitogen and antigen induced B and T cell responses of peripheral blood mononuclear cells from the harbour seal (*Phoca vitulina*). *Vet Immunol Immunopathol* 37: 217-230 (1993).

Van Bressem MF, Visser IKG, De Swart RL, Örvell C, Stanzani L, Androukaki E, Siakavara K and Osterhaus ADME. Dolphin morbillivirus infection in different parts of the Mediterranean Sea. *Arch Virol* 129: 235-242 (1993).

Visser IKG, Van Bressem MF, De Swart RL, Van de Bildt MWG, Vos HW, van der Heijden RWJ, Saliki JT, Örvell C, Kitching P, Kuiken T, Barrett T and Osterhaus ADME. Characterization of morbilliviruses isolated from dolphins and porpoises in Europe. *J Gen Virol* 74: 631-641 (1993).

De Swart RL, Ross PS, Vedder LJ, Timmerman HH, Heisterkamp SH, Van Loveren H, Vos JG, Reijnders PJH and Osterhaus ADME. Impairment of immune function in harbor seals (*Phoca vitulina*) feeding on fish from polluted waters. *Ambio* 23: 155-159 (1994).

Langeveld JPM, Casal JI, Osterhaus ADME, Cortes E, De Swart RL, Vela C, Dalsgaard K, Puijk WC, Schaaper WMM and Meloen RH. First peptide vaccine providing protection against viral infection in the target animal: studies of canine parvovirus in dogs. *J Virol* 68: 4606-4513 (1994).

Ross PS, De Swart RL, Visser IKG, Murk W, Bowen WD and Osterhaus ADME. Relative immunocompetence of the newborn harbour seal, *Phoca vitulina*. *Vet Immunol Immunopathol* 42: 331-348 (1994).

De Swart RL, Ross PS, Timmerman HH, Vos HW, Reijnders PJH, Vos JG and Osterhaus ADME. Impaired cellular immune response in harbour seals (*Phoca vitulina*) feeding on environmentally contaminated herring. *Clin Exp Immunol*, in press.

De Swart RL, Harder TC, Ross PS, Vos HW and Osterhaus ADME. Morbilliviruses and morbillivirus diseases of marine mammals. *Infect Agent Dis*, in press.

De Swart RL, Ross PS, Timmerman HH, Van Loveren H, Reijnders PJH, Vos JG and Osterhaus ADME. Impairment of immune function in harbour seals (*Phoca vitulina*) feeding on fish contaminated through the food chain. *Hum Exp Toxicol*, in press. (abstract).

De Swart RL, Ross PS, Timmerman HH, Hijman WC, De Ruiter E, Liem AKD, Brouwer A, Van Loveren H, Reijnders PJH, Vos JG and Osterhaus ADME. Short-term fasting does not aggravate immunosuppression in harbour seals (*Phoca vitulina*) with high body burdens of organochlorines. *submitted*

De Swart RL, Ross PS, Vedder LJ, Boink FBTJ, Reijnders PJH, Mulder PGH and Osterhaus ADME. Haematology and clinical chemistry values of harbour seals (*Phoca vitulina*) fed environmentally contaminated herring remain within normal ranges. *submitted*

Harder TC, Harder M, De Swart RL, Osterhaus ADME and Liess B. Major immunogenic proteins of two distinct phocid herpesvirus species are antigenically related to polypeptides of herpesviruses of terrestrial carnivores. *submitted*

Osterhaus ADME, De Swart RL, Vos HW, Ross PS, Kenter MJH and Barrett T. Morbillivirus infections of aquatic mammals: newly identified members of the genus. *Vet Microbiol*, in press.

Osterhaus ADME, De Swart RL, Vos HW, Verhoef J and Zarnke RL. Prevalence of canine distemper virus and phocine distemper virus specific serum antibodies in marine mammals from Alaska and russia, 1978-1994. *manuscript in preparation*

Ross PS, De Swart RL, Reijnders PJH, Van Loveren H, Vos JG and Osterhaus ADME. Contaminant-related suppression of delayed-type hypersensitivity and antibody responses in harbor seals fed herring from the Baltic Sea. *Environ Health Perspect* 103: 162-167 (1995).

Ross PS, De Swart RL, Timmerman HH, Vedder LJ, Van Loveren H, Vos JG, Reijnders PJH and Osterhaus ADME. Suppression of natural killer cell activity in harbour seals (*Phoca vitulina*) fed Baltic Sea herring. *submitted*

Ross PS, De Swart RL, Van der Vliet H, Willemsen L, De Klerk A, Van Amerongen G, Brouwer A, Van Loveren H, Vos JG and Osterhaus ADME. Impairment of cellular immune responses and host resistance to rat cytomegalovirus (RCMV) in offspring of rats exposed during pregnancy and nursing to Baltic Sea herring oil or 2,3,7,8-TCDD. *manuscript in preparation*

Ross PS, De Swart RL, Van der Vliet H, De Klerk A, Timmerman HH, Van Binnendijk RS, Lutke-Schipholdt IJ, Brouwer A, Vos JG, Van Loveren H and Osterhaus ADME. Host resistance to rat cytomegalovirus (RCMV) in adult PVG rats fed herring from the contaminated Baltic Sea. *manuscript in preparation*

Vos JG, Bloksma N, De Heer C, De Swart RL, Ross PS and Van Loveren H. Immunotoxicity of anthropogenic trace materials in the environment. In: *Proceedings 5. Statuskolloquium des Projectes Umwelt und Gesundheit (PUG) des Forschungszentrum Karlsruhe, 15/16-03-1995, in press.*

Vos JG, De Heer C, De Swart RL, Ross PS and Van Loveren H. Immunotoxicity of polychlorinated biphenyls and related compounds. In: *Proceedings joint international symposium "New trends in allergy IV" and "Environmental allergy and allergotoxicology III". Springer Verlag, in press.*