Seals, pollution and disease:

environmental contaminant-induced immunosuppression

Peter S. Ross

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Zeehonden, vervuiling en infectieziekten:
immuunsuppressie als gevolg van blootstelling
aan milieuvervuilende stoffen

met een samenvatting in het Nederlands

Proefschrift

Ter verkrijging van de graad van doctor

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in het openbaar te verdedigen

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Peter Stuart Ross

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Promotores: Prof. Dr. A.D.M.E. Osterhaus
Prof. Dr. J.G. Vos

Copromotor: Dr. H. van Loveren

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Dedicated to the natural inhabitants of the Wadden Sea.

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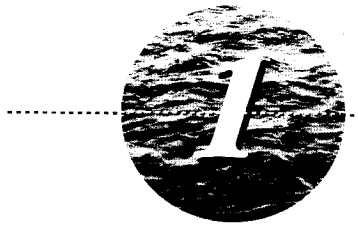

General Introduction

Partly taken from:

Peter S. Ross, Ilona K.G. Visser, Heinz W.J. Broeders, Marco van de Bildt, W. Don Bowen, and Albert D.M.E. Osterhaus

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During the last 50 years, the ubiquitous contamination of the global environment has resulted in detectable levels of many classes of anthropogenic chemicals in wildlife inhabiting even remote areas (200,244,282). Classes of particular biological concern include the persistent, water-insoluble polyhalogenated aromatic hydrocarbons (PHAHs), including the polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) and polychlorinated biphenyls (PCBs). Having fulfilled their role as heat transfer and dielectric fluids (e.g. PCBs), or having been created as undesired by-products of pesticide production (e.g. PCDDs) or combustion processes (PCDDs and PCDFs), these chemicals have made their way slowly but steadily into soils, water and air. These compounds are chemically stable, lipophilic and accumulate readily in the aquatic food chain where they largely resist metabolic breakdown. Environmental mixtures of the PHAHs are complex, since classes consist of 209 theoretical PCB congeners, 75 PCDD congeners, and 135 PCDF congeners (Figure 1).

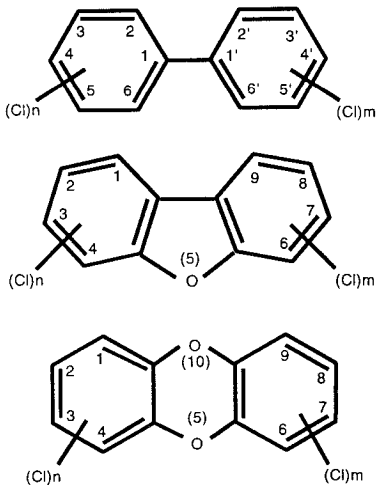

Figure 1: Generalized chemical structures of the PCBs, PCDFs and PCDDs, with possible Cl binding sites identified numerically, as well as the ortho- (o), meta- (m), and para- (p) positions being identified for PCBs.

The transport of PHAHs in the environment depends largely on the interaction between the solubility of the different congeners, which is related to the degree and pattern of chlorination, and the geophysical processes to which they are exposed in the environment at the source of introduction. While PHAHs tend to be highly insoluble

in water, organic particles provide a surface upon which they can bind and be readily transported in the water column (85,285). This may lead to subsequent deposition in sediments and to uptake by benthic or pelagic organisms at a low trophic level, such as shellfish, zooplankton and crustaceans (195,258). Atmospheric transport has been recognized as an important route for the movement of PHAHs in the environment, reflecting the relatively high volatility and low aqueous solubility of these compounds (174). Both direct introduction by incineration and combustion processes, and indirect introduction by volatilization from water surfaces contribute to atmospheric PHAHs, allowing them to be transported over large distances. PHAHs in air and surface water samples are readily detected in the remote Arctic and Antarctic environments, reflecting the "global distillation" of these compounds (9,109,200).

Following the discovery that PCBs and the pesticide dichlorodiphenyl-trichloroethane (DDT) had become widespread contaminants (132), concern was voiced about the potential threat to wildlife (38). The subsequent DDT-related extirpation of raptors and other piscivorous bird populations from many areas of North America and Europe (87) led to tighter production and disposal regulations for DDT and PCBs in most industrialized countries in the 1970s. Subsequently, DDT, and to a lesser extent, PCB, concentrations declined in many environmental compartments, as well as in the fatty tissues of wildlife species (3). These declines, however, largely reflected a redistribution towards environmental sinks, rather than a real degradation in the ecosystem. The environmental cycling of stable PHAHs, leakage from industrial storage sites, use in many developing nations, and low-temperature combustion processes slowed the decline during the 1980s. Recent data suggest that levels of PHAHs have stabilized in human and wildlife populations in industrialized areas (122,182), reflecting an equilibrium in environmental cycling.

While PHAHs represent a class of environmental contaminants of concern in many parts of the world as a result of their chemical characteristics and toxic nature, other contaminants including a number of pesticides and metals can also present a risk to wildlife populations. However, significant metal contamination is generally restricted to areas in the vicinity of dense human population and industry. In addition, their limited potential for biomagnification tends to preclude their potential for toxic effects in wildlife at high trophic levels (88). Likewise, the bioaccumulation of lipophobic compounds can be expected to be low, thereby limiting their potential for toxicity to localized areas.

Biological effects of environmental contaminants in wildlife

Bioaccumulation in the aquatic food chain results in high concentrations of many of the lipophilic PHAH contaminants in organisms occupying high trophic levels (Figure 2). Consistent with this, most of the biological effects of PHAH

contaminants have been observed in top predators, and organisms of particular concern include the piscivorous birds, otters, seals and whales. As a result of the chemical nature of many PHAHs, they can readily influence many physiological processes. The effects of DDT on fecundity were related to its structural similarity to the estrogen molecule, and its ability to disturb the dynamics of calcium metabolism in fish-eating birds. As a result, egg-shell thinning reduced the survival rates of developing embryos (116). While the mechanisms remain elusive, PCBs, PCDDs and PCDFs have been shown to interfere with a number of physiological processes in birds inhabiting relatively contaminated areas of North America and Europe. Effects observed include liver enzyme induction (26), embryotoxicity (248), skeletal deformities (87,95) and reduced parental attentiveness (194).

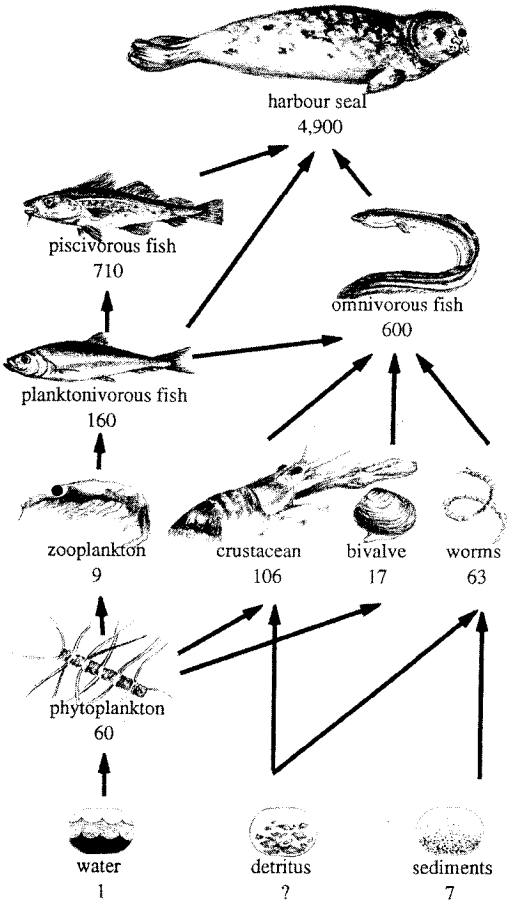

Figure 2: The harbour seal food chain in the Dutch Wadden Sea. Occupying a high trophic level, the harbour seal accumulates high levels of lipophilic PHAHs from its diet (adapted from A.D. Vethaak, personal communication; drawn by H. Gurney).

While studies of marine mammals are constrained by obvious difficulties, epidemiological evidence has implicated PHAHs in a number of bioeffects in free-ranging populations (Table 1). Although conclusive cause-and-effect studies on the effects of PHAHs in free-ranging marine mammals are difficult, results of an earlier semi-field study in which harbour seals were fed environmentally-contaminated fish strengthened the evidence of PHAH-related reproductive toxicity suggested by epidemiological studies (197). In this latter study, serum vitamin A and thyroid hormone levels were also affected by contaminants (30).

Table 1: Epidemiological evidence of contaminant-induced bioeffects among marine mammal populations.

effect	location	species	reference
abortions, premature pupping	California, USA	California sea lions (*Zalophus californianus*)	63
tumours, decreased fecundity	Québec, Canada	Beluga whales (*Delphinapterus leucas*)	164
decreased fecundity	Wadden Sea, The Netherlands	harbour seals (*Phoca vitulina*)	196
impaired reproduction	Baltic Sea	ringed seals (*Phoca hispida*)	112
skeletal lesions	Baltic Sea	harbour seals, grey seals (*Halichoerus grypus*)	17,171
reduced testosterone levels	north Pacific Ocean	Dall's porpoises (*Phocenoides dalli*)	235

Immunotoxicity of environmental contaminants

Among the broad range of physiological processes affected by contaminants, the immune system has been shown to be particularly sensitive to the toxic action of many PHAHs. Earlier studies found PHAHs to be immunotoxic in mice (274), rats (274), guinea pigs (276), rabbits (144) and monkeys (246). Other environmental contaminants have also been shown to be potentially immunotoxic, including lead (140), cadmium (125), methyl mercury (128), nickel (230), organotin compounds (228), hexachlorobenzene (278), polycyclic aromatic hydrocarbons (PAHs) (286), and a number of pesticides (133,234). The chemical characteristics of many metals, PAHs

and pesticides have largely precluded their bioaccumulation in the food chain (88,181) and may limit any effects on wildlife to local areas of contamination. The ready bioaccumulation of PHAHs even in fauna inhabiting remote areas and the relative immunotoxicity of these compounds suggest that PCBs, PCDDs and PCDFs pose the greatest immunotoxic threat to wildlife occupying high positions in the aquatic food chain.

The mechanism of PHAH injury to the immune system has been shown to be largely mediated by the cytosolic Aryl hydrocarbon (*Ah*)-receptor found in mammalian cells (223,224). While no physiological ligand has been identified, 2,3,7,8-TCDD has been found to bind readily to the *Ah*-receptor (Figure 3). The resulting intracellular TCDD-receptor complex induces enzyme production by binding to the dioxin regulatory element (DRE) in the nucleus (287), which in turn, leads to biological responses which are poorly understood. However, the high binding affinity of TCDD for the *Ah*-receptor relative to other PHAH congeners, coupled with relationships

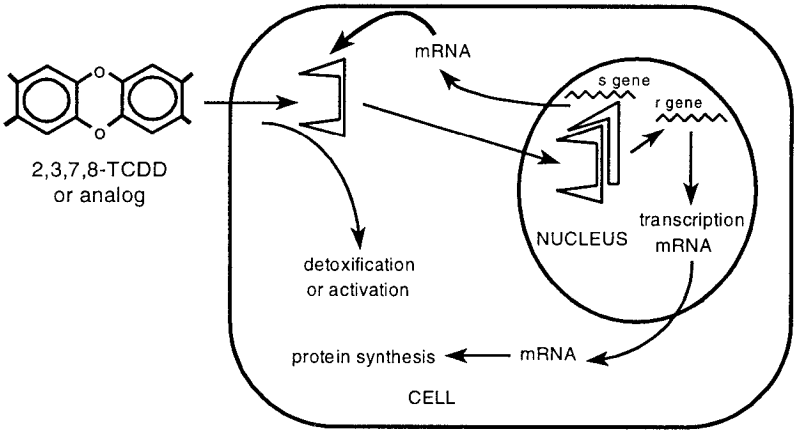

Figure 3: The Aryl hydrocarbon (*Ah*)-receptor in mammalian cells has been demonstrated to mediate the immunotoxic action of TCDD-like compounds, although the precise mechanism remains unclear (adapted from 214). mRNA (messenger ribonucleic acid); s gene (structural gene); r gene (regulatory gene).

between chemical structure and immunological effects in laboratory animals, support the idea of a common underlying mechanism of action in PHAH-induced immunotoxicity. The toxicities of complex contaminant mixtures can be simplified by a knowledge of the toxicities of the individual PHAH congeners relative to TCDD (214). The PCB, PCDD and PCDF congeners which structurally resemble TCDD are each assigned a TCDD toxic equivalent factor (TEF), and additive toxic equivalents (TEQs) calculated from concentrations measured in the mixture (212). Because certain

PHAH congeners may act in a synergistic or antagonistic manner, the use of TEQs to define complex mixtures should be applied with a degree of caution and considered primarily as an indicator of the overall *Ah*-receptor-mediated toxicity of a complex mixture. In addition, certain PHAH-induced toxicities have been shown to be partly or entirely non *Ah*-dependent, such as vitamin A and thyroid hormone deficiencies, and neurotoxic and developmental effects (32,170).

While the immunotoxic action of PHAH chemicals is poorly understood, it is likely that multiple targets are involved. Developing leukocyte progenitors in the bone marrow (83) and thymus appear to be sensitive to TCDD (100). Thymus atrophy, for example, is induced by one-time doses between 1 and 5 µg/kg body weight in the rat (56). The underlying cause of thymic atrophy is most likely related to a diminished maturation of T-cell precursors (55,100), or thymocytes, and a reduced seeding of the thymus by bone marrow thymocyte progenitors (82-84). Immunotoxicity at the level of the thymus has repercussions for the mature cellular immune response, with impaired T-lymphocyte functionality and impaired T-dependent immunity being common findings in studies of TCDD-exposed laboratory animals (273). Additionally, exposure to TCDD has been shown to impair B-lymphocyte function, although the concentrations required for such an effect are higher than those which lead to effects on T-cell function (252).

The developing immune system of mammals has been shown to be particularly sensitive to TCDD-induced immunotoxicity following exposure during gestation and nursing (247,274). While TCDD transfer from mother to offspring in laboratory rodents appears to take place primarily via the milk, a small degree of transplacental transfer also takes place (177,239). Since seals and other marine mammals occupying high trophic levels are exposed to relatively high levels of PHAH mixtures in milk (2), the risk for immunotoxic effects on the developing immune system may be high in animals born in contaminated environments.

Host resistance to numerous pathogens has been shown to be affected by PHAH-induced immunotoxicity in studies of laboratory rats and mice. Using different models, rodents exposed to relatively low doses of TCDD or related PHAHs have been shown to be less resistant to bacterial (245), parasitic (156) and virus infections (89), in many cases leading to elevated morbidity, mortality or increased loads of pathogens in immunosuppressed individuals. Since the end result of contaminant-induced immunosuppression can vary among pathogens, the selection of a set of appropriate host resistance models is important in laboratory studies (260).

The harbour seal immune system and anti-viral immunity

One of the major roles of the immune system of animals is to provide a functional barrier against invading and ever-present pathogens. The skin and mucous

membranes provide an essential physical defence against microorganisms, which is complemented by a complex network of integrated non-specific and specific effector mechanisms (Figure 4). Leukocytes participating in the non-specific arm of the immune system include granulocytes, which efficiently phagocytize bacteria; natural killer (NK) cells, which lyse cells altered by virus infection or malignant transformation; and macrophages, which phagocytize and digest microorganisms and debris, resulting in presentation of antigen for the development of immunological memory. The specific immune response is based upon a complex network of cells and mediators which results in the recognition of foreign antigens, thus providing an efficient system of immunological memory. Key players in this system include T-helper cells, a lymphocyte subpopulation that supports the immune response through cell-to-cell communication; T-cytotoxic cells, a lymphocyte subpopulation that participates in this communication and can specifically lyse cells presenting foreign antigen fragments on their surface; and antibody-producing B-cell derived plasma cells, a lymphocyte subpopulation which also participates in this communication and secretes

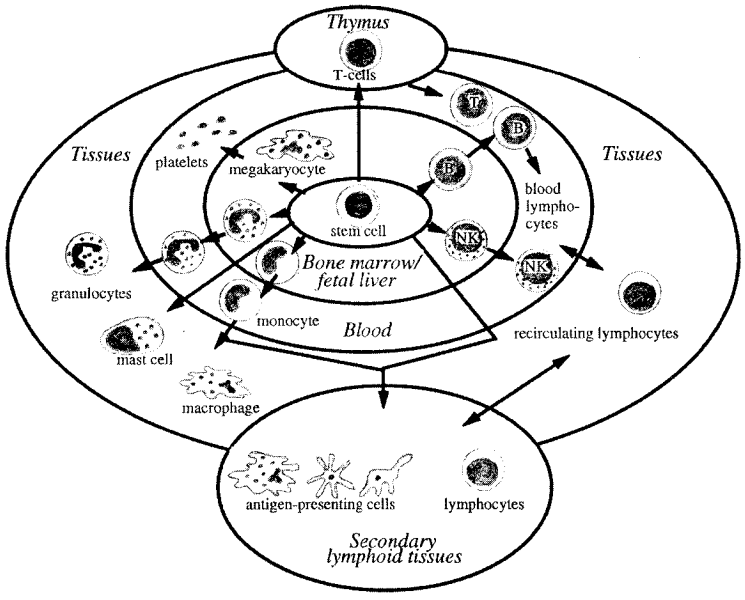

Figure 4: A diagrammatic representation of developing leukocytes. While granulocytes, natural killer (NK) cells, and B-lymphocytes migrate directly to the blood and periphery following development in the bone marrow, T-lymphocyte precursors migrate from the bone marrow to the thymus for further maturation (adapted from 202; drawings by H. Gurney).

specific antibodies. Cellular communication is largely carried out by cytokines, molecules which have multiple functions and simultaneously provide communication among different combinations of cell populations.

Upon exposure to a new pathogen, the physical barriers as well as non-specific immune responses form the first line of defence against infection. Early responses in virus infections typically include a peak in serum gamma-interferon and NK cell activity between two and four days following infection (284). Subsequently, a usually effective specific response is mounted by the interaction of T-helper, T-cytotoxic, and B-lymphocytes which is aimed at clearance, or least, control of the virus infection (81). The relative contributions to virus clearance by the non-specific and the specific immune responses is not always clear and differs among viruses. For example, the immunological control of lymphocytic choriomeningitis (LCMV) infection in mice has been shown to be highly dependent upon a specific cytotoxic T-lymphocyte response, while the control of murine cytomegalovirus (MCMV) infection has been shown to be largely dependent upon the response of NK cells in early infection (283,284). The complexity of the immune response *in vivo* and the dynamic interaction between virus replication and the resulting immune response underline the need for a broad approach to immunotoxicity testing.

Comparative immunological studies suggest that there is great similarity among mammalian species, reflecting an evolutionary conservation of essential defensive mechanisms. Despite this, differences do exist among effector mechanisms in different species. The order Pinnipedia evolved from an ancestor of the modern day order Carnivora. There is little reason to assume that the immune system of pinnipeds differs greatly from that of terrestrial carnivores that have been studied (139). Preliminary studies have described gross structural similarities in lymphoid tissues and antibody structure (41,219) between pinnipeds and other carnivores (39,42). Since the placentation type has been shown to be endotheliochorial (71) as in dogs and cats, the transfer of maternal antibodies may be expected to follow a similar pattern.

Despite the dearth of knowledge on the immune system of pinnipeds, the adaptation of immune function tests used for humans and other mammals has proven successful. In a first approach to a functional assessment of a pinniped immune system, concanavalin A (Con A) was used in lymphocyte stimulation tests with blood obtained from harbour seal (*Phoca vitulina*) mothers and their pups during the lactation period on Sable Island, Canada (209). Subsequently, it was shown that the mitogens Con A, phytohaemagglutinin A (PHA), and to a lesser extent pokeweed mitogen (PWM), stimulated harbour seal T-lymphocytes, while PWM and lipopolysaccharide (LPS) of *Salmonella typhimurium* stimulated B-lymphocytes (58). The use of protein A of *Staphylococcus aureus* to quantify total (209) and specific immunoglobulin G (268) levels in the serum of seals has been a useful tool given the lack of specific reagents available for seal immunoglobulins. The recent development of monoclonal antibodies

against harbour and grey seal immunoglobulins has enabled the determination of both specific and total antibody levels in serum (142a,142b). The identification and quantification of interleukin 6 (IL-6) in the serum of PDV-infected harbour seals and grey seals (*Halichoerus grypus*) followed the initial description of this cytokine (142c), although its functional role in the seal was not addressed. Further developments in the assessment of immune function in harbour seals are described in this thesis.

Absence of mortality in PDV-1 infected seals in Canada: grounds for speculation?

The mass mortality of harbour seals in Europe in 1988 resulting from infection with phocid distemper virus-1 (PDV-1) has been extensively described (73,186,191). PDV-1 is a newly-identified member of the genus *Morbillivirus*, and is distinct from phocid distemper virus-2 (PDV-2), which resulted in the deaths of several thousand Baikal seals (*Phoca sibirica*) in 1987-88 (98,187,265) and proved to be a strain of canine distemper virus (CDV). The 1988 PDV-1 epizootic in Europe, which left about 60% of the harbour seal population dead, led to speculation about the origin and nature of the virus, and the potentially predisposing role that pollution may have played in weakening the immune systems of the seals (73). During the European epizootic, there were no reports of unusual mortalities or sick seals on the east coast of Canada, which suggested at that time that Canadian seal populations were not exposed to PDV-1. In fact, harbour seal pup production on Sable Island, a major breeding site in eastern Canada, doubled between 1978 and 1990 (W.T. Stobo, Bedford Institute of Oceanography, Dartmouth, Canada, personal communication), suggesting no unusual additional mortality in the population during that period.

In a routine veterinary screen, we found virus neutralizing antibodies to CDV in 64% of grey seal (16/25) and 60% of harbour seal (3/5) serum samples collected from harbour and grey seals on Sable Island, Nova Scotia, Canada (43°55'N; 60°00'W) in January of 1989. In addition, two out of five serum samples from captive adult female harbour seals at Dalhousie University (Halifax, Nova Scotia) showed the presence of CDV-neutralizing antibodies. Captured on Sable Island in May of 1988, these seals showed no signs of illness in captivity, and were isolated for the five-month period before blood was sampled, suggesting infection before that time.

In an effort to identify the virus that had induced these antibodies, and lacking adequate volumes of the original samples from 1988 and 1989, we collected further serum samples from nine adult female harbour seals on Sable Island in May of 1991. These samples were first screened in a CDV enzyme-linked immunosorbent assay (ELISA) described elsewhere (268). Again, CDV-positive samples were found: three of the nine serum samples were ELISA-positive (titre >40). These three samples, along with one of the negative samples, were tested for antibody specificity in virus neutralization (VN) assays. Although antibodies to different morbilliviruses cross-react

to a certain degree in this assay, their relative specificities can be estimated by differential VN assays (265). We used a panel of eight members or provisional members of the genus *Morbillivirus* in the VN assays. The panel tested included CDV, the previously identified seal morbilliviruses PDV-1 and PDV-2, the recently isolated porpoise and dolphin morbilliviruses (PMV and DMV) (14), measles virus (MV Bussel strain), rinderpest virus (RPV RBOK strain), and peste des petits ruminants (PPRV Nigeria 75-1). Virus neutralization assays were undertaken as described elsewhere (265), with titres reflecting the ability of heat-inactivated serum to neutralize 30 $TCID_{50}$ of virus cultured in Vero cells. Whereas the control serum (CDV ELISA-negative) did not neutralize any of the viruses, all three CDV-ELISA positive serum samples showed neutralizing activity to most or all of the morbilliviruses tested. Although there were considerable differences among the titres, they were consistently highest against PDV-1. Transformation of titre values on the basis of PDV-1 = 1.0 demonstrates the relative specificity of the serum antibodies in the Canadian harbour seals for the viruses tested (Figure 5).

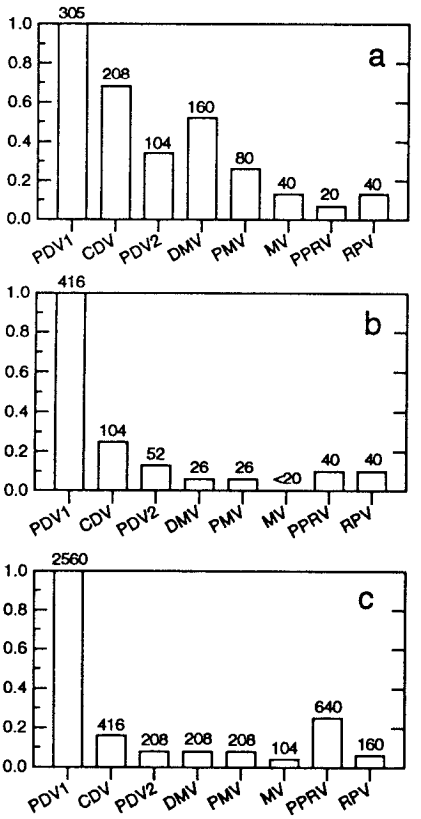

Figure 5: Virus neutralizing serum antibody titres of three female harbour seals sampled on Sable Island, Canada, against a panel of eight members or provisional members of the genus *Morbillivirus* in 1991.

The serological data suggest that the virus which infected the Canadian seals is most closely related, if not identical, to the virus which caused the 1988 mass mortality in Europe. Our collective results also suggest that the virus was enzootic in the harbour and probably grey seal populations of southeastern Canada. It has probably been so since May of 1988 or before, although we cannot exclude the possibility that a different morbillivirus had induced the antibodies found in the samples from 1988 and 1989. Since the European outbreak began in the spring of 1988, further questions may be posed about the origin of PDV-1. Dietz and others (72) found canine distemper neutralizing antibodies in 12 out of 40 harp seals (*Phoca groenlandica*) from the west coast of Greenland in 1985-86. The authors suggested that the virus responsible for these antibodies was PDV-1, and the subsequent mass migration of harp seals in 1986-87 southwards to northern Europe may have led to the 1988 epizootic. Aside from one low-titred sample collected from a harbour seal in 1986, no antibodies against CDV were found in European harbour seals before 1988 (188).

Available data indicate that there are approximately 13,000 harbour seals (163), 100,000 grey seals (293), and 2,500,000 harp seals (201) in eastern Canadian waters, representing a significant pool available for virus infection. Given our evidence of a past PDV-1 like infection among these seals, the following questions arose:

* why was there no mass mortality or other sign of disease observed in Canadian seals?
* when and how was the PDV-1-like virus introduced into the Canadian populations of harbour and grey seals?
* did harp seals play a role as a reservoir and carrier, bridging the seal populations of Europe and North America?

More extensive historical and epidemiological mapping of antibody profiles from seals in Greenland and North America would help to define the origin and movement of distemper viruses in seals. In fact, PDV-1 specific antibodies have recently been detected in serum samples collected on the east coast of North America from harbour seals in the early 1980s (A. Osterhaus, personal communication). Serum antibodies to morbilliviruses have recently been found in different marine mammal species inhabiting North American waters (78,93). The enzootic nature of PDV-1 in North American pinnipeds may explain why little or no morbillivirus-associated mortality was observed. The introduction of morbilliviruses into naive populations has been shown to lead to events of mass mortality. Clear examples of this phenomenon include large-scale mortalities among native North Americans related to the introduction of measles virus by early European explorers and settlers (178) or distemper virus in Serengeti's lions (108). On the other hand, differences between conditions during the infection in North America and in Europe may have led to a differential effect of the same

virus. One may hypothesize about the role of environmental conditions such as food availability or climatological factors, differences in population density, and genetic differences in the host seal populations (73,150). A possible difference in PDV strains between North America and Europe could also have led to differences in the outcome of epizootics. Finally, the contribution of immunotoxic compounds found at relatively high levels in European seals to the course of the 1988 PDV-1 epizootic has been the subject of extensive speculation (104). Studies presented in this thesis have specifically addressed the relationship between dietary exposure to complex mixtures of environmental contaminants as found in European coastal waters and immune function in harbour seals.

Outline of this thesis

In this thesis, the effects of environmental contaminants on immune function in harbour seals were studied. We initially established that PDV or a very similar virus had infected relatively uncontaminated harbour seals on Sable Island, Canada, without leading to any evident mass mortality (this chapter). Since little information existed on immune function in the harbour seal, we undertook preliminary studies on Sable Island in order to apply and adapt existing immunological techniques to a carefully controlled group of free-ranging seals (Chapter 2). We were able to demonstrate that existing methods can provide valuable information on the developing immune system of harbour seals. A broad approach was used to evaluate both functional aspects and maternal transfer of immunity in the newborn harbour seal pup. We subsequently initiated a captive feeding project in The Netherlands in which two groups of 11 harbour seals each were fed either herring from the relatively uncontaminated Atlantic Ocean or herring from the contaminated Baltic Sea. We routinely monitored immune function parameters in these animals by studying NK cell activity (Chapter 3 and 4), specific T-lymphocyte function, *in vitro* (Chapter 3 and 5), and delayed-type hypersensitivity (DTH) responses *in vivo* (Chapter 6).

Since obvious legal and ethical constraints limited us from carrying out challenge tests in the seals, we carried out two parallel feeding experiments using laboratory rats. In the first of these, recently weaned rats were fed a freeze-dried diet of the same Atlantic and Baltic herring used in the seal study for a period of 130 days, at which point immune function and host resistance to rat cytomegalovirus (RCMV) were assessed (Chapter 7). Rats were exposed to the same contaminant profile and a similar contaminant level as the seals, with a correction for body weight. The availability of specific reagents enable the evaluation of thymus and spleen lymphocyte subpopulations. Since the developing immune system has been shown to be particularly sensitive to the toxic actions of PHAHs, and seals inhabiting contaminated areas are also exposed perinatally, we carried out a second experiment in which

pregnant rats were given oil extracted from the two herring diets between day 6 of gestation and the weaning of the rat pups (Chapter 8). During this time, the developing rat fetuses, and subsequently, the nursing pups, were exposed to the mixture of contaminants present in the Baltic Sea herring and, to a lesser extent, the Atlantic Ocean herring. While attention has been paid to the presence of a complex mixture of environmental contaminants in the Baltic Sea herring, we have largely concentrated on the *Ah*-related PHAH components because of the unequivocal evidence of their immunotoxic potential and their presence in high concentrations in marine mammals.

Relative immunocompetence of the newborn harbour seal, Phoca vitulina

Peter S. Ross, Rik L. De Swart,
Ilona K.G. Visser, Lies J. Vedder, Willem Murk,
W. Don Bowen, and Albert D.M.E. Osterhaus

Veterinary Immunology and Immunopathology 42: 331-348 (1994)

Abstract

The immune system of many mammalian species is not fully developed at birth, with newborns obtaining temporary immunological protection from maternal antibodies. Little is known of the immune system of the harbour seal, and developmental aspects of its immune system have not been systematically studied. We collected blood and milk samples from nine free-ranging mother-pup pairs throughout the lactation period on Sable Island, Canada, in an effort to characterize developmental aspects of the immune system of this newborn pinniped. Pup lymphocytes responded stronger to the mitogens Concanavalin A, Phytohaemagglutinin, and pokeweed mitogen than the lymphocytes of their mothers. In contrast to newborn cats and dogs, newborn seal pups developed high specific antibody responses after immunization with an inactivated rabies vaccine. Circulating levels of total IgG in newborn pups were low (3% of maternal levels), but increased rapidly after colostrum intake (to 65% of maternal levels after 15 days). A similar pattern of increase in pup serum was observed for phocine distemper virus specific antibodies which had been detected in the serum and milk of mothers, suggesting that the transfer of colostral antibodies is an important feature of temporary protection for the pup. We speculate that the relative immunocompetence of the harbour seal at birth reflects an adaptation to its relatively short nursing period and limited maternal care.

Introduction

Harbour seals (*Phoca vitulina*) are found in mid to northern latitudes in the Pacific and the Atlantic Oceans. They spend much of their time at sea, and come ashore predominantly during the breeding season in May to June in North America and June to July in Europe. The newborn harbour seal spends approximately 24 days with its mother, during which time the pup grows rapidly, with a mean mass gain of 800 g per day (28). At birth, the seal pup may be subjected to often severe environmental conditions. Weather conditions and topography, in particular, affect their chances of survival (11,22,232). The influence of infectious diseases on the survival of young pinnipeds is less well documented, but many bacterial and viral infections have been identified among seal pups (10,11,267). How these infections relate to the immunological status of the seals is more difficult to ascertain, as little information exists.

Interest in the immunology of marine mammals increased considerably following the 1988 phocine distemper virus (PDV) epizootic among harbour seals (*Phoca vitulina*) and grey seals (*Halichoerus grypus*) in Europe, and concerns about the possible role of environmental pollutant-induced immunosuppression (62,73,186).

Additional mass mortalities among Baikal seals (*Phoca sibirica*) in 1987 (98,187), and striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea in 1990-91 (256,267), have generated further scientific interest in the state of health of marine mammals, and in factors which may predispose them to disease.

Mammals are generally born with a poor specific immunologic response to pathogens, and rely upon a combination of non-specific defences and temporary protection from passively acquired maternal antibodies for protection against early infections (13). Many studies have identified clear deficiencies in the immune system of mammalian neonates, including low lymphokine production (288), poor neutrophil chemotaxis (218), poorly developed responses of lymphocytes to mitogens (94,118), and inadequate antibody responses to antigens (130,152). A suppressive action of T-cells may play a role in the diminished immune response of newborns (50). Macrophage function also appears to be impaired, possibly as a result of inhibitory factors present in the serum of the neonate (166). However, some studies document relatively good responses for some parameters, including mitogen-induced lymphocyte proliferation (106,289). The lack of specific memory cells in newborn mammals is, in part, made up for by the transfer of specific antibodies from the mother. This transfer may take place transplacentally, via colostrum, or a combination of both, depending on the type of placentation in different mammalian species. The specificities of these transferred antibodies are a direct reflection of those present in the mother's serum.

In a study of the northern fur seal (*Callorhinus ursinus*), Cavagnolo and Vedros (41) found low levels of immunoglobulins G, M, and A (IgG, IgM, IgA respectively) in pup serum at birth, and steadily rising levels during early life. Carter *et al.* (39) found low IgG levels in grey seal pups aged less than one week, and slowly rising levels over the first six weeks of life. Information on the cellular immune responses of seals is limited, but in a preliminary study, we showed that perpheral blood mononuclear cells (PBMC) from harbour seal pups showed strong proliferative responses to Concanavalin A (Con A) as compared to lymphocytes of their mothers at mid-lactation (209). No information exists as to the ability of young pinnipeds to mount an immunological response to antigens *in vivo*, or as to the relative importance of maternally-derived immunoglobulins to pup serum antibody levels.

The study described here focuses on the development of passively and actively acquired immunocompetence in newborn harbour seals during the lactation period, and the ability of seal pups to mount antibody responses is compared with that of young dogs and cats.

Methods

Sample collection

Free-ranging harbour seal mothers and their pups were studied during May-June 1991 on Sable Island, Nova Scotia, Canada (43°55'N; 60°00' W). Daily monitoring

and tagging of all newborn pups on Sable Island enabled us to capture pups which had yet to suckle for the first time, as well as their mothers. To confirm that pups had not yet suckled, a 0.64 cm diameter KI-100 foal stomach tube (Kalaijan Industries, Inc., Long Beach, CA, U.S.A.) was inserted into the stomach of the pup, and a 60 cc syringe (Becton Dickinson, Rutherford, NJ, U.S.A.) used to evacuate any contents. Pups with colostrum present in their stomachs were excluded from the study. Those pups with empty stomachs were marked, weighed and their blood sampled from the extradural vein. Three 10 ml heparinized Vacutainers and one 2 ml ethylenediaminetetraacetic acid (EDTA) tube (Becton Dickinson) were filled and inverted gently 15 times. The same series of blood samples was also taken from the mothers. Following blood sampling, mothers were lightly sedated with 10-15 mg of Diazepam (Sabex, Boucherville, Qc, Canada), administered intravenously, and given 15-30 IU of Oxytocin (Phoenix Pharmaceutical, Inc., St. Joseph, MI, U.S.A.) in the gluteal muscle to permit a milk sample to be collected. At each capture, a 30 ml sample of milk was evacuated by suction using a 60 cc syringe with the end removed. Pairs were recaptured for sampling at 2, 5, 10, and 15 days post-parturition. Mothers and pups were held for about 10-15 minutes at each capture and released together. Body weights of harbour seal pups doubled by 15 days of age (from a mean (±SE) of 10.9 ± 0.4 kg to 21.7 ± 1.1 kg). Thus, healthy pups with normal weight gain patterns were studied.

Milk was aliquoted in 5 ml samples and stored at -20°C until analysis. Blood samples were kept at room temperature until processing in a field laboratory within four hours. EDTA samples were used for a total leukocyte count. Erythrocytes were lysed using 50 µl whole blood in 1 ml 2% acetic acid solution, and leukocytes counted using a haemocytometer (Spotlite, American Hospital Supply Corporation, McGaw Park, IL, U.S.A.). One drop of blood was smeared onto a glass microscope slide, air dried, fixed with 95% methanol and stored at room temperature until stained with May-Grünwald/Giemsa (123). Leukocyte subpopulations were classified as band cells, neutrophils, lymphocytes, monocytes, eosinophils, and basophils, and counted.

Peripheral blood mononuclear cells (PBMC) were collected and cryopreserved at two points in lactation from both mothers and pups: at birth and 10 days post-parturition. Heparinized blood was layered on a Histopaque-1077 (Sigma Chemical Company, St. Louis, MO, USA) and centrifuged for 30 minutes at 400xg. The PBMC layer was aspirated and cryopreserved as outlined elsewhere (209). Briefly, cells were suspended in RPMI-1640 medium (Sigma Chemical Company) containing 20% heat inactivated fetal calf serum (FCS), 10% dimethylsulfoxide, 100 IU/ml penicillin and 100 µg/ml streptomycin. They were then frozen at a controlled rate in a portable liquid nitrogen container and stored at -140°C or colder until assayed.

Lymphocyte stimulation assays

Cryopreserved PBMC were thawed quickly at 37°C and resuspended in 10 ml ice cold culture medium consisting of RPMI-1640 supplemented with 10% heat inactivated FCS, penicillin (100 IU/ml), streptomycin (100 µg/ml), L-glutamine (2 mM) and 2-mercaptoethanol (2 x 10^{-5} M). Cells were washed once (10 minutes at 400xg), resuspended in 10 ml culture medium, and kept at 37°C for four hours. Viable cells were counted on the basis of trypan blue exclusion. PBMC were cultured in 96-well, round-bottomed culture plates (10^5 cells/well; Greiner Labor Technik, Nurtingen, Germany) in culture medium at 37°C in a humidified atmosphere of 5% CO_2. Mitogens were added at concentrations found to be optimal in preliminary experiments on cryopreserved cells and in previous studies of harbour seal PBMC (58,209). These were 2.5 µg/ml Con A (ICN Biomedicals, Costa Mesa, CA, USA), 20 µg/ml phytohaemagglutinin (PHA-M; Boehringer, Mannheim, Germany), and 1.0 µg/ml pokeweed mitogen (PWM; ICN Biomedicals). PBMC were cultured for 96 hours and ^{3}H-thymidine was added for the last 18 hours. Cells were harvested and cell associated radioactivity was measured in a Betaplate liquid scintillation counter (LKB, Wallac, Finland).

Measurement of total immunoglobulin concentrations

A protein A (from *Staphylococcus aureus;* Boehringer) based enzyme-linked immunosorbent assay (ELISA) was used to estimate total IgG levels in the serum and milk of seals. In a variation of the previously described system (209), high-binding, flat-bottomed 96-well ELISA plates (Costar, Cambridge, MA, USA) were coated with 1.0 µg/ml of protein A (Boehringer) in carbonate buffer (pH 9.6) at 37°C for 2 hours. Plates were washed twice in 0.01% Tween 20-water solution (Tween 20: Merck-Schuchardt, München, Germany), and blocked at 37°C for one hour with ELISA buffer consisting of 1% bovine serum albumin (BSA) Fraction V (Sigma), 0.1% Tween 20 (Merck-Schuchardt), 0.1% Triton (Merck-Schuchardt), and 5% NaCl in phosphate buffered saline (PBS; 0.001 M). Twofold serial dilutions of serum samples in ELISA buffer were added to the wells, and plates incubated at 37°C for 1 hour. Plates were washed twice, and protein A coupled to horseradish peroxidase (Amersham International, Amersham, United Kingdom) in ELISA buffer was added to each well. Plates were incubated at 37°C for 1 hour, washed twice, and developed as described elsewhere (35). Plates were read at 450 nm using a Titertek Multiskan MCC-340 spectrophotometer.

Seal serum samples were run over a sodium dodecyl sulphate polyacrylomide gel electrophoresis (SDS-PAGE) gel. One ml of NH_4SO_4 precipitated harbour seal serum (prepared as outlined in 103) was buffered with 250 µl phosphate buffer (pH 8.1, 0.5 M) and applied to a 3.5 ml protein A-Sepharose (CL-48; Pharmacia, Uppsala, Sweden) column as outlined in (79). The non-binding fraction and citrate (pH 3.0, 0.1 M)-eluted binding fraction were collected separately. These two fractions, plus seal

serum and high molecular weight protein standards (BRL), were run in a PhastGel (Pharmacia) homogenous 12.5% SDS-PAGE and stained with Coomassie Brilliant Blue.

Measurement of naturally occurring, virus-specific antibodies

Seal serum and milk samples were tested for antibodies against morbilliviruses with a canine distemper virus (CDV) ELISA, using methods described elsewhere (268). PDV was used in VN assays as described previously (265). Briefly, the VN assays tested the ability of heat inactivated serum sample dilutions to neutralize 30 $TCID_{50}$ of virus cultured in Vero cells. Vero cell culture followed a 1.5 hour coincubation of 30 $TCID_{50}$ PDV with twofold serial dilutions of serum starting at 1:20. VN assays could not be performed on milk samples because of the toxicity of the samples to Vero cells.

Immunization with rabies vaccine

Four age groups of seal pups on Sable Island were immunized with an inactivated human rabies vaccine (Connaught Laboratories Ltd., Toronto, Canada) without adjuvant. Pups independent of the mother-pup study were used in this experiment. Newborn (n=5), three-day-old (n=6), six-day-old (n=6), and ten-day-old (n=6) pups were immunized, and blood was sampled precisely ten days later. A serum separation tube (Becton Dickinson) was filled with blood taken from the extradural vein. The sample was centrifuged for 20 minutes at 500xg within four hours of sampling, and the serum was aspirated and frozen at -20°C for later assay for rabies virus specific antibodies. In a follow-up experiment, 6 healthy harbour seals older than eight months, that were housed at the Seal Rehabilitation and Research Centre, were immunized and sampled following the same protocol as used on Sable Island. These served as a reference group for the immunized pups.

In two parallel experiments, specific pathogen free (SPF) kittens were immunized with the same rabies vaccine and SPF Beagle dog pups were immunized with another rabies vaccine with a similar potency (National Institute of Public Health and the Environment, Bilthoven, The Netherlands) without adjuvant in an effort to compare the patterns of humoral responses of seal pups to these better studied species of carnivores. Dog pups and kittens were rabies antibody negative as determined in an ELISA pre-screen. Dog pups were immunized at three different ages: newborn (n=3), six days (n=3), and ten days (n=3) post-parturition, and a group older than eight months (n=4). SPF kittens were immunized at four different ages: newborn (n=4), three days (n=5), six days (n=5), and ten days (n=5) post-parturition. Serum samples were collected ten days following immunization, and were frozen until assayed for rabies virus specific antibodies.

Measurement of rabies virus specific serum antibodies

Serum samples of immunized animals were assayed for rabies virus-specific antibodies using a rapid focus fluorescent inhibition test (RFFIT) for the detection of rabies virus neutralizing antibodies. The RFFIT was undertaken as described elsewhere (292), using the Pasteur strain of rabies virus, and the results were expressed in international units.

Results

Profiles of circulating leukocytes

Pups were born with significantly lower total leukocyte counts than their mothers (Figure 1). However, pups acquired similar total leukocyte counts by two days of age. Differential counts suggested that the observed rise between birth and two days

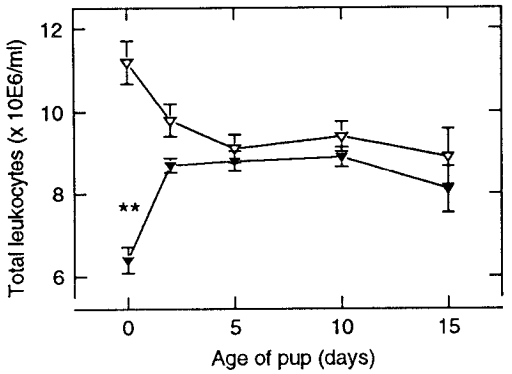

Figure 1: Mean total leukocyte counts (± SE) in harbour seal pups (solid symbols) and their mothers (open symbols) during the first 15 days of the nursing period (base on a sample size of nine mothers and nine pups). Significant differences between mothers and pups are indicated for time point by *($p<0.05$) or **($p<0.01$), as measured by a *t*-test.

of age reflected an increase in the numbers of lymphocytes and neutrophils, and to a lesser extent in the numbers of monocytes (Figure 2). Low numbers of circulating neutrophils, lymphocytes and monocytes contributed to the low total leukocyte counts at birth, with a sudden rise in neutrophils taking place on day two post parturition. However, the overall increase in lymphocyte and monocyte numbers throughout lactation appeared to be the principal factor in the increased total leukocyte count, as the percentage of neutrophils declined later in the lactation period. Eosinophil counts were low when compared to other cell types, and showed fluctuation through lactation.

Lymphocyte proliferation assays

The functionality of harbour seal lymphocytes was tested by determining their proliferative capacity upon stimulation with three different mitogens. Harbour seal pup PBMC showed two to four times higher proliferative responses to Con A, PHA and PWM than those of their mothers at birth and at ten days of age (Figure 3).

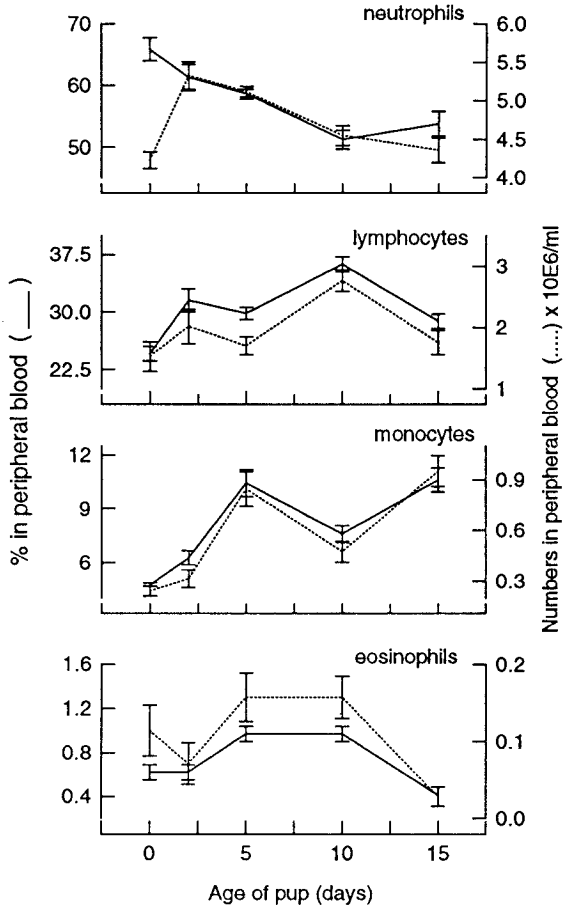

Figure 2: Differential leukocyte counts of harbour seal pups (mean ± SE; n=9) during the first 15 days of the nursing period, expressed as percentages of total cells counted (solid lines), and as concentration in blood (dotted lines).

Responses of pup PBMC tended to be higher at ten days of age than at birth, though these differences were not significant.

Transfer of maternal protein A binding immunoglobulins to pups

The SDS-PAGE of seal serum enabled us to assess the efficacy of protein A in binding seal IgG heavy and light chains. Protein A was shown to bind the majority of IgG in harbour seal serum, with the non-binding fraction having little detectable light or heavy chains of IgG (Figure 4). Pups were born with about 3% of maternal serum protein A binding IgG concentrations, with this figure rising to about 65% at 15 days of age (Figure 5). Colostrum contained about 45% of protein A binding IgG

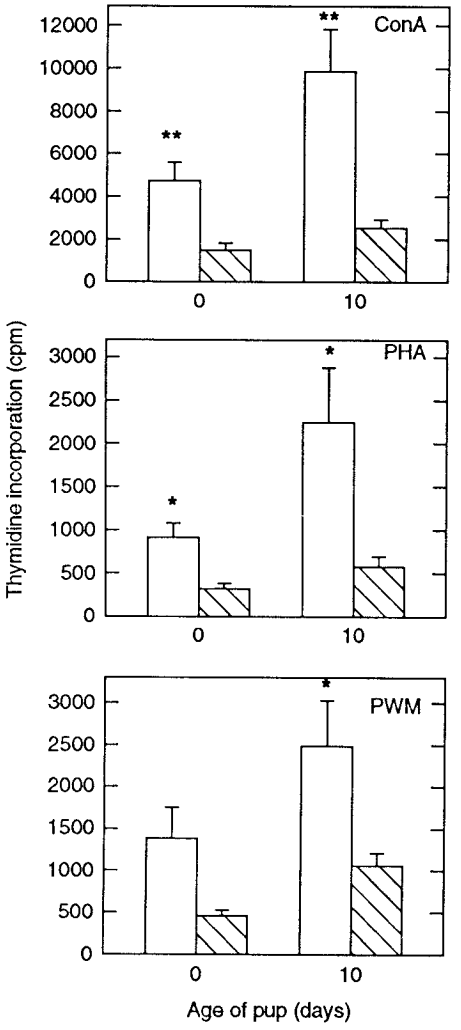

Figure 3: Mean proliferative responses (± SE) of harbour seal PBMC *in vitro* from pups (open bars; n=8 at day 0; n=5 at day 10) at two points during the nursing period, and from their mothers (hatched bars; n=7 at day 0; n=8 at day 10). PBMC were stimulated with the plant lectins Con A (2.5 µg/ml), PHA (20 µg/ml), and PWM (1.0 µg/ml) and results are expressed as mean ± SE counts per minute (cpm) of ^{3}H-thymidine incorporation. Significant differences between mothers and pups for time points are indicated by * ($p<0.05$) or ** ($p<0.01$), as measured by a *t*-test.

levels as compared to those found in the maternal serum, whereas milk later in lactation had relatively low concentrations, declining to about 5%.

Transfer of specific antibodies to pups

To investigate in more detail the contribution of maternal antibodies to the serum antibodies in the pups, we screened mother's serum for specific antibodies acquired through a previous infection, and subsequently screened their pups in order to differentiate between antibody transfer via colostrum and milk and antibody

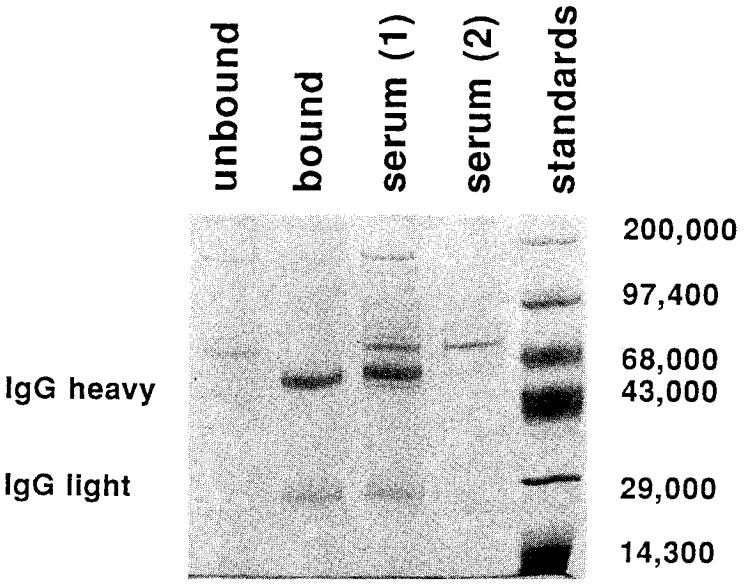

Figure 4: SDS-PAGE of seal serum, with fractions including protein A bound, protein A unbound, two samples of harbour seal serum, and standards. The heavy and light chain fractions of IgG can be clearly seen in the protein A bound fraction, but are not visible in the unbound fraction.

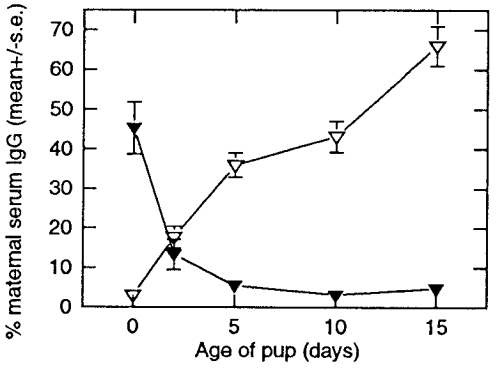

Figure 5: Mean ± SE IgG levels in pup serum (open symbols; n=9) and in their mother's milk (closed symbols; n=9) from birth through 15 days of age, expressed as a percentage of the mean maternal IgG concentration. IgG was measured by a protein A ELISA.

produced by the pup. Three of the nine mothers studied had CDV reactive serum antibodies as demonstrated by the CDV ELISA without showing evidence of infection at the time of sampling (results not shown). Pups of these mothers had very low CDV ELISA titres at birth (mean of 2% of maternal titres), but by 10-15 days of age, the

same pups had titres approaching those of their mothers (mean of 63% of maternal titres). Colostrum had a high concentration of specific antibodies, with levels at 96% of those in maternal serum, whereas milk at day 15 post-parturition contained 32%. Virus neutralization data substantiated the protein A based CDV ELISA data, with pups being born with low levels of PDV-neutralizing antibodies (mean of 9% of maternal serum values), and attaining maternal levels towards ten days of age (Figure 6).

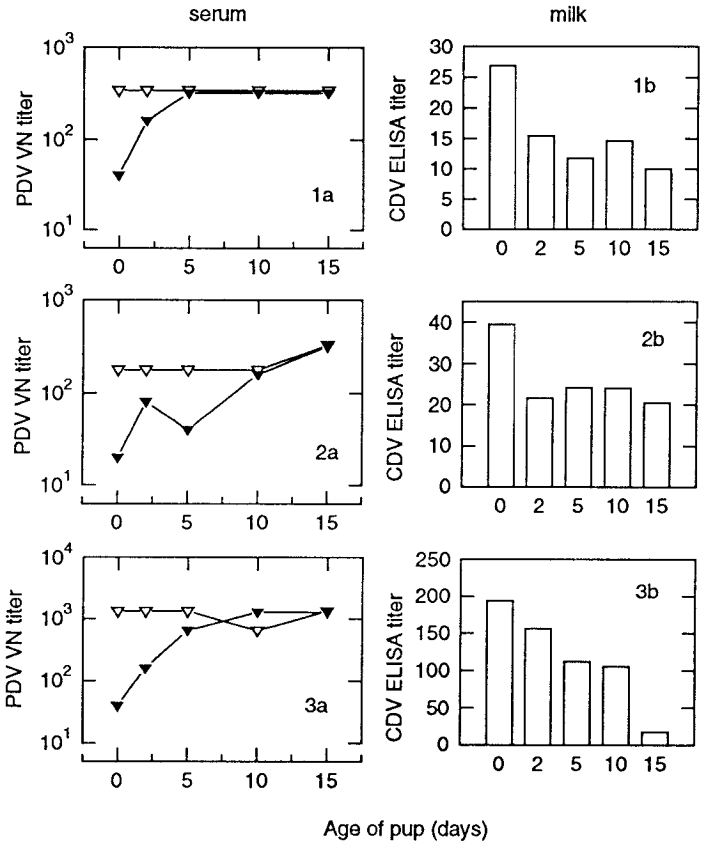

Figure 6: Virus neutralization (VN) titres against PDV-1 in three CDV ELISA positive harbour seal mothers (open symbols) and their pups (closed symbols) from birth to 15 days of age (mother-pup pairs shown as 1a, 2a, 3a), and CDV ELISA titres for milk (1b, 2b, 3b).

Antibody response to rabies immunization

Seal pups responded strongly to rabies immunization within the 10 day incubation time, as measured by the RFFIT (Figure 7). Seal pups in the three-day-old age group were best able to mount a response, having a titre which was significantly higher than that of the older seals tested. The pattern of antibody responses clearly contrasts those observed in the dogs and the cats. Dog pups of the same age categories (no three-day-old group was tested) did not mount strong rabies virus specific

responses compared to adult dogs (Figure 7). Like dog pups, young kittens displayed a relatively poor response to rabies immunization at birth relative to seal pups, with a slight increase at ten days of age (Figure 7).

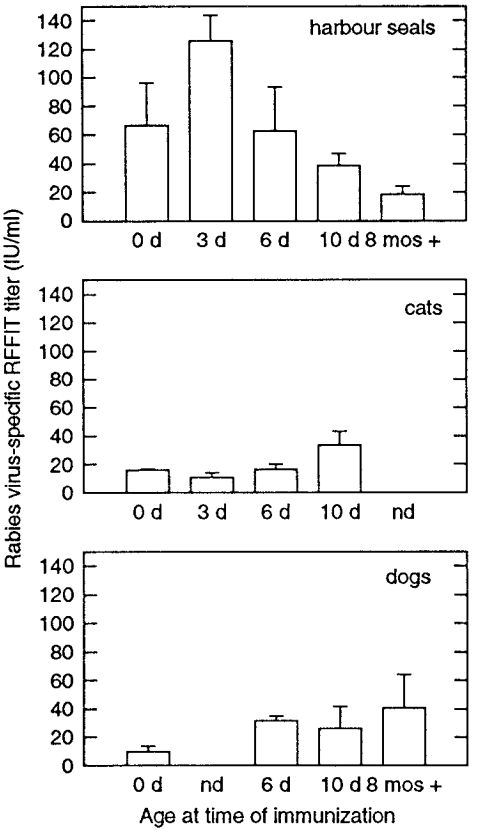

Figure 7: Antibody responses of harbour seals (n=5, 6, 6, 6 for each age group, respectively), kittens (n=4, 5, 5, 5 for each age group, respectively), and Beagle pups (n=3 for each age group, and n=4 for the group aged 8 months or over) to inactivated human rabies vaccine ten days following immunization. Values represent mean ± SE International Units (IU) as determined by a rapid focus fluorescence inhibition test (RFFIT). nd = not done. Seal pups aged three days had a significantly higher antibody response than those aged eight months or more ($p<0.001$).

Discussion

The results of our *in vitro* and *in vivo* tests suggest that the newborn harbour seal is born with a functionally competent immune system, although serum antibodies of the IgG class are virtually absent. High levels of total protein A binding IgG and PDV-specific antibodies in milk at parturition suggest that colostrum is an important source of IgG for newborn harbour seals. The low levels of total IgG in the serum of seal pups at birth, followed by their rapid increase during lactation provide further support for this view. Protein A has been used extensively in immunologic studies, and has been shown to bind to the Fc region of IgG in many mammals (97,148). It is also

routinely used for the measurement of both specific (268) and total IgG levels (209) in the harbour seal. Our SDS-PAGE results support the use of protein A as an immunologic reagent in the harbour seal, as the majority of IgG in seal serum bound to protein A beads. Although all mammals rely on maternal immunoglobulins for a certain degree of temporary protection after birth, the relative importance of transplacental versus colostral transfer varies according to type of placentation. Transplacental transfer of IgG in the harbour seal seems to be minimal, consistent with their endotheliochorial placentation (71) which is largely impermeable to IgG (250). Dogs and cats are other examples of mammals with endotheliochorial placentation, and are born with 5-10% of maternal serum IgG concentrations (250). However, on the basis of total serum levels of IgG in harbour seal pups, we could not initially exclude that the rapid rise in IgG levels shortly after birth was due to endogenously-produced immunoglobulins. We therefore measured antigen-specific antibodies in milk and pup serum where the mother had been identified as antibody-positive as a consequence of a previous infection and their pups showed no signs of infection. The patterns of specific antibody titres in colostrum/milk and in pup serum, as measured by both a protein A based ELISA and a biologically based VN assay, strongly suggest that maternal immunoglobulins are responsible for a large part of the observed increases in pup serum. As the pattern of these maternally derived specific antibodies was similar to our observed pattern of total IgG increase in the pups, it seems reasonable to conclude that the serum IgG in the 15-day-old pups originates predominantly from the absorption of colostral/milk antibodies and that there is limited endogenous production of IgG during that time.

The increase in IgG levels observed in our study of harbour seal pups during lactation is more dramatic than those reported for other pinniped species. Cavagnolo and Vedros (41) found that IgG increased to 26% of maternal levels in the northern fur seal by 35 days of age. This may be related to species differences in maternal lactation strategy, as the northern fur seal pup nurses less frequently, and over a longer period of three months (92). Serum levels at birth were comparable to those in our study, being 5% of maternal values. Carter *et al.* (39) also found a slow rate of increase in IgG levels in grey seal pups, attaining only 15% of maternal values by 4-5 weeks of age. Sanders and Ortiz (216) found IgG levels to rise from 9% at two days of age to 39% of maternal values at weaning in the northern elephant seal, *Mirounga angustirostris.*

The protection of young mammals from virus infections by specific maternal antibodies has been demonstrated in several species (131,165,169,192,238). The three harbour seal newborns that obtained PDV-specific antibodies via colostrum/milk in our study have essentially been passively immunized against infection by morbilliviruses. We previously showed that the virus which elicited these antibodies in Canadian waters was closely related, if not identical, to PDV, the virus responsible

for the 1988 European epizootic among harbour seals (210). Although little is known of patterns in seals, in other mammals the half life of maternal IgG in newborn serum averages approximately 12-18 days. This implies that protection against specific pathogens can last several months, depending on the original titre in the milk (192,289).

The strong proliferative responses of pup PBMC to the three mitogens reflect the capacity of both T- and B-lymphocytes of the newborn seal to respond to polyclonal antigens. Con A and PHA elicit specific T-lymphocyte responses in mammals, while PWM stimulate both B- and T-lymphocytes (99,172). The specificity of these mitogens in stimulating harbour seal T- and B-lymphocytes has recently been confirmed by De Swart *et al.* (58). Responses of newborn lymphocytes in other mammalian species vary, but are not completely consistent with the patterns observed in our study seals. We must assume that the responses of mothers' PBMC to mitogens are representative of the "normal" activity of adult seal PBMC, but cannot entirely rule out that the physiological and/or hormonal stress of the breeding period affected their immune responses (209). In dogs, lymphocyte responses to PHA and PWM are significantly lower at birth than in adults (145). Gerber and Brown (94) found canine lymphocytes to be non-responsive to PHA from birth to four weeks of age. Holan *et al.* (118) found that newborn mice lymphocytes were unresponsive to Con A, and cited immature T-lymphocytes and neonatal suppressor cell activity. Hammerberg *et al.* (105) found that newborn pigs had relatively low PWM-induced lymphocyte proliferation, and that other aspects of humoral immunity were also impaired. Studies of human newborns, however, showed that their lymphocytes respond well to mitogens (7,36,110).

The ability of the newborn harbour seal pup to respond to a primary antigen, tested here by the antibody response after immunization with inactivated rabies vaccine, was strong, whether it be compared to the response found in older seals (>eight months of age), or pups and kittens of any age. While dog bitches had been identified as rabies antibody negative in an ELISA pre-screen, subsequent RFFIT identified very low levels of rabies virus neutralizing antibodies in their serum. These may have been the result of a vaccination of the bitches more than five years previously, and may have resulted in very low levels of VN antibodies in the serum of the dog pups (0.2 IU/ml or less) prior to the immunization experiment. Although we cannot entirely exclude a negative influence of these maternal antibodies on the responses of dog pups, the very low levels observed before immunization, coupled with the similar response pattern in kittens, suggest that the dog pups responded normally to immunization.

The apparent immunocompetence of the harbour seal neonate relative to other mammals may reflect a combination of its large birth mass relative to mothers, its long gestation period (estimated at 9-10 months (27)), its short period of suckling (mean

weaning age is 24 days), and its precocial nature at birth. This is in line with the observations of Banks (13), who noted that mammals with short gestation periods (i.e. less than 30 days) had poor immune responses compared to those with longer gestation periods. However, gestation length alone cannot fully explain immunological differences among newborn mammals. Humans, for example, have a gestation length similar to harbour seals, yet are born with some noted immunological deficiencies (8,288). The seal pup must independently cope with its environment within a very short time of birth, and an active cellular immune system, coupled with temporary protection from maternally derived antibodies may be a vital component of their precocial nature.

Conclusion

The newborn harbour seal is born with low levels of circulating immunoglobulins. The high colostral concentrations of total and specific immunoglobulins, and subsequent increase in pup serum concentrations through the nursing period suggest that obtaining colostrum early in lactation is an important aspect of temporary immunological defence. Newborn harbour seals appear to have a relatively competent immune system which is primed for antigenic challenge. This was evidenced by *in vivo* antibody responses to immunization with an inactivated rabies vaccine and by strong lymphocyte proliferation in response to *in vitro* stimulation with mitogens (Con A, PHA and PWM). The relative immunocompetence of the harbour seal pup appears to be in contrast with a number of observations of other mammalian species. We suggest that this reflects an evolutionary adaptation to its short nursing period and limited maternal care.

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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

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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 (164,196,244), 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 (96). 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 polychlorinated biphenyls PCBs (17,111,112,171). 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 (235). In a semi-field 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 (197).

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 (280). These chemicals include polychlorinated biphenyls (PCBs), dibenzo-*p*-dioxins (PCDDs) and dibenzofurans (PCDFs) (273), hexachlorobenzene (HCB) (269), dieldrin (86), ß-hexachloro-cyclohexane (ß-HCH) (53), and dichlorodiphenyl-trichloro-

ethane (DDT) (12). 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 (98,187,267), striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea from 1990 onward (75,267), and harbour seals in northwestern Europe in 1988 (189,267), has led to extensive speculation about the possible contribution of environmental pollutants to these outbreaks of infectious diseases, by causing an impairment of immune function (104,181,190,217,225). In addition, morbillivirus infections have been observed in seals inhabiting less polluted areas without causing any evident mortality (72,210).

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.

Methods

Seals and diets

In a semi-field 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 (seven females and four males in each group), and the diet of the first group was changed to herring caught in a polluted coastal area of the Baltic Sea (about 100 km off the southwest coast of Finland). The seals were housed at the Seal Rehabilitation and Research Centre in Pieterburen, in two similar basins with approximately 40 m^3 water and haul-out platforms of approximately 24 m^2.

Diets were similar as regards overall quality, and were both supplemented weekly with 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 (153,259). 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 (TEQs) were calculated using the international toxic equivalency factors (TEFs) for dioxins as reported by Van Zorge *et al.* (264), and the proposed TEFs for coplanar and mono-ortho coplanar PCBs as given by Safe (212), 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 (77). 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 (29). White blood cell (WBC) counts were determined in whole blood using EDTA as an anti-coagulant, with an automated haematology analyzer (Sysmex E-5000) with differentiation of leukocyte subsets. Samples were kept shielded from direct 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 (58), 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 (261). 10^6 YAC-1 cells were labelled 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 (58). 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) (58). Proliferation was quantified by measuring the incorporation of ^{3}H-labelled thymidine on day 4 for Con A, PWM and PHA, and on day 5 for LPS. Means of control cultures were subtracted from means of stimulated cultures prior to statistical analyses.

Statistical analysis

Accumulated longitudinal data were analyzed with ANOVA split plot analyses with time, sex and diet as factors (167), 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).

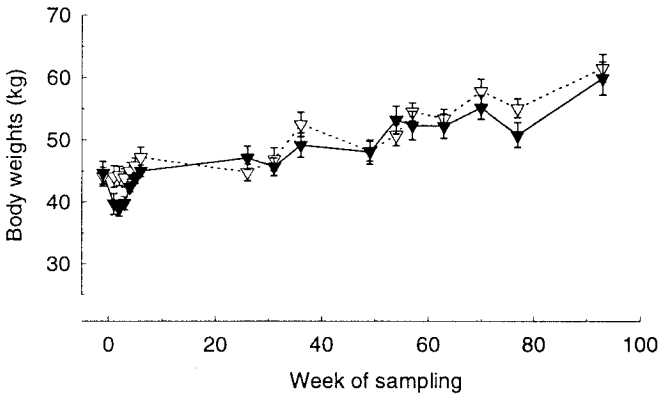

Figure 1: Mean body weights of harbour seals feeding on herring from the Baltic Sea (closed symbols) or the Atlantic Ocean (open symbols) over the course of the feeding experiment. Each group consists of seven females and four males. Vertical error bars indicate standard errors of the means. Week 0 represents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

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 *Ah*-receptor binding organochlorines in TCDD toxic equivalents were 288 ng TEQ per day per seal in group 1 and 29 ng TEQ per day per seal in group 2. Since the animals were fed in groups and not individually, only estimates of the daily intakes of organochlorines per seal could be determined.

Table 1: Estimated daily intakes of organochlorines by seals feeding on herring from the Baltic Sea (group 1) or the Atlantic Ocean (group 2) in µg/day and in ng TEQ day.

Compounds	Estimated daily intakes			
	Group 1		Group 2	
	µg/day	ng TEQ/day	µg/day	ng TEQ/day
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.* (259), 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.* (153); [3] n.a. = not applicable.

Comparison of haematological data

Vitamin A levels proved to be significantly lower in serum of the seals of the first group ($P<0.01$, Figure 2), confirming results of a previous experiment with a similar setup in which reproductive disorders had been observed (29,197). 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, PBMC were

isolated, and a series of *in vitro* functional immunological assays was carried out. 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 (31,193). 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 (29).

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 (284). 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 (58). T-cells, especially cytotoxic T-lymphocytes (CTLs), are known to be of crucial importance in the clearance of virus infections (81), which has also been documented for morbillivirus infections (254). 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 (261,273), and reduced proliferative responses of lymphocytes to mitogens have been observed after exposure to PCBs, PCDDs, PCDFs, Dieldrin, and ß-HCH (53,86,273).

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 (25). 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.

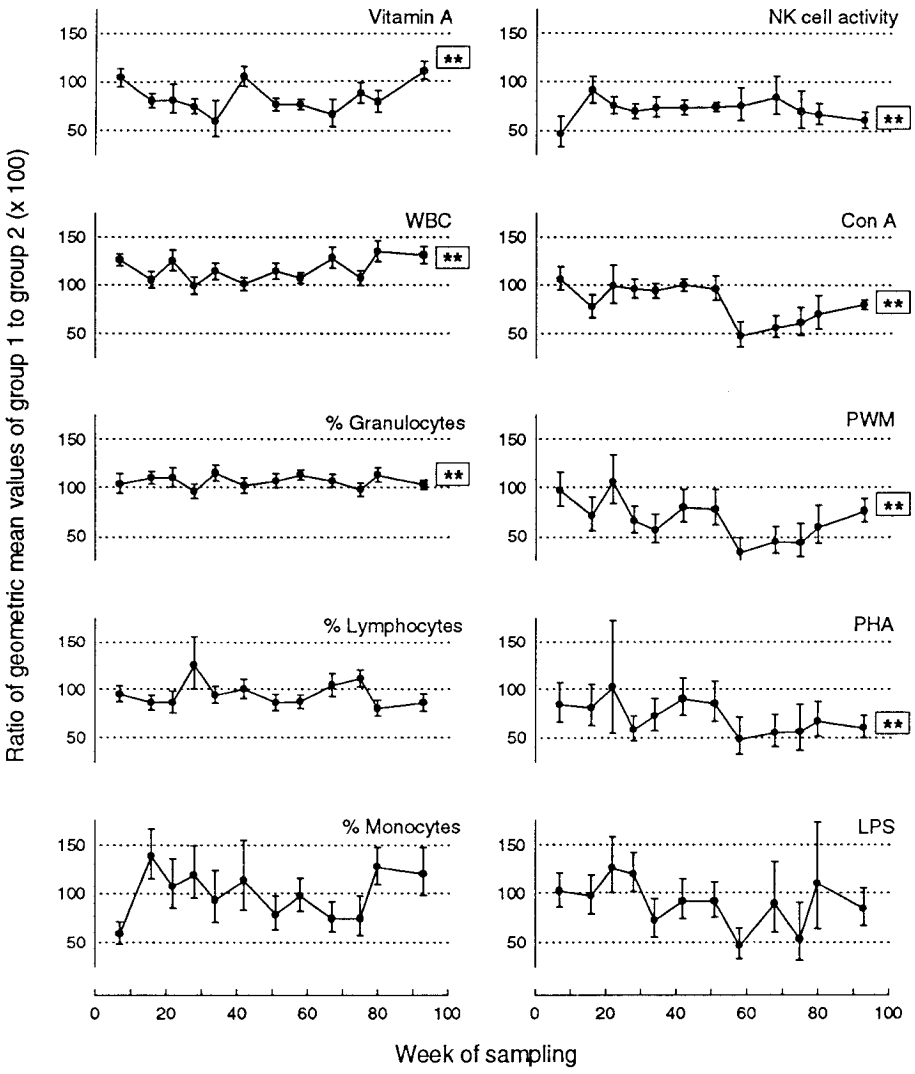

Figure 2: Differences in means of serum vitamin A levels, haematological and immunological values between harbour seals feeding on herring from the Baltic Sea (group 1) or the Atlantic Ocean (group 2). Values are shown as ratios of geometric mean values of group 1 (n=11) to group 2 (n=11). Vertical error bars are 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 is 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 ANOVA. Results of the same assays carried out 21 weeks before the start of the experiment led to the following ratios (± SE): 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.

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 (179). However, as both NK cells and T-cells play an important role in the immune response against virus infections, it is not unlikely that a functionally impaired NK and T-cell response led to increased susceptibility to morbillivirus infections in marine mammals, and thus contributed to the severity and extent of the recent epizootics.

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

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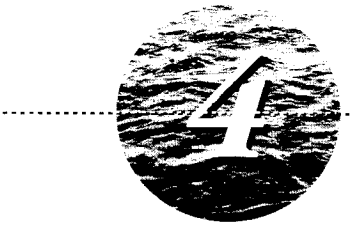

Suppression of natural killer cell activity in harbour seals (Phoca vitulina) fed Baltic Sea herring

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

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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 leukocytes 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 (73,185,267). 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 (154,256). The high trophic status of many marine mammal species predisposes these animals to accumulating high

concentrations of lipophilic organochlorines, including polychlorinated biphenyls (PCBs), dichlorodiphenyl-trichloro-ethane (DDT) and, to a lesser extent, polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) (225,226,243).

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 (197) and vitamin A and thyroid hormone levels (29). Organochlorine chemicals have been linked to skeletal lesions among grey (17) and harbour seals (171) in the Baltic Sea. PCBs have been correlated with pathological lesions which inhibited reproduction in ringed seals in the Baltic Sea (112). While contaminants have not been conclusively shown to have played a contributory role in virus epizootics (4,104,225), 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 (273). In preliminary findings, we observed a reduction in T lymphocyte responses and natural killer (NK) cell activity of PBMC isolated from harbour seals fed herring from the Baltic Sea as compared to controls (62), as were *in vivo* specific delayed-type hypersensitivity (DTH) and antibody responses to the antigen ovalbumin (204).

NK cells are lymphocytes which represent an important first line of defence against both virus infections and tumour cells (for reviews see 151,284). 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) (43) and humans (e.g. antibodies against $CD16^+CD56^+$) (180), much of the research on these lymphocytes has relied upon a functional definition. Routine testing for NK 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. NK 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 (284). 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 NK 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 (62), by describing the specific natural cytotoxic activity of peripheral blood mononuclear cells (PBMC) isolated from both the Atlantic and Baltic groups of seals. Since natural killer (NK) cells have not been described for harbour seals, we first undertook a series of assays to determine the feasibility of measuring natural cytotoxicity of leukocytes isolated from harbour seals, and to characterize this activity in harbour seals from comparative knowledge of NK cells in the other mammals.

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 (62). 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 nutrient 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 (59,62).

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 (204). 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 leukocytes

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 (58). 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 x 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 x 10^6 YAC-1 cells were radiolabelled 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. NK cell assays consisted of a co-incubation of 1 x 10^6, 5 x 10^5, and 2.5 x 10^5 seal PBMC (effector cells) with 1 x 10^4 radiolabelled YAC-1 target cells in a final well volume of 200 µl in 96-well round-bottomed 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 toxic equivalent (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 observations), and such an evaluation was carried out in order to exclude a methodological artefact in observed cytotoxicity results. For scanning microscopy, 10×10^6 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 (62).

Blubber biopsy samples were used for congener-specific analyses of planar

(IUPAC numbers 77, 126 and 169), mono- (IUPAC numbers 118, 156, and 189) and di-ortho (IUPAC number 180) PCBs. Analyses were undertaken using methods described elsewhere (23,259). All 2,3,7,8 chlorine-substituted dioxin (n=7) and furan (n=10) congeners were measured using methods described elsewhere (153). 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 (6) and for dioxins and furans (264). Individual values for all 22 seals are presented here, while means of the two groups have been presented elsewhere (204).

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). 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

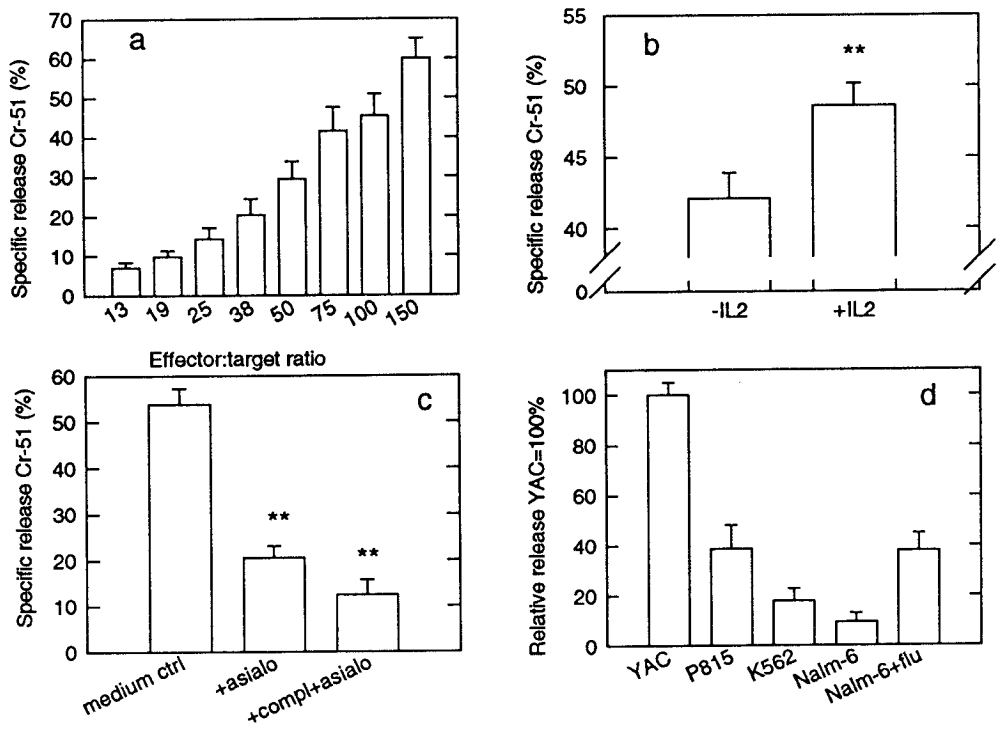

Figure 1: Natural cytotoxicity assays were undertaken using PBMC from harbour seals: a) using YAC-1 target cells at three effector:target cell ratios; b) with recombinant human interleukin-2 (rhIL-2) using a 100:1 effector:target ratio; c) with medium only, the antibody anti-asialo GM1, or a mixture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

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

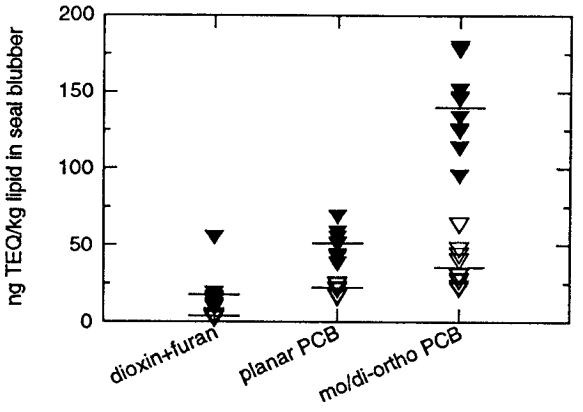

Figure 2: Contaminant burdens in blubber sampled from individual seals fed Atlantic herring (open symbols) or Baltic herring (closed symbols) following two years on the respective diets. Contaminants were grouped here as i) dioxin (PCDD) and furan (PCDF) TEQs; ii) planar PCB TEQs; and iii) mono- and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

$p<0.01$ 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 ± SE 3.5; 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 NK cells described in other mammals, including mice (52), rats (57), chickens (137), dogs (143), cattle (37), pigs (199), horses (46) and humans (115,249). The classic pattern of natural killer cell-induced cytotoxicity, as observed at different effector:target ratios (151) was apparent when PBMC from our seals were co-incubated with radiolabelled YAC-1 tumour cells (i.e. higher numbers of effector cells, higher release of ^{51}Cr by radiolabelled 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 (114). 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

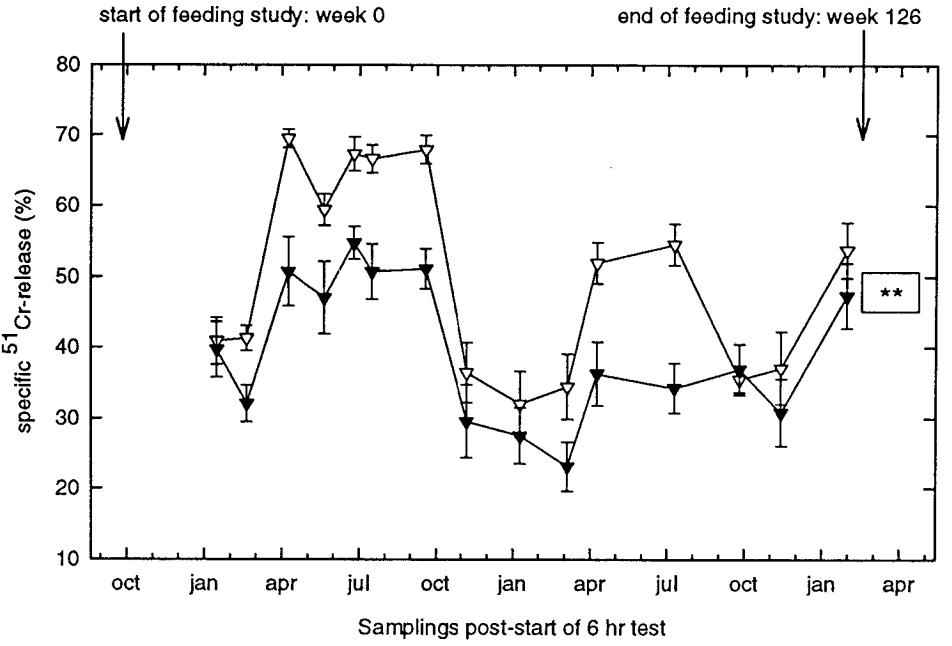

Figure 3: Natural cytotoxic activity of PBMC isolated from harbour seals fed herring from either the relatively contaminated Baltic Sea (solid symbols) or the relatively uncontaminated Atlantic Ocean (open symbols). Activity was measured as the specific release of ^{51}Cr from YAC-1 target cells in a six-hour co-incubation with seal PBMC at an effector:target ratio of 100:1. Data points represent the mean ± 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 ($p<0.01$).

(136), and has been shown to suppress or abolish NK cell activity when administered *in vivo* (136) or *in vitro* (127). Human gamma-Interferon did not enhance or block natural cytotoxicity, indicating a non-responsiveness of seal leukocytes, which may be expected for this non-autologous cytokine. Pre-incubation with autologous interferon leads to enhanced NK cell cytotoxicity in mice (74), but can also protect virus-infected target cells against lysis (34). Since NK cells in mammals comprise a population of leukocytes 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 NK 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 (128), to 20 mg/kg diet bis(tri-n-butyltin)oxide (TBTO) or 150 mg/kg hexachlorobenzene (HCB) (261), and to 50 mg Aroclor 1254 (PCB)/kg in feed over a ten-week period (240). Exposure to 2,3,7,8-TCDD has led to both an increase (90) or to no change (291) in baseline NK 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 titres and increased mortality following pathogenic challenge (121,156) (for review see 279).

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 (161) 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 (161). 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.

NK 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 (18). NK-deficient young mice (24) and beige mice (221) are less resistant to mouse cytomegalovirus (MCMV). Stein-Streilen and Guffee (233) 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.* (283) found that mice administered anti-asialo GM-1 had higher mortality rates and virus titres 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 (40).

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 (273) 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 (204), 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 leukocyte 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 (251) compared to T-lymphocytes (168), 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.

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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

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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 (RV) antigen 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 polio virus-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 (160,211,273). 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-trichloro-ethane (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 (134,157,244), 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 (267). 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 USA in 1987 (154). 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 (119,224). 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 (TEQs) (212,215). 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 (120,273).

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 (62,204). Haematological studies showed increased white blood cell (neutrophils) and red blood cell counts in these animals (61,62). Here we report effects of the different diets on cellular and humoral immune responses of these animals.

Methods

Seals

Twenty-two 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 (61,62,204). 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 (61,62,204). 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 (62).

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 (58,62). 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 x 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 (58). 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-labelled 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 USA. 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 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 (58). 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 titres were measured in plasma using direct enzyme linked immunosorbent assays (ELISAs) as previously described (58). 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 labelled protein A. Previously, we have shown that protein A predominantly binds phocine IgG (208).

Results are shown as 50% titres (sample dilution at which extinction at 450 nm is reduced to 50% of the maximal signal). Poliovirus type-specific neutralizing plasma antibody titres were determined by a routinely used microneutralization test as previously described (76).

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 (204), 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.

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

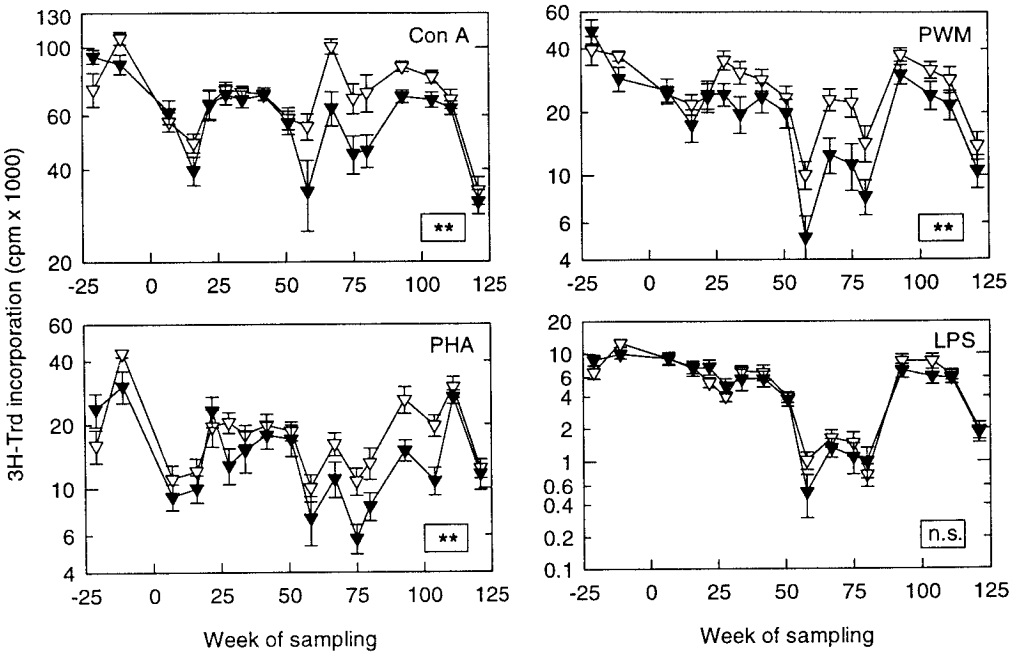

Figure 1: Mitogen-induced proliferative responses of PBMC obtained from harbour seals fed on Atlantic herring (open symbols) or Baltic herring (closed symbols), measured as ^{3}H-Trd incorporation after subtraction of controls. Data shown are mean cpm ± SE of 11 animals per group (seven females and four males each). Asterisks indicate a significant difference between the two groups over time (repeated measures ANOVA, $p<0.01$). n.s.: not significant.

as stimulator cells. Since this assay was developed during the feeding 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 titres 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

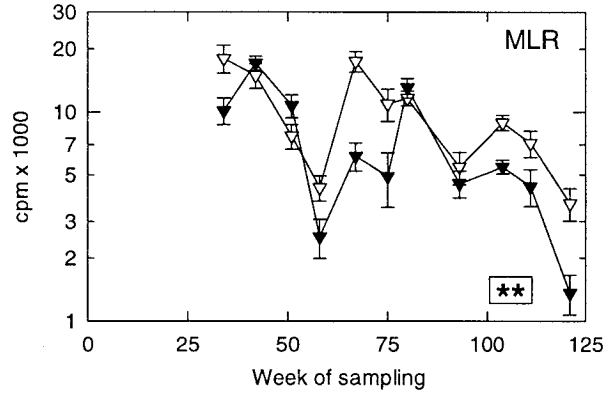

Figure 2: Mixed lymphocyte reactions (MLR) of PBMC obtained from harbour seals fed on Atlantic herring (open symbols) or Baltic herring (closed symbols), measured as ^{3}H-Trd incorporation after subtraction of controls (mean cpm ± SE). Asterisks indicate a significant difference between the two groups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

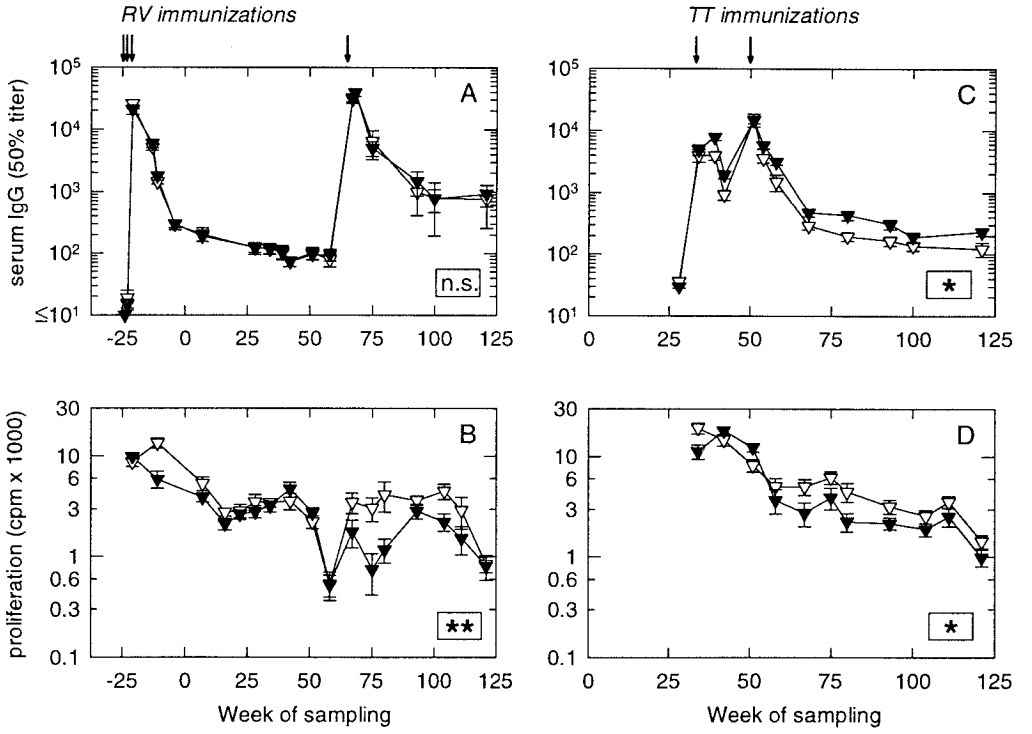

Figure 3: Specific serum antibody titres (plots A and C) and proliferative responses (plots B and D) of seals fed on Atlantic (open symbols) or Baltic (closed symbols) herring, following immunization with the primary antigens rabies virus antigen (RV, plots A and B) or tetanus toxoid (TT, plots C and 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.

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). TT-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 titres against poliovirus type 1 (Mahoney), type 2 (MEF) and type 3 (Saukett) were not significantly different when the seals of both groups were compared (Figure 4). No polio virus-specific proliferative responses could be measured using a concentrated antigen preparation, as has been observed previously using human PBMC (253).

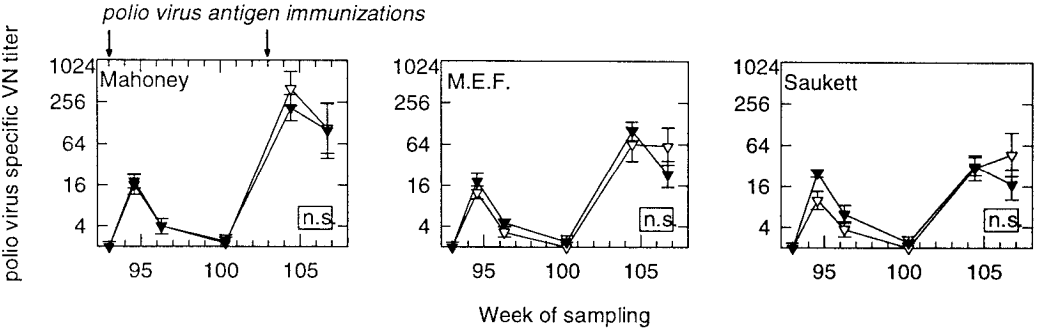

Figure 4: Virus neutralizing serum antibody titres against polioviruses type 1 (Mahoney), type 2 (MEF) and type 3 (Saukett) in seals fed on Atlantic (open symbols) or Baltic (closed symbols) herring, after immunization with trivalent polio vaccine. Immunizations are indicated by arrows. Data shown are mean ± SE. n.s.: not significant.

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

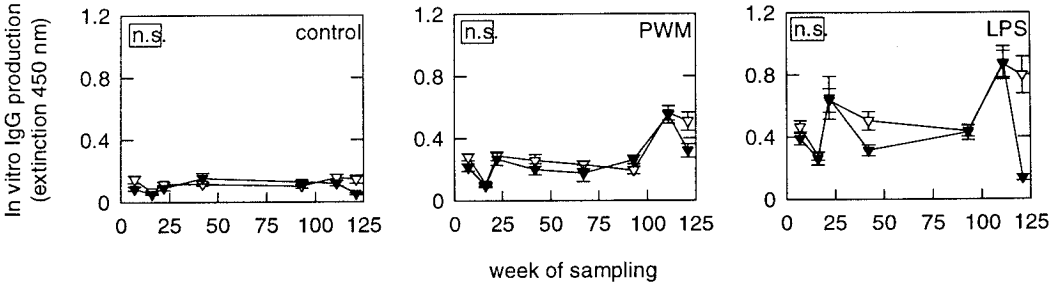

Figure 5: *Ex vivo/in vitro* total IgG production by PBMC obtained from harbour seals fed on Atlantic herring (open symbols) or Baltic herring (closed symbols), after stimulation with PWM or LPS (top plot: medium control). Data shown are protein A binding antibodies in culture supernatants six days after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

previous observations, which indicated an impairment of *ex vivo/in vitro* NK cell activity and T-cell mitogen-induced lymphoproliferative responses and *in vivo* DTH responses in seals fed on Baltic Sea herring (62,204). 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 (120,273). 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 (62). 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 short-term doses as low as 0.5 µg/kg, including impaired lympho-proliferative responses (120,273). 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 (273).

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 (211,290). 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 (204), 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 (269). 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 (102,281), while dimethyldioctadecyl-ammonium bromide (DDA), the adjuvant to the ovalbumin immunizations, is an adjuvant which is particularly potent in inducing cell-mediated immune responses (117). 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 (237), and indications of immunological differences were reported in the high fish consumption group (236). 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 (273), 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 (179,255). 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 (189), 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.

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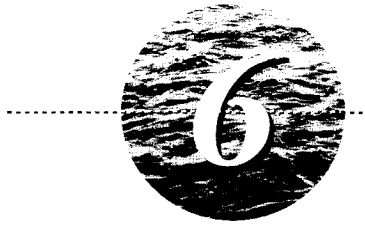

Contaminant-related suppression of delayed-type hypersensitivity and antibody responses in harbour seals fed herring from the Baltic Sea

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

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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 polychlorinated biphenyls (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 (273), 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) (273). Fish-eating animals including gulls, cormorants (87), seals (1), carnivorous whales (164,173), and certain groups of humans (124,237) 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 (87) and seals (17,171), and reproductive impairment in seals (112,197).

Mass mortalities among harbour (*Phoca vitulina*) and grey (*Halichoerus grypus*) seals in Europe in 1988 (73,185,266), Baikal seals (*Phoca sibirica*) in 1987-88 (98,187), bottlenose dolphins (*Tursiops truncatus*) in the Gulf of Mexico in 1987-88

(146), and striped dolphins (*Stenella coeruleoalba*) in the Meditteranean Sea in 1990-91 (256), 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 (73,186). Although 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 (104); PDV or a very similar virus had infected Canadian harbour seals before the European disaster, with no apparent mortality (113,210); and harbour seals living in less contaminated areas of Britain had apparently lower mortality rates during the PDV epizootic than those from polluted areas (225). 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 (62,205). 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.

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 (62). They had been captured as recently weaned pups from the relatively uncontaminated northeast 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 nutrient 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 (62). 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 (62). 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 (61,62). 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 pre-screen, 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, Basel, 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).

After the pre-screen, 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 pre-screen. 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 at 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, 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 (23,259). 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 (153). 2,3,7,8-TCDD TEQs were calculated on the basis of Toxic Equivalent (TEFs) designated for dioxins and furans (264) and for PCBs (6). 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 after 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 titres 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 (62). 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.

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% ± 0.6% (SE) of the total TEQ values, compared to only 7% ± 0.6% (SE) for the dioxins and furans.

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 lipid	
	Atlantic seals	Baltic seals
PCB (mono and di-ortho)	35.5 ± 3.66	140.0 ± 7.95
PCB (planar)	22.2 ± 1.00	51.1 ± 3.04
dioxins & furans (total)	4.1 ± 0.19	17.7 ± 4.14
TEQs (total)	61.8 ± 4.13	208.7 ± 11.60

Ovalbumin-specific DTH responses

In a pre-screen 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. After 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 ($r=-0.64$; $p<0.01$).

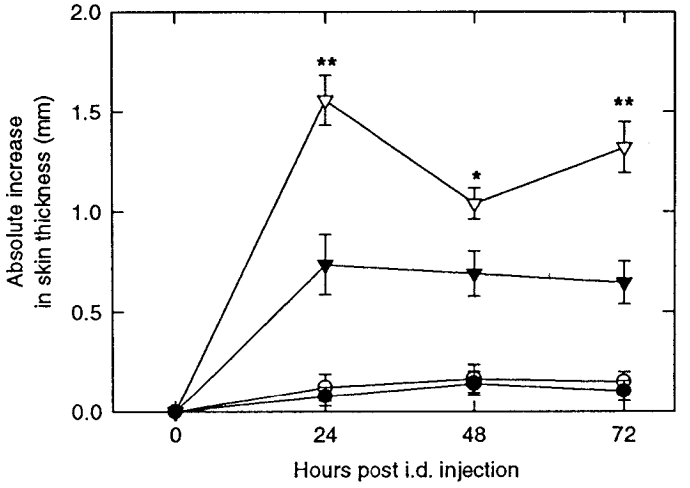


Figure 1: Nine days after immunization with ovalbumin and the adjuvant DDA, harbour seals of both Atlantic (open triangles) and Baltic (solid triangles) groups exhibited a delayed-type hypersensitivity (DTH) response to an intradermal skin challenge using ovalbumin. Seals fed the relatively contaminated 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 occurred 24 hours following injection. A control injection of 100 µl saline resulted in only a very small swelling for both Atlantic (open circles) and Baltic (solid circles). 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 (* $p<0.05$; ** $p<0.01$).

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

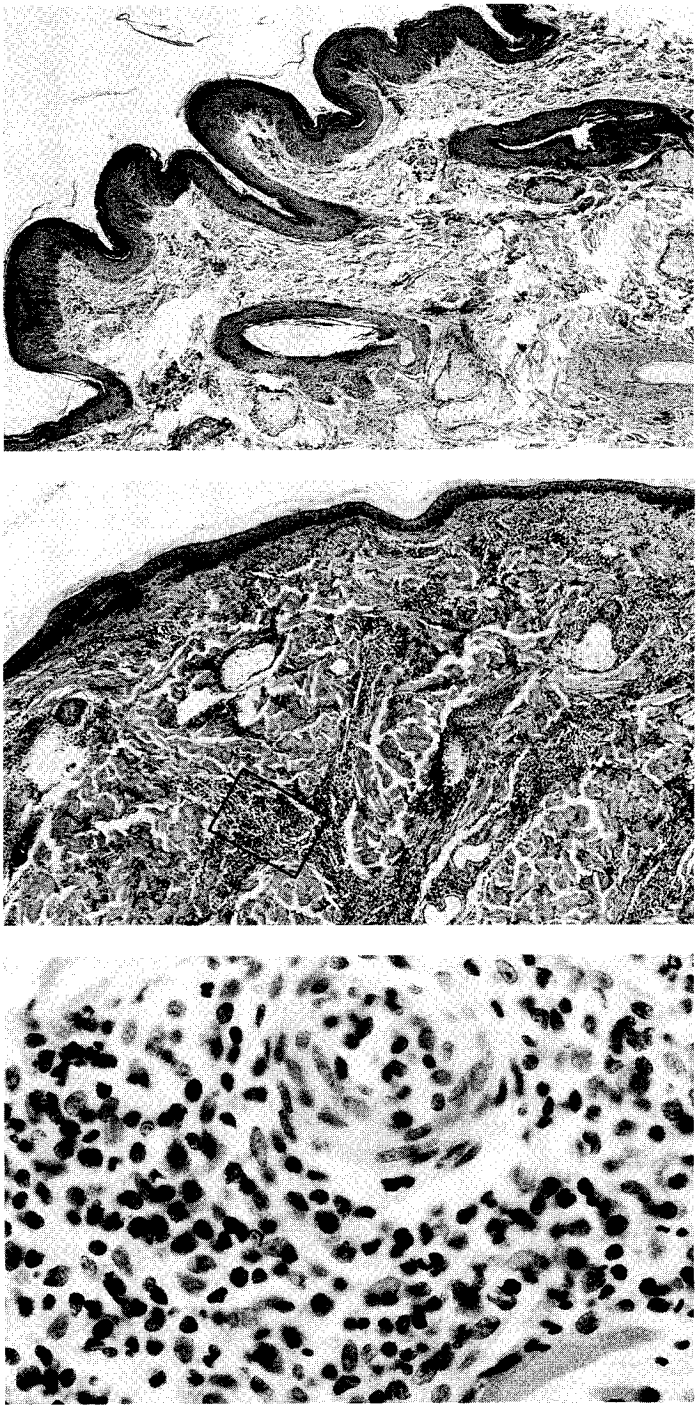


Figure 2: Biopsies of seal skin from (top) a control sample (40x H&E), (middle) a positive skin test 72 hours after intradermal injection with the recall antigen ovalbumin (40x H&E), and (bottom) an enlargement of an indicated section (see outlined square) of the same cut (200x H&E). The enlarged section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

antigen. In a blood sampling four weeks subsequent to the immunization, seals of both groups had mounted antibody responses to ovalbumin, titres 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).

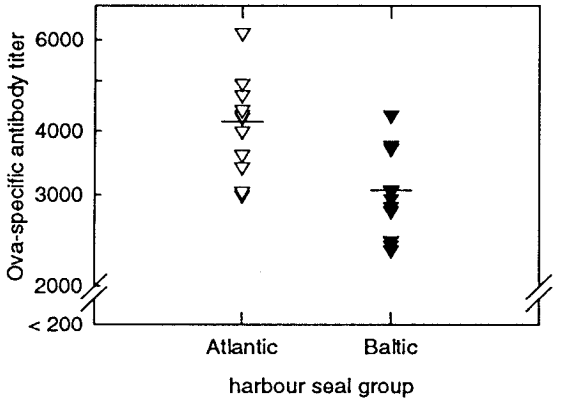


Figure 3: Serum antibody titres mounted against ovalbumin four weeks after immunization were significantly lower (independent *t*-test; $p<0.01$) in the Baltic Sea group of seals (solid symbols) as compared to the Atlantic group (open symbols). Seals of both groups had no detectable antibodies to ovalbumin 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 (117). The skin reaction to ovalbumin was characterized by a mononuclear infiltrate in the dermis, as observed in classical DTH responses in other species studied (126). 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 (172), including the harbour seal (58). These results not only strengthen our previous evidence of a contaminant-induced suppression of T-lymphocyte function in the harbour seals (62), but also lend support to the use of *ex-vivo/in vitro* tests of immune function.

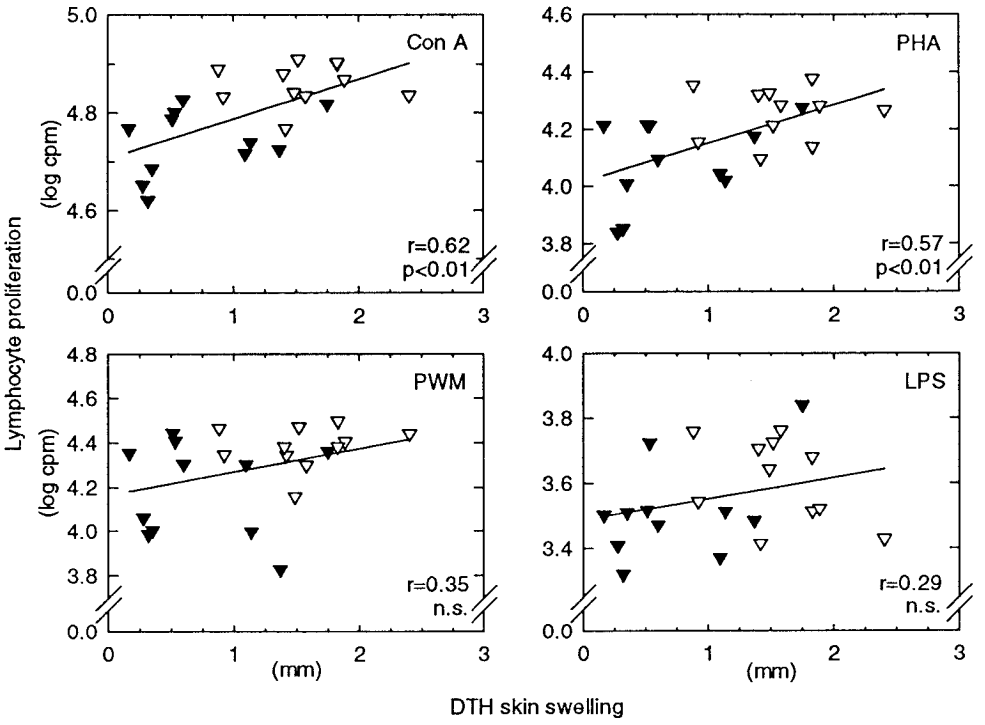


Figure 4: Correlations between *in vitro* tests of immune function conducted previously and DTH responses. Good correlations existed between DTH and lymphocyte stimulation by the mitogens Con A (r=0.62, p<0.01) and PHA (r=0.57, p<0.01), but not PWM (r=0.35, not significant) and LPS (r=0.29, not significant), implicating T-lymphocytes in the DTH response. Mean counts per minute were calculated 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 symbols) Atlantic seals; (solid symbols) Baltic seals.

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.

Impairment of DTH reactions following exposure to TCDD has been observed in Guinea pigs receiving eight weekly doses of 0.04 µg/kg (275); C57BL/6 mice receiving 4 weekly doses of 4 µg/kg (47) or a one time dose of 50 µg/kg (158); 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 (80). 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 (276). Vos and Moore (274) 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 significant impairment of DTH responses (45). Impairment in B-cell responses has been observed in adult animals, with lower antibody responses to various antigens reported following exposure to PCBs (273) and dioxins (275). 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.* (158), 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 (273).

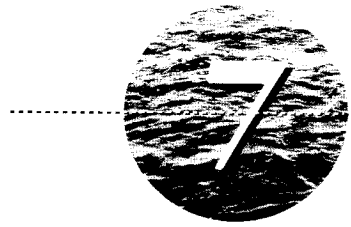
Many different classes of potentially immunosuppressive contaminants bioaccumulate in the Baltic Sea ecosystem, making it impossible to identify any one contaminant as responsible for the impaired immune responses in our study. Evidence for the mediation of immunotoxicity via the *Ah*-receptor in animals and the high affinity of 2,3,7,8-TCDD and its dioxin and PCB analogs for this cytosolic receptor (215) 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 (208,209), and correlative approaches similar to those used in wildlife toxicology (257) 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.



Host resistance to rat cytomegalovirus (RCMV) and immune function in adult PVG rats fed herring from the contaminated Baltic Sea

Peter S. Ross, Henk van Loveren, Rik L. De Swart, Helen van der Vliet, Arja de Klerk, Helga H. Timmerman, Rob S. van Binnendijk, Abraham Brouwer, Joseph G. Vos, and Albert D.M.E. Osterhaus

(submitted)

Abstract

The immunotoxic potential of many classes of environmental contaminants has been well established in laboratory studies, with much attention being focussed on Aryl hydrocarbon (*Ah*)-receptor binding polychlorinated biphenyl (PCB), polychlorinated dibenzo-*p*-dioxin (PCDD), and polychlorinated dibenzofuran (PCDF) congeners. In a semi-field study, we previously showed that harbour seals (*Phoca vitulina*) fed herring from the contaminated Baltic Sea had lower natural killer cell activity, T-lymphocyte functionality and delayed-type hypersensitivity responses than seals fed herring from the relatively uncontaminated Atlantic Ocean. While ethical and practical constraints preclude in-depth studies in seals, specific reagents and a wider array of immune function tests allow such studies in laboratory rats. We therefore carried out a feeding study in rats aimed at extending our observations of contaminant-induced immunosuppression in harbour seals. The same two herring batches used in the seal study were freeze-dried, supplemented and fed to female adult PVG rats for a period of 4½ months. An additional dietary control group was fed standard pellet food. Daily contaminant intakes of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxic equivalents (TEQs) were estimated to be 0.3 ng/kg body weight and 1.6 ng/kg in the Atlantic and Baltic groups, respectively. At the end of the feeding experiment, no contaminant-related changes in thymus or spleen CD4/CD8 cellularity, natural killer cell activity, or mitogen-induced proliferative responses of thymus or spleen cells could be detected. A novel model was established to assess the specific T-cell response to rat cytomegalovirus (RCMV). When applied to the feeding study, no differences between the Atlantic and Baltic groups in the RCMV-induced proliferative T-lymphocyte responses could be detected, but virus titres in salivary glands of infected rats of the Baltic Sea group were higher. These elevated RCMV titres suggest that the dietary exposure to low levels of contaminants may have been immunotoxic at a level which our immune function tests could not otherwise detect. In addition, lower plasma thyroid hormone levels in the Baltic Sea group of rats confirmed that exposure to this environmental mixture of contaminants led to adverse health effects.

Introduction

The immunotoxic potential of the polyhalogenated aromatic hydrocarbons (PHAHs) including the polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) has been well established in laboratory animals (273). However, exposure regimes in such studies usually use single compound and rarely approach the kinds of contaminant mixtures and levels to which humans and wildlife are exposed. The persistent and lipophilic

nature of organochlorines leads to biomagnification in the food chain, predisposing animals at the higher trophic levels to accumulating high concentrations of these compounds (241). In the case of wildlife, many bioeffects in populations of fish-eating birds and seals inhabiting polluted areas of North America and Europe have been identified, including mixed function oxidase induction (26), reproductive impairment (87,111,197), embryotoxicity (248); skeletal malformations (17,87,171), vitamin A deficiencies (29) and thyroid hormone deficiencies (29). While direct cause-and-effect relationships are difficult to identify, evidence from these semi-field and epidemiological studies have tended to primarily implicate planar and mono-ortho PCBs in many of the observed effects.

Little is known about the immunotoxic effects of environmental mixtures of anthropogenic contaminants, but the ubiquitous presence and the immunotoxicity of PCBs, PCDDs and PCDFs suggest that these PHAHs may present a risk to the immunocompetence of certain wildlife species. While exposure to relatively simple mixtures and dose regimes of PHAHs has been shown to lead to a wide range of immunological effects in laboratory animals, thymus atrophy and diminished T-lymphocyte responses are particularly sensitive indicators of immunotoxicity (273). The mechanism of immunotoxic action of different PCB, PCDD and PCDF congeners has been found to be largely mediated via the cytosolic aryl hydrocarbon (*Ah*)-receptor (176,223,224). With some caution, therefore, the immunotoxic potential of complex environmental PHAH mixtures may be simplified and expressed on the basis of their affinity for the *Ah*-receptor relative to that of 2,3,7,8-TCDD, and expressed on a toxic equivalent (TEQ) basis (212,213).

The occurrence of virus-induced mass mortalities among marine mammal populations in recent years (73,184) has led to concerns that environmental contaminants in the marine food chain had been immunotoxic to affected animals. In a captive feeding study, we recently demonstrated that harbour seals (*Phoca vitulina*) fed herring from the contaminated Baltic Sea developed impaired immune responses compared to those fed herring from the Atlantic Ocean. Seals of the Baltic group had significantly lower *in vitro* natural killer (NK) cell activity (205), mitogen- and antigen-induced T-lymphocyte proliferative responses (60,62), and *in vivo* antibody and delayed-type hypersensitivity (DTH) responses to ovalbumin (204). The determination of contaminant levels in blubber biopsies taken towards the end of the study revealed that seals of the Atlantic group had mean TEQ concentrations of 62 ± 4.1 ng/kg lipid, while seals of the Baltic group had mean concentrations of 209 ± 11.6 ng/kg lipid (204). We concluded that free-ranging seals in contaminated coastal waters have compromised immune function and may therefore be more vulnerable to infectious disease events, such as the phocine distemper virus-related mass mortality of harbour seals and grey seals (*Halichoerus grypus*) in Europe in 1988 (189). However, legal, ethical and methodological constraints restricted our protocol largely to

experiments using blood samples and specific responses to immunizations. In addition, the availability of specific reagents for a comprehensive assessment of immune function in seals is limited, which further impeded our ability to study mechanistic alterations in the immune system of the Baltic group of seals.

Immunotoxicological studies are routinely carried out in laboratory rodents (273). Because the rat is predominantly used in toxicological studies, its use in immunotoxicology can provide additional information for risk assessment of chemicals or contaminant mixtures. Tiered approaches have been described for rats, in which a cross-section of histopathological and functional assays is used to functionally characterize different lymphoid compartments, including the thymus, spleen, lymph nodes, and blood cells (263,279). The availability of reagents specific for leukocyte subpopulations and their products (e.g. immunoglobulins) and the ability to carry out host resistance tests represent two methodological approaches not possible in seals.

2,3,7,8-TCDD-induced immunosuppression has been shown to impair host resistance in laboratory rodents to many agents, including *Salmonella* bacteria (245), endotoxin (272) and influenza virus (121,291). While exposure to bis(tri-*n*-butyltin)oxide led to elevated virus titres in the salivary glands of rats infected with rat cytomegalovirus (RCMV) (91), nothing is known about the effects of the TCDD-related compounds on the RCMV-specific cellular response or the outcome of this virus infection. RCMV, like cytomegaloviruses of other animal species, causes a chronic, largely subclinical, infection in its natural host (33).

Here we describe a system in which the specific T-lymphocyte response to RCMV can be assessed in infected rats, involving an *in vitro* stimulation of lymphocytes by autologous paraformaldehyde-fixed RCMV-infected rat embryo cells. This newly developed RCMV-response model, in conjunction with an array of non-specific tests of immune function, was used in a feeding study to determine whether a diet containing herring from the contaminated Baltic Sea was immunotoxic to laboratory rats, as was the case in our previous study of harbour seals. In addition, we measured plasma levels of thyroxine, as this thyroid hormone is a well established and relatively sensitive marker of PHAH exposure (30,31,149).

Methods

Diets

Herring from either the relatively uncontaminated Atlantic Ocean or the contaminated Baltic Sea was used in rat feeding experiments, as previously described for our study of harbour seals (62). For the rat study, herring was first freeze-dried in order to preserve its freshness and to facilitate the production of a finely ground diet. This ensured the uniformity of the diet and served to avoid any selectivity on the part of rats. For this, frozen whole Atlantic and Baltic herring were ground and freeze-dried. Once ready, the freeze-dried herring was again ground to a powder and stored

in sterile 1 L glass bottles at -20°C until use. Two dietary supplements were designed following an analysis of the nutritional quality of the two herring batches (Hope Farms, Woerden, The Netherlands). This was aimed at ensuring an adequate energy and nutritional uptake and to prevent a protein intake level that would be too high in a diet consisting solely of fish. The Atlantic herring diet consisted of 33% freeze-dried Atlantic herring powder and a specially prepared supplement consisting of 0.25% standard vitamin mix, 0.40% choline Cl 50%, 5% cellulose, 10% corn starch and 51.4% cerelose. The Baltic herring consisted of 54% freeze-dried Baltic herring powder, 0.25% standard vitamin mix, 0.40% coline Cl 50%, 5% cellulose, 10% corn starch and 30.4% cerelose. Freeze-dried herring was thawed immediately prior to use and then mixed with the supplements. Differences in the herring content between the two diets reflected a compensation made for differences in the lipid content of the two fish batches, as described for the seal study (62). Standard irradiated rat pellet food (#1210 SP; Hope Farms) was fed to rats of a third dietary control group for the duration of the experiment.

Congener-specific determination of dietary contaminant levels

Freeze-dried Atlantic and Baltic herring were analyzed for coplanar PCBs (IUPAC numbers 77, 126 and 169) using methods described elsewhere (259). Mono-ortho (IUPAC numbers 105, 114, 118, 123, 156, 157, 167 and 189) and di-ortho (IUPAC numbers 170 and 180) PCB concentrations were determined by multidimensional gas chromatography using methods described elsewhere (54). Concentrations of all 2,3,7,8-substituted PCDD (n=7) and PCDF (n=10) congeners were determined as described elsewhere (153). Values of TEQs were then determined for each of these congeners using recently described toxic equivalent factors (TEFs) for PCBs (6), and for PCDDs and PCDFs (264). Dichlorodiphenyl-trichloro-ethane (DDT) and hexachlorobenzene (HCB) levels were determined as previously described (23). Residue levels are expressed on a lipid weight basis, and the estimated contaminant intake per rat was calculated by determining their daily intake of lipid and multiplying this by the concentrations of the residues in the herring.

Rats

All experiments were carried out under the supervision of the Animal Ethics Committee of the National Institute of Public Health and the Environment (Bilthoven, The Netherlands), consistent with the guidelines of the European Community Council Directive on the use of laboratory animals in experiments (86/609/EEC).

We selected the inbred PVG rat strain (PVG/OlaHsd; Harlan-Olac, Zeist, The Netherlands) for use in the feeding study since an inbred rat was required for the development of the RCMV model. However, because little is known of the sensitivity of this rat strain to the immunotoxic actions of TCDD-like compounds, we carried

out a preliminary experiment in which thymus atrophy served as the endpoint. For this, we compared the dose response of TCDD-induced thymus atrophy in PVG rats to that of the better studied Wistar rat (Rivm:WU(CPB); National Institute of Public Health and the Environment). Twenty-five eight-week old specific pathogen free (SPF) rats of both strains were housed in pairs in cages placed in negative pressure closed barrier isolators. Following one week of acclimation, rats were given a one-time dose of 2,3,7,8-TCDD (Dow Chemical, Midland, USA) in 1 ml olive oil at a concentration of 0, 0.25, 1, 4, 16, 32, or 64 µg/kg body weight by oral gavage. Eight days later, rats were sacrificed and thymus weights determined.

Subsequently, 24 recently weaned, female, SPF PVG (Harlan-Olac) rats were housed in pairs in filter top cages for the long-term feeding study. Following one week of acclimation and *ad libitum* water and standard rat pellet diet, rats were randomly divided into three groups of eight rats each and body weights recorded. For the rest of the experiment, all rats received an *ad libitum* supply of water and either the Atlantic herring diet, the Baltic herring diet, or pellets as a dietary control. All rats were fed three times per week, and average food consumption per rat was determined. Body weights were determined once a week.

RCMV infection *in vivo* and necropsy

All rats were injected intraperitoneally with 1 x 10^5 plaque forming units (PFU) RCMV (obtained from C. Bruggeman, University of Limburg, The Netherlands) on day 119 of the feeding study and sacrificed on day 130 for assessment of immune function using non-specific and RCMV-specific tests. Heparinized blood was drawn from the dorsal aorta and plasma stored at -86°C. Body, thymus, spleen, liver and salivary gland weights were recorded, and thymus and spleen were aseptically removed and placed in culture medium consisting of RPMI medium (GIBCO, Grand Island, USA) containing 10% heat inactivated fetal calf serum (FCS; PAA, Linz, Austria) and 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine (hereafter: culture medium). Salivary glands were removed, and a 1:10 weight:volume suspension was homogenized in Basal Eagle Medium (GIBCO) containing 2% FCS and the same antibiotic recipe as for culture medium and frozen at -86°C (91).

Preparation of cell suspensions

Cell suspensions were prepared by crushing the thymus and spleen tissue through a 70 µm nylon cell strainer (Becton Dickinson, Rutherford, USA) and the removal of connective tissue with glass wool. Subsequent washing, rinsing and cell culture steps were carried out using culture medium. Cells were counted using a Coulter Counter (Coulter Electronics, Luton, UK). Spleen cells were further purified by Ficoll (Pharmacia LKB Biotechnologie, Uppsala, Sweden) 1.077 g/ml density gradient separation.

Flow cytometric analysis of thymus and spleen cell suspensions

Cell subpopulations were identified and analyzed using a fluorescence activated cell scanner (FACS; Becton Dickinson, Rutherford, USA). Thymus and spleen cell suspensions were analyzed for cells with $CD4^+$ and $CD8^+$ surface markers using the appropriate monoclonal antibodies. Briefly, cells were incubated with FITC-labelled ER2 (Serotec, Oxford, UK) and biotynilated OX-8 (Serotec), followed by R-phycoerythrin-conjugated streptavidin (Jackson Immunoresearch Laboratories, West Grove, USA). Samples were analyzed by FACS after gating based on the forward and side scatter profiles.

Mitogen-induced proliferation of thymus and spleen cells

Lymphocyte stimulation assays using the mitogens concanavalin A (Con A; final concentration 2 µg/ml; Janssen Chimica, Beerse, Belgium), phytohaemagglutinin (PHA; final concentration of 1:60 dilution; Wellcome, Dartford, UK), and pokeweed mitogen (PWM; final concentration of 1:60 dilution; GIBCO) were undertaken using both thymus and spleen suspensions as described elsewhere (270). Cells were adjusted to concentration and 4×10^5 were placed in triplicate wells of a 96-well, round-bottom cell culture plate (Greiner, Nürtingen, Germany) along with the relevant mitogen solution. Cellular proliferation was assessed by measuring the incorporation of ^{3}H-thymidine following 72 hours of culture.

Natural killer cell activity

Natural killer (NK) cell activity was assessed as described elsewhere (57). Briefly, spleen cells were tested for natural cytotoxic activity against ^{51}Cr-labelled YAC-1 tumour cells at a 100:1 effector:target ratio in a four hour coincubation following overnight incubation at 37°C to remove adherent cells.

Total immunoglobulin titres (IgM, IgG)

Total plasma IgM and IgG titres were determined as described elsewhere (271), and titres were defined as the plasma dilution at which the maximum absorbance signal obtained from pooled plasma samples from each necropsy day was reduced by 50%.

Specific cellular immune response to RCMV infection

A model was set up in the rat for the assessment of the specific cellular immune response to RCMV. This consisted of an *in vitro* coincubation of fixed, RCMV-infected rat embryo cells (stimulator cells) with cells isolated from the spleens of rats previously infected with the same virus (effector cells).

Rat embryo cells (REC) from the inbred PVG rat strain were prepared and cryopreserved as described elsewhere (91). Monolayers of REC were established by placing approximately 1×10^7 thawed REC in culture medium supplemented with 40

mg/ml Gentamycin in a 225 cm^2 flat-bottomed cell culture flask. Following 24 hours in culture, monolayers were infected with RCMV at a multiplicity of infection of 10 PFU per cell for one hour at 37°C in phosphate buffered saline, and subsequently incubated with culture medium for a further 18 hours. At this point, a cell scraper (Costar, Cambridge, USA) was used to loosen any cells not already in suspension from the RCMV-induced cytopathic effect. The cells were then washed and fixed in 1.0% paraformaldehyde in a pellet of 1 x 10^7/ml and blocked with 0.2 M glycine as described elsewhere (254). Non-RCMV infected control stimulator cells were prepared in the same manner.

RCMV-specific lymphocyte stimulations

RCMV-specific stimulations involved a coincubation of 1.5 x 10^5 effector cells (2 x 10^5 for the feeding study) with either 3 x 10^3 fixed RCMV-infected REC or 3 x 10^3 fixed non-infected REC in 200 µl culture medium per well of a 96-well round-bottom cell culture plate (Greiner). In preliminary experiments, pooled cell suspensions of lymph node and peripheral blood were also cultured in triplicate with both RCMV-infected stimulator cells or non-infected controls. Duplicate plates were placed in a 37°C 5% CO_2 humidified incubator. Cellular proliferation was quantified in one of the duplicate plates by determining ^{3}H-thymidine incorporation between days 5 and 6. The second of the duplicate plates was used for FACS analysis of expanded $CD4^+$ and $CD8^+$ subpopulations as described above. For this, two gates were used: a gate encompassing total lymphocyte and monocyte populations, and a gate encompassing only the blast cells. The latter were identified as those lymphocytes with low side scatter and high forward scatter.

Evaluation of the RCMV stimulation model

In preliminary experiments, four groups of six adult female SPF PVG rats were injected intraperitoneally with 1 x 10^5 PFU RCMV in 1 ml saline solution on day -17 (17 day infection), day -11 (11 day infection), day -6 (6 day infection), or not at all (0 day infection). All rats were sacrificed at the same time (day 0), and necropsies were carried out under aseptic conditions. Blood was pooled by group and peripheral blood mononuclear cells (PBMC) isolated by Ficoll (Pharmacia) density gradient separation. Spleen cell suspensions were further purified by Ficoll (Pharmacia) density gradient separation and cervical posterior lymph node cells were isolated as described above for other tissues. Spleen cell suspensions for each animal, plus pooled PBMC and pooled lymph node cell suspensions, were counted using a Coulter Counter (Coulter Electronics) and adjusted to concentration.

RCMV titres in salivary glands

Virus titres in the salivary glands of rats from the feeding study were determined as described elsewhere, and expressed as total PFU in the salivary glands (91).

Plasma thyroid hormone

Plasma thyroxine (total T4; TT4) was determined using a chemiluminescent immunoassay (Amersham, Little Chalfont, UK) as described elsewhere (175).

Statistics

Results of each assay on each necropsy day were analyzed using a one-way ANOVA, followed by independent two-sided *t*-tests between the Atlantic group (contaminant control) and the other groups (Baltic and pellet) if this proved significant in the ANOVA. Owing to a non-normal distribution of results, RCMV titres in salivary glands were analyzed using a Wilcoxon's signed rank test.

Results

TCDD-induced thymic atrophy in PVG versus WU rats

A one-time dose of 2,3,7,8-TCDD administered orally resulted in a dose-dependent thymic atrophy in both PVG and WU rats (Figure 1). This was statistically significant at 1 µg/kgbw in PVG rats and 4 µg/kgbw in the WU rats, indicating that the PVG rat is relatively sensitive to the immunotoxic action of TCDD and is a suitable inbred strain for use in the feeding study described here. Slight effects of the TCDD treatment on body weight for both rat strains were observed at the two highest dose levels (32 and 64 µg/kg), resulting in decreased weights of 10-15% (data not shown).

RCMV-induced T-cell response: a model for immunotoxicological studies

The coincubation of fixed, RCMV-infected stimulator cells and cells isolated from the organs of previously infected rats led to strong lymphocyte proliferative responses that were detectable both visually and by ^{3}H-thymidine incorporation (Figure 2). Spleen cell cultures displayed the highest specific proliferation, while lymph node cell cultures did not show a response until 11 days post *in vivo* infection. PBMC showed no response at all. Stimulations were RCMV-specific, as non-infected stimulator cells did not induce a significant proliferation in samples from infected rats. Furthermore, cells from non-infected rats (day 0) were not stimulated in the presence of either infected or non-infected stimulator cells. The highest proliferative responses of spleen cells were observed in rats infected for the longest period (17 days). Blast cells of both $CD4^{+}CD8^{-}$ and $CD4^{-}CD8^{+}$ phenotypes were detected following the *in vitro* coincubation of spleen cells from infected rats with stimulator cells (Figure 3a). Despite the expansion of both major T-lymphocyte subpopulations, there was a progressive shift towards $CD8^{+}$ cell expansion in rats during the course of infection (Figure 3a). This preferential $CD8^{+}$ expansion is apparent in a comparison of $CD4^{+}$:$CD8^{+}$ ratios of blast cells in culture in the presence of RCMV-infected stimulator cells, as compared to $CD4^{+}$ cells (see Figure 3b).

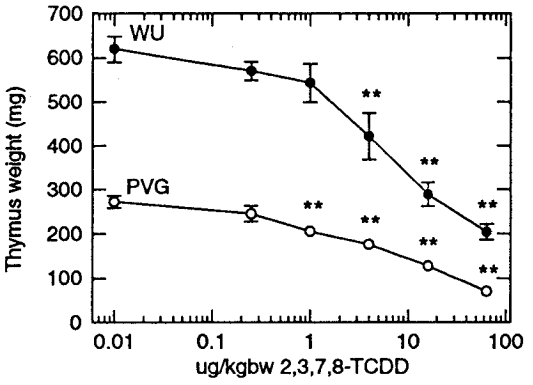


Figure 1: Preliminary studies: Absolute thymus weights in WU (solid symbols) and PVG (open symbols) rats eight days following oral administration of different doses of 2,3,7,8-TCDD.

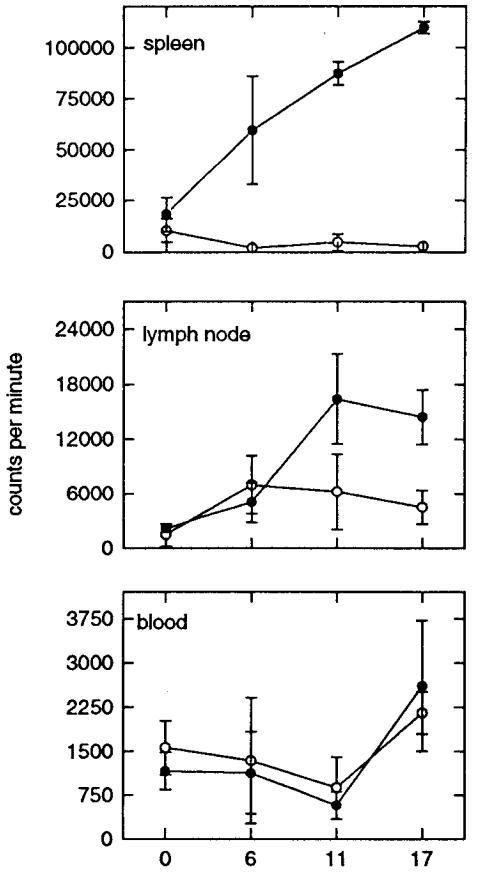


Figure 2: Preliminary studies: RCMV-specific proliferative responses of rat lymphocytes isolated from three lymphoid compartments (spleen cells, pooled posterior cervical lymph node cells, and pooled PBMC) following different *in vivo* infection incubation times. Rats were infected with RCMV for 6, 11 or 17 days prior to necropsy, or not at all (0 days), and cells from the identified compartment were coincubated with 3 x 10^3 fixed RCMV-infected rat embryo cells. Proliferation of lymphocytes was measured by the incorporation of ^{3}H-thymidine and expressed as counts per minute (cpm). Open symbols represent cultures with control (non-RCMV infected) stimulator cells. Closed symbols represent cultures with RCMV-infected stimulator cells. Values for spleen cells represent the means ± SE for six animals per group, and mean ± SE for triplicate pooled cultures of lymph node cells and PBMC.

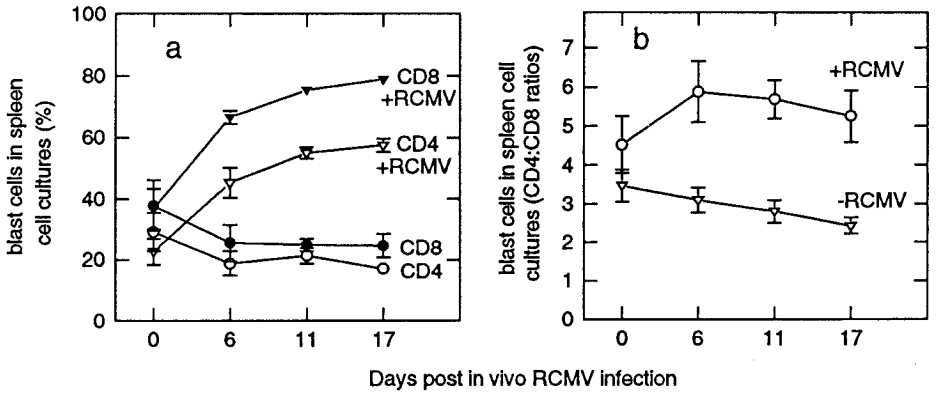


Figure 3: Preliminary studies: Following six days in culture, RCMV-induced expanded lymphocytes were labelled for flow cytometric analysis for the three groups of rats infected with RCMV for different lengths of time or not at all: a) the percent of total CD8 (T-cytotoxic) cells (solid symbols) and total CD4 (T-helper) cells (open symbols) that were identified as blast cells following *in vitro* stimulations between spleen cells and either RCMV-infected stimulator cells (triangles) or control (non-infected stimulator) cells (circles) in rats infected with RCMV for different infection times; b) the CD4:CD8 ratios in the blast cell population following culture with either RCMV-infected stimulator cells (circles) or control non-RCMV-infected cells (triangles).

Feeding study with RCMV-infected rats exposed to dietary environmental contaminants

Toxicological and gross health parameters

Herring from the contaminated Baltic Sea had elevated levels of all contaminants measured, as compared to herring from the relatively uncontaminated Atlantic Ocean (Table 1). The feeding study began when the rats were approximately eight weeks old and continued until they were 26 weeks old. Rats consumed an average of 159 g and 137 g of herring lipid in the Atlantic and Baltic groups, respectively, during the course of the feeding experiment. Rats in the Baltic Sea group had consumed roughly four times higher levels of TEQ than those of the Atlantic group by day 130 of the feeding experiment (Table 2). PCDDs and PCDFs accounted for approximately 40% of the total TEQ in the Baltic herring, with 2,3,4,7,8-PCDF accounting for the majority of this contribution (results not shown). PCBs accounted for almost 60% of the total TEQ in the Baltic Sea herring, with 2,3',4,4',5 PeCB (IUPAC number 118) and 2,3,3',4,4',5 HxCB (IUPAC number 156) accounting for the majority of the mono- and di-ortho PCB contribution, and 3,3'4,4',5 PeCB (IUPAC number 126) accounting for the majority of the non-ortho PCB contribution (results not shown).

Table 1: Analysis of chemical residues in herring (ng/g lipid):

Compounds	Atlantic	Baltic
ΣPCB	1209	7135
mono-ortho PCBs	63	408
di-ortho PCBs	16	193
non-ortho PCBs	0.88	1.88
ΣPCDD (2,3,7,8-substituted)	0.02	0.10
ΣPCDF (2,3,7,8-substituted)	0.07	0.23
ΣDDT	39	222
HCB	31	89
ß-HCH	<10	140
dieldrin	154	340

Rats readily consumed the two herring diets and showed no overt signs of diet-related health problems. There were no differences in gross health parameters between rats of the Atlantic and the Baltic groups (Table 3). However, at the end of the feeding study, rats consuming the standard pellet diet were heavier than those in the two herring diet groups. Thymus and spleen weights were also slightly higher in the pellet group, though these could be largely accounted for by the differences in body weights among groups.

Non-specific tests of immune function in rats

NK cell activity was readily measurable in spleen cell suspensions of all three groups (Figure 4). No significant differences in NK cell activity among the three groups were detected.

Rats of the Baltic group had reduced total thymocyte numbers and thymocyte $CD4^+$:$CD8^+$ ratios, but elevated numbers of $CD4^+CD8^+$, $CD4^+CD8^-$ and $CD4^-CD8^+$, as compared to those in the Atlantic group (Table 4). However, since the results observed in Baltic rats were similar to those of the pellet group, an effect of contaminants could not be determined. There were no significant differences in $CD4^+$ or $CD8^+$ subpopulation patterns in the spleens among the three groups (Table 5).

There were no significant differences in spleen or thymus cell proliferation induced by Con A, PHA or PWM between Atlantic and Baltic groups, though responses were considerably higher in the group consuming standard pellet food (Figure 5).

Table 2: Estimated cumulative intake of contaminants per rat during the feeding study:

	Atlantic		Baltic	
Compound	µg/kgbw	ng TEQ/kgbw	µg/kgbw	ng TEQ/kgbw
ΣPCBs	1001	(24)	5199	(91)
mono-ortho PCBs	52	5.2	297	37
di-ortho PCBs	13	0.52	138	5.1
non-ortho PCBs	0.72	18	1.37	49
ΣPCDDs (2,3,7,8)	0.02	4.2	0.07	11
ΣPCDFs (2,3,7,8)	0.06	12	0.171	52
ΣDDT	32	n.a.	162	n.a.
HCB	26	n.a.	65	n.a.
ß-HCH	n.d.	n.a.	102	n.a.
dieldrin	127	n.a.	248	n.a.
total TEQ (ng)		**40**		**154**

Table 3: Gross parameters of health in rats fed Atlantic or Baltic herring or pellets:

	ANOVA	Atlantic	Baltic	Pellet
Body weight: start (g)	ns	71.4 ± 4.7	70.9 ± 3.5	73.8 ± 4.4
Body weight: end (g)	*	192 ± 5.4	188 ± 3.4	208 ± 5.6 +
Growth (since birth)	ns	2.75 ± 0.15	2.71 ± 0.15	2.87 ± 0.17
Thymus (mg)	ns	143 ± 8.5	148 ± 4.6	144 ± 6.4
Thymus:body weight index (x1000)	**	0.73 ± 0.03	0.79 ± 0.02	0.69 ± 0.03++
Spleen (mg)	**	347 ± 11.3	359 ± 4.8	421 ± 9.7 ++
Liver (g)	ns	7.6 ± 0.27	8.1 ± 0.22	7.4 ± .35

ANOVA: ns, not significant; * $p<0.05$; ** $p<0.01$; *t*-test: different than Atlantic group; + $p<0.05$; ++ $p<0.01$.

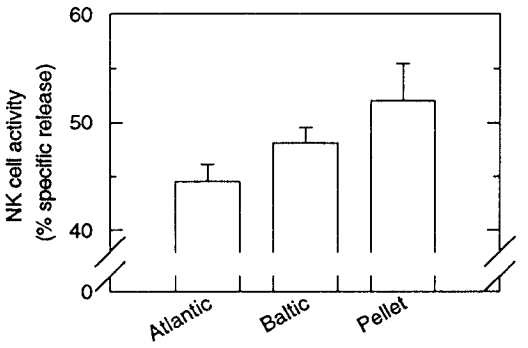


Figure 4: Feeding experiment: Natural killer cell activity as measured as the specific release of ^{51}Cr by YAC target cells using a 100:1 effector:target ratio in a four hour coincubation. Bars represent mean ± SE of results from eight animals per group. No significant differences were detected.

Table 4: Cellularity of the thymus:

	ANOVA	Atlantic	Baltic	Pellet
# cells in thymus	**	192 ± 10.6	151 ± 5.3 ++	144 ± 5.8 ++
#CD4/thymus	**	6.4 ± 0.52	11.3 ± 0.46++	12.22 ± 0.85++
#CD8/thymus	**	3.2 ± 0.31	7.3 ± 0.34++	7.7 ± 0.39 ++
#CD4+CD8+/thymus	**	37.7 ± 3.34	69.8 ± 2.8++	71.1 ± 4.63++
ratio CD4:CD8	**	2.0 ± 0.06	1.6 ± 0.04++	1.6 ± 0.07 ++

ANOVA: ns not significant; * p<0.05; ** p<0.01; *t*-test: different than Atlantic group; + p<0.05; ++ p<0.01.

Table 5: Cellularity of the spleen:

	ANOVA	Atlantic	Baltic	Pellet
# cells in spleen	ns	324 ± 34.5	325 ± 50.9	410 ± 12.9
#CD4/spleen	ns	74.3 ± 9.22	64.5 ± 11.96	83.0 ± 2.92
#CD8/spleen	ns	50.6 ± 6.59	45.5 ± 8.83	55.2 ± 2.76
ratio CD4:CD8	ns	1.5 ± 0.04	1.4 ± 0.05	1.5 ± 0.05

ANOVA: ns not significant; * p<0.05; ** p<0.01; *t*-test: different than Atlantic group; + p<0.05; ++ p<0.01.

No significant differences in total plasma IgM or IgG levels were observed (Table 6), suggesting no effect of contaminants on this parameter.

Host resistance of PVG rats to RCMV

No differences were observed between the Atlantic and Baltic groups of rats in RCMV-specific proliferative T-lymphocyte responses, though these responses were higher in the pellet group (Figure 6). RCMV titres in the salivary glands of the Baltic rats were significantly higher than those in the Atlantic group of rats (Figure 7). Rats fed standard rat pellet had the highest RCMV titres of the three groups, but these

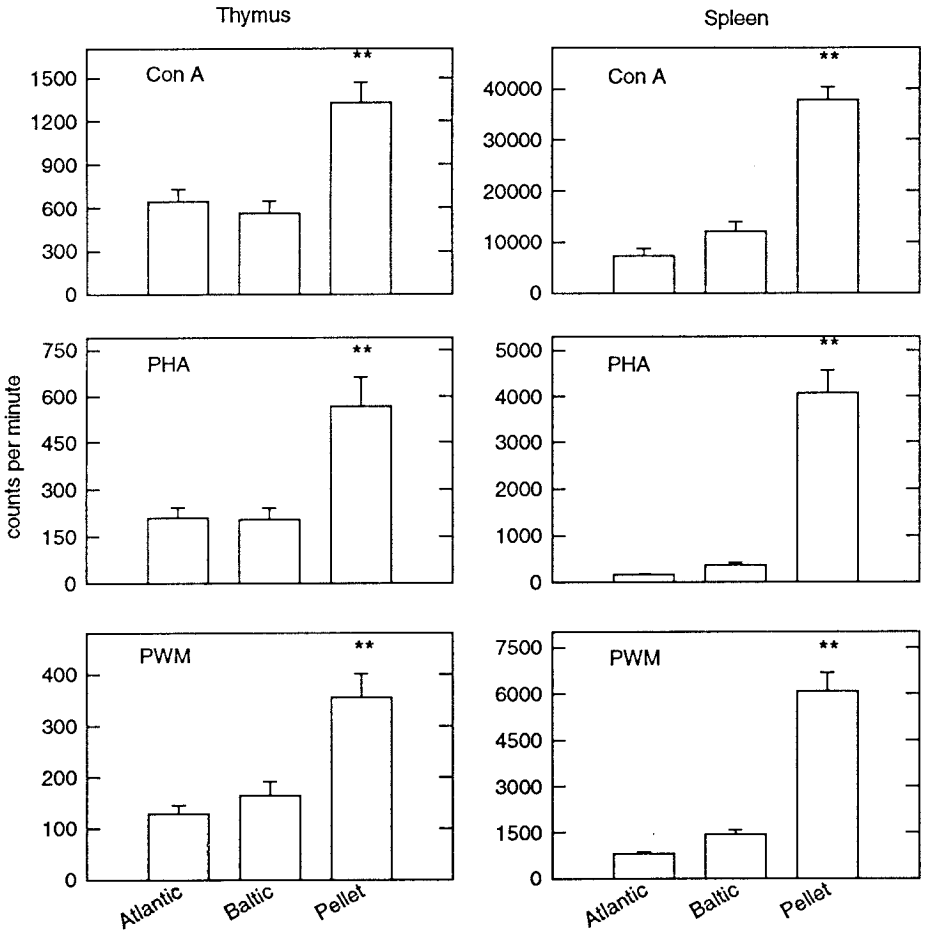


Figure 5: Feeding experiment: Proliferative responses of lymphocytes isolated from the thymus (left column) and spleen (right column) of rats after stimulation with the mitogens Con A, PHA pr PWM. Rats had been fed a diet of either i) Atlantic herring; ii) Baltic Sea herring; or iii) standard rat pellets for a period of 4½ months. Bars represent mean ± SE for total cpm in cell culture for eight animals. Among group differences were measured by univariate ANOVA; between group differences identified by independent *t*-test by comparison with the Atlantic group are indicated by asterisk (** $p<0.01$).

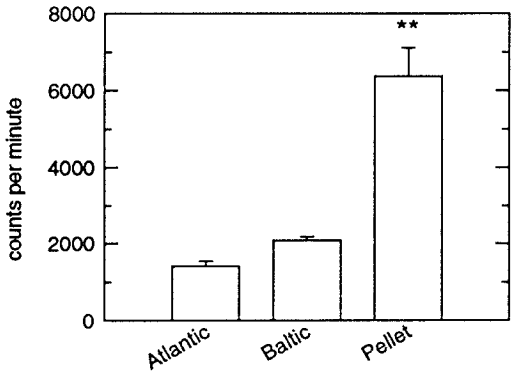


Figure 6: Feeding experiment: The proliferative responses of spleen cells to fixed RCMV-infected stimulator cells following 130 days on the Atlantic, Baltic, or rat pellet diets and 13 days of *in vivo* infection with RCMV. Bars represent means ± SE of eight animals. Significant differences are indicated following univariate ANOVA and asterisks identify significance of difference (* $p<0.05$; ** $p<0.01$) as compared to the Atlantic group.

results cannot be used in a direct comparison with the two herring-fed groups because of major nutritional differences.

Plasma thyroid hormone

Plasma TT4 levels were significantly lower in the Baltic Sea group, as compared to the Atlantic or the pellet group (Table 7). Levels were similar between the rats of the Atlantic group and the dietary control group.

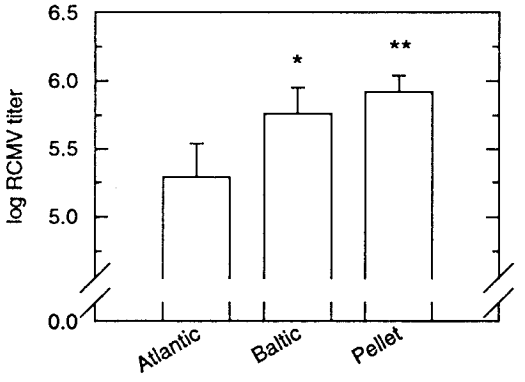


Figure 7: Feeding experiment: RCMV titres in the salivary glands of rats fed Atlantic, Baltic or pellet diets for 130 days, and following 13 days of infection with RCMV. Bars represent mean ± SE of eight animals. Significant differences are indicated following Wilcoxon's signed rank tests and asterisks identify significance of difference (* $p<0.05$; ** $p<0.01$) as compared to the Atlantic group.

Discussion

Chronic exposure to an environmental mixture of contaminants through fish consumption led to decreased plasma thyroid hormone levels but not to any measurable changes in immune function in adult rats fed herring from the Baltic Sea. NK cell activity, and mitogen- and RCMV-induced lymphocyte proliferative responses

Table 6: Total plasma immunoglobulin titres:

	ANOVA	Atlantic	Baltic	Pellet
IgG titre ($x10^3$)	ns	109 ± 59	168 ± 52	180 ± 39
IgM titre ($x10^3$)	ns	9.3 ± 1.0	9.0 ± 0.6	10.0 ± 0.4

ANOVA: ns not significant; * p<0.05; ** p<0.01; *t*-test: different than Atlantic group; + p<0.05; ++ p<0.01.

Table 7: Plasma thyroid hormone levels:

	ANOVA	Atlantic	Baltic	Pellet
Plasma TT4 (nmol/L)	*	37.9 ± 1.3	32.2 ± 1.5 +	37.5 ± 1.1

ANOVA: ns not significant; * p<0.05; ** p<0.01; *t*-test: different than Atlantic group; + p<0.05; ++ p<0.01.

in the two groups of rats fed herring from either the relatively uncontaminated Atlantic Ocean or the contaminated Baltic Sea were not significantly different. However, significantly higher RCMV titres in the salivary glands of the Baltic group of rats suggest that contaminants may have affected parameters of immune function that are important in the control of this virus infection which we either did not measure or could not detect using functional tests. Owing to the nutritional differences between the two herring-fed groups and the pellet-fed group, a direct comparison of these results is not possible. The Baltic Sea herring contained a complex mixture of TCDD-like and other compounds, but available evidence made us expect the greatest immunotoxic potential to come from the *Ah*-dependent PHAH congeners in the Baltic herring. Assuming an *Ah* basis for any potential immunotoxicity of the Baltic herring, our initial experiments using thymus atrophy as an endpoint in rats acutely exposed to TCDD suggested that levels may have been too low to result in detectable immune alterations in the Baltic group of rats. The cumulative exposure levels in our feeding study were at the edge of those which caused thymus atrophy in PVG rats exposed to a one-time dose of TCDD.

However, the daily intake of dietary TCDD toxic equivalents on a body weight basis in the 4½ month feeding study was similar to that which led to immunosuppression in our previous harbour seal studies which lasted 2½ years. In these latter semi-field studies, we observed decreases in NK cell activity (205) and T-cell responses (60,62,204) in the group of seals fed herring from the Baltic Sea as compared to the group fed Atlantic herring, with differences becoming apparent within four months. While the cumulative dose may have been limiting in the rat study, its

shorter lifespan relative to seals suggest that the exposure regime was comparable. Species differences in sensitivity to the immunotoxic effects of TCDD-like compounds may also be the basis for these observations. Among the laboratory rodents studied, the adult rat has been found to be relatively insensitive in this respect (229,275). Although nothing is known of the comparative sensitivity of the harbour seal to TCDD, our findings suggest that they are more sensitive than the PVG rat.

We cannot exclude that differences in immune function between the pellet group on the one hand, and the two groups fed herring on the other, were related to the presence of contaminants in the latter. However, the contaminant levels in the Atlantic herring were far below those of the Baltic group and no dose-related pattern of effects on immune function were detected. It is more likely that other differences between the pellet and the two herring diets, such as nutritional characteristics, may have positively or negatively influenced immune function (138), and masked any effect of contaminants in the Baltic group of rats. The lower body weights of rats in the two herring diets attest to such influences.

Previous feeding studies using laboratory mice have demonstrated that contaminated fish can be immunotoxic. C57Bl/6 mice fed a 33% diet of Coho salmon from Lake Ontario for four months had impaired serum antibody responses to SRBC, but no effects on peripheral lymphocyte subpopulation numbers or cytotoxic T-lymphocyte (CTL) activity were detected (49). No analyses were undertaken for PCDD or PCDF levels, but Lake Ontario salmon contained 2.9 μg/g lipid of total PCBs, being somewhat less than the 4.4 μg/g in our Baltic Sea herring. Differences between their observations and those obtained in our rat study may reflect the relative sensitivity of this mouse strain, differences in the contaminant mixture and levels (contaminants accumulated in the Lake Ontario food chain as opposed to that of the Baltic Sea), and the selection of immune function tests carried out.

Since one of the key advantages of using a laboratory animal model is the ability to assess immune function in the context of host resistance, we designed the RCMV model for the evaluation of virus-specific T-cell responses in the PVG rat. In our initial experiments, we observed an expansion of both $CD4^+$ and $CD8^+$ lymphocytes when spleen or lymph node cells were cultured with fixed, RCMV-infected autologous cells. The high proliferative responses of spleen lymphocytes relative to those isolated from lymph node or blood may reflect the spread of RCMV-specific precursor cells from the spleen to the periphery over time, the potentially better culture conditions afforded by the spleen cell subpopulations and/or the possible continued presence of antigen in the *in vitro* spleen cell cultures. The preferential expansion of $CD8^+$ lymphocytes *in vitro* may indicate that this system reflects CTL activity in RCMV-infected rats, as has been demonstrated in other systems (254). Should this be the case, CTL activity appears not to have been affected by the low doses of contaminants to which the rats were exposed here. Previous studies have found that CTL activity in

mice was affected by a total dose of 2,3,7,8-TCDD as low as 16 ng/kgbw (47,176), while others have observed no effect on CTL activity at doses as high as 3 µg/kgbw in mice (107) or 30 µg/kgbw in rats (198).

In the PVG rat model, elevated RCMV loads in salivary glands in the Baltic group of rats did not correlate with immune function parameters. While reduced host resistance often translates to changes in immune function, the converse is not necessarily true, as measured immunosuppression is a relatively poor predictor of decreased host resistance (159). The high virus loads in the salivary glands of the pellet group appear to be consistent with this fact. A comparison between the herring-fed groups and the pellet-fed group is hampered by the substantial differences in the characteristics of the respective diets.

The lower thyroxine levels in the rats fed Baltic herring demonstrated that the relatively low exposure levels did lead to a biological effect, consistent with previous observations in several species, including the harbour seal (29,62). Thyroid hormones are well established markers of exposure to PCDDs, PCBs and some of their metabolites (31,149). Thyroxine levels are hypothesized to be reduced in animals exposed to these compounds by a combination of a facilitated excretion through induction of UDP-glucuronyl transferase (15) and competitive binding to the serum transport protein for T4, transthyretin (32). In another chronic feeding study, *Ah*-responsive C57Bl/6 mice fed a 33% diet of salmon from Lake Ontario for four months exhibited hepatomegaly, elevated ethoxyresorufin-O-deethylase (EROD) enzyme levels, decreased thyroxine and triiodo-L-thyronine (48). A two-month diet of Lake Ontario salmon also resulted in thyroid disorders and lower serum T4 levels in rats (231).

We conclude that, at least in the present study design, the rat may not be an adequately sensitive animal to mimic the effects of chronic low level exposure to environmental contaminants in seals. However, the increased RCMV titres in Baltic rats point to the utility in combining immune function tests with host resistance tests. Since dosages used here were low, an acute exposure to immunotoxic chemicals may provide a detectable effect on functional responses which may, in turn, lead to altered host resistance. The approach used here, based on a combination of RCMV titre determination in the salivary glands (91) and the assessment of RCMV-specific T-cell responses would be interesting for this purpose.

Adult rats fed a diet of herring from the Baltic Sea did not exhibit any marked changes in immune function, but higher RCMV loads in their salivary glands following infection suggest a possible immunotoxicity which may have been masked by other dietary influences or was otherwise undetectable in our tests. This, combined with the lower TT4 levels, indicate that chronic exposure to low levels of dietary contaminants in Baltic Sea herring can have adverse health effects. Since the developing immune system has been shown to be more sensitive to the effects of

TCDD-like compounds (247,274), perinatal exposure of laboratory rats to these chemicals may exacerbate a chronic exposure-induced immunotoxicity in such a system. In line with this hypothesis, we are currently conducting immunotoxicological studies in which PVG rats are perinatally exposed to the contaminants present in the Baltic Sea herring.

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Impaired cellular immune response in offspring of rats exposed during pregnancy and nursing to Baltic Sea herring oil or 2,3,7,8-TCDD

Peter S. Ross, Rik L. De Swart, Helen van der Vliet, Linette Willemsen, Arja de Klerk, Geert van Amerongen, Jan Groen, Abraham Brouwer, Dennis C. Morse, Henk van Loveren, Albert D.M.E. Osterhaus, and Joseph G. Vos

(submitted)

Abstract

We have previously demonstrated that harbour seals (*Phoca vitulina*) fed herring from the contaminated Baltic Sea developed impaired natural killer (NK) cell activity, T-lymphocyte function and delayed-type hypersensitivity (DTH) responses. In an effort to extend these observations, we carried out a parallel study using laboratory rats, for which additional immunological reagents and techniques are available. Pregnant PVG rats were given oil extracted from herring from the relatively uncontaminated Atlantic Ocean, the contaminated Baltic Sea, or the Atlantic Ocean spiked with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), on a daily basis between day 6 of gestation and the weaning of their pups. The daily intakes of Aryl hydrocarbon (*Ah*)-receptor dependent toxic equivalents (TEQs) for mothers were 0.3 ng/kgbw in the Atlantic group, 2.1 ng/kgbw in the Baltic group, and 134 ng/kgbw in the 2,3,7,8-TCDD positive control group. We assessed immune function and host resistance to rat cytomegalovirus (RCMV) in offspring aged 11, 25, 46 or 59 days. Rat pups of the Baltic and TCDD groups had impaired cellular immune responses, as evidenced by lower mitogen-induced thymus and spleen lymphocyte proliferation *in vitro*, and lower CD4:CD8 cell ratios in the thymus, than pups of the Atlantic group. Differences were most pronounced in pups aged 11 days, and most evident in pups of the TCDD group. Although many non-specific immune function parameters recovered with time, antigen-specific responses *in vivo* were affected in rats aged 46 and 59 days. The RCMV infection-associated increase in NK cell activity was significantly lower in rats of both the Baltic and TCDD groups. RCMV-specific T-lymphocyte proliferative responses *in vitro* were reduced in the TCDD group. RCMV-specific serum antibody titres did not differ among groups at 12 days post-infection, but were significantly lower in both Baltic and TCDD groups at 25 days post-infection. No differences were detected in RCMV titres in the salivary glands of these infected rats at the end of the experiment, suggesting that immunological recovery with age masked possible immunotoxic effects. Plasma thyroxine levels were unaffected in Baltic rat pups, but were lower in the young pups of the TCDD group, where there was also an age-dependent recovery. The recovery in immune function likely reflected the half-life of TCDD in rats and the waning exposure levels in the rapidly growing pups with time. The consistent dose-dependent pattern of effects in both non-specific and specific immune function (Atlantic < Baltic < TCDD) suggests a common underlying mechanism of immunotoxicity in Baltic and TCDD groups of rats, and implicates the *Ah*-receptor binding contaminants in the effects of the Baltic Sea mixture of pollutants. While harbour seals appear to be more sensitive than the PVG rat to the immunotoxic effects of the contaminants present in the Baltic Sea herring, the concordance in results obtained in both species suggests that extrapolation of certain immunotoxic effects from the rat to the seal is possible.

Introduction

The immunotoxic potential of organochlorine chemicals has been well established in laboratory animal studies, with polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) being of particular concern (273). However, little is known of the effects of complex environmental mixtures of these polyhalogenated aromatic hydrocarbons (PHAHs) and other compounds. Fish-eating animals occupying high trophic levels in the aquatic food chain often have high burdens of these persistent lipophilic contaminants, and may be at particular risk to their immunotoxic effects. While the mass mortality among harbour (*Phoca vitulina*) and grey (*Halichoerus grypus*) seals in northern Europe in 1988 was shown to be caused by a newly identified morbillivirus, phocine distemper virus or PDV (73,189), pollution-induced immunotoxicity could not be ruled out as a contributing factor. We subsequently demonstrated that subadult harbour seals fed herring from the contaminated Baltic Sea had impaired natural killer (NK) cell activity (205) and T-lymphocyte responses *in vitro* (60,62) and *in vivo* (204), and speculated that contaminants played a role in the 1988 mass mortality.

Following our harbour seal study, several questions remained unanswered as a result of legal, ethical, and methodological constraints in carrying out immunological studies in seals. In the first of two parallel studies established to extend our findings in seals, adult PVG rats were fed a mixture of freeze-dried herring prepared from the same two supplies used in the seal study. Despite similar intakes of contaminants between rats and seals on a body weight basis, there was no evidence of immune alterations in the rats following 4½ months on the respective diets (206). However, higher virus titres in the salivary glands of rat cytomegalovirus (RCMV)-infected rats fed the Baltic Sea herring suggested a possible immunotoxic effect which we could not detect using our functional assays. Plasma thyroxine levels were significantly lower in the Baltic group, supporting the idea of a biological effect of PHAH exposure.

Several studies have indicated that, compared to other species, the adult rat is relatively insensitive to the immunotoxic effects of low levels of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds (229). However, the developing immune system of mammals, including that of the rat, has been shown to be particularly sensitive to the immunotoxic action of TCDD (80,227,274). Maternal exposure to two doses of 1 µg/kgbw of TCDD during gestation and three doses of 1 µg/kgbw during lactation resulted in thymus atrophy and reduced PHA-induced spleen cell stimulation in 25 day-old male rat pups (274). In another study, a combined pre- and postnatal exposure to four doses of 5 µg/kgbw resulted in more profound and long-lasting effects than those observed in rats exposed only post-natally (80).

Since wildlife species are not only exposed to lipophilic immunotoxic chemicals during adulthood, but also perinatally, the developing immune system of seals inhabiting contaminated areas may be particularly vulnerable to the effects of

environmental contaminants in their diet. The second of our parallel rat studies, presented here, involved a daily exposure of pregnant, and subsequently nursing, female rats to oil extracted from Atlantic and Baltic herring batches used in both of our previous studies. A third group received a mixture of Atlantic herring oil and TCDD and served as a positive control. All rats received standard rat pellet food in order to limit the variables that could affect immune function to contaminant exposure. We assessed immune function parameters at four time points in the offspring of these rats, and evaluated these in the context of host resistance to RCMV infection.

Methods

Herring oil

Oil was prepared from North Atlantic herring or Baltic Sea herring by heating in water to 100°C (National Institute for Fisheries Research, Ijmuiden, The Netherlands). The lipid fraction was mechanically removed, centrifuged once and the supernatant extracted. This oil was mixed with the antioxidant 0.02% butyl-hydroxytoluene (BHT) and 30 ml aliquoted in 50 ml brown glass bottles. Bottles were then filled with argon gas, sealed and stored at -20°C until use.

Determination of dietary PCB, PCDD and PCDF levels

Atlantic and Baltic herring oil was analyzed for congener-specific planar PCBs (IUPAC numbers 77, 126 and 169) using methods described elsewhere (259). Mono-ortho (IUPAC numbers 105, 114, 118, 123, 156, 157, 167 and 189) and di-ortho (IUPAC numbers 170 and 180) PCB concentrations were determined by multidimensional gas chromatography using methods described elsewhere (54). Concentrations of all 2,3,7,8 chlorine-substituted PCDD (n=7) and PCDF (n=10) congeners were determined using methods described elsewhere (153). Values of TCDD toxic equivalents (TEQs) were then determined for each of these congeners using recently described toxic equivalent factors (TEFs) for PCBs (6), PCDDs and PCDFs (264).

Study design

Pregnant rats were divided into three groups and given relatively uncontaminated Atlantic herring oil or contaminated Baltic Sea oil or Atlantic oil containing 2,3,7,8-TCDD. This oil was administered by oral gavage on a daily basis from day 6 of gestation to the weaning of the pups (a total of 41 days). Immune function was assessed in four female pups from each nest at different time points after birth: pups aged 11, 25, 46 and 59 days (n=8 per group per necropsy). Rat pups of the latter two age groups were infected with RCMV at age 34 days and used in a host resistance study. In addition, one 21-day-old male per nest was used for a study of delayed-type hypersensitivity (DTH) responses.

Rat study

Animals were housed and cared for under the supervision of Animal Ethics Committee of the National Intitute of Public Health and the Environment (Bilthoven, The Netherlands), according to the regulations of the European Community Council Directive on the care of laboratory animals (86/609/EEC).

Eight-week-old, specific pathogen free (SPF), behaviourally receptive adult female PVG (inbred) rats (PVG/OlaHsd; Harlan-Olac, Zeist, The Netherlands) were bred overnight and subsequently housed separately in sterile filter top cages. From day 6 of the theoretical pregnancy onwards, all rats (n=45) received by oral gavage 1 ml/day of Atlantic or Baltic herring oil, or a positive control consisting of 27.68 ng 2,3,7,8-TCDD (Dow Chemical, Midland, USA) per ml Atlantic herring oil. Rats received an *ad libitum* supply of water and standard irradiated rat pellet food (#1210 SP; Hope Farms, Woerden, The Netherlands) for the duration of the feeding study. Pregnancy was assessed by weight gain in late gestation, and a minimum of eight successful nests per exposure group were ultimately used in the study. With the exception of the day of and the day following birth, oil was administered to the mothers on a daily basis until their pups were weaned at 24 days of age.

On the day following birth, rat pups were sexed, weighed on a pooled sex basis, and nests adjusted to four females and three males each. One female pup per nest was later used in each of two necropsies for assessment of immune function and two host resistance studies using RCMV. One male per nest was used to study DTH responses to ovalbumin. Other males were used for a separate study.

***In vitro* tests of immune function**

For the first two immune function necropsies (age of pups 11 and 25 days), one female pup was sacrificed from each of eight nests from each group. Body, thymus, spleen and liver weights were recorded, and the thymus and spleen were placed aseptically in culture medium consisting of RPMI 1640 (GIBCO, Grand Island, USA), 10% heat inactivated fetal calf serum (FCS; PAA, Linz, Austria), 100 IU/ml penicillin, 100 µg/ml streptomycin and 2 mM glutamine. Cell suspensions were prepared as described elsewhere (206), counted and adjusted to concentration.

Cell suspensions of both thymus and spleen were analyzed for CD4 and CD8 T-lymphocyte subpopulations by surface markers. Using a double staining, CD4 cells were labelled using FITC-labelled ER-2 (Serotec, Oxford, UK) and CD8 cells labelled with biotynilated OX8 (Serotec) monoclonal antibodies as described previously (206). A fluorescence-activated cell scanner (FACS; Becton Dickinson, Rutherford, USA) was used to measure triplicate samples of 10,000 cells. Analysis of mononuclear cell populations was carried out using gates based on the basis of forward and side scatter characteristics.

Mitogen-induced lymphocyte stimulations were undertaken using thymus and spleen cell suspensions as described previously (270). Briefly, 2 x 10^6 thymus cells or

8 x 10^5 spleen cells were stimulated with the mitogens concanavilin A (Con A; final concentration of 2 µg/ml; Janssen Chimica, Beerse, Belgium), phytohaemagglutinin (PHA; final concentration of 1:60; Wellcome Foundation, Dartford, England) or pokeweed mitogen (PWM; final concentration of 1:60; GIBCO) were placed in 96-well round-bottomed cell culture plates (Greiner, Nürtingen, Germany). Plates were placed in 37°C 5% CO_2 humidified incubators, and lymphocyte proliferation was assessed by ^{3}H-thymidine incorporation after 72 hours of culture.

Natural killer (NK) cell activity in spleen cell preparations was assayed following removal of adherent cells by overnight incubation of the spleen cells at 37°C as described elsewhere (57). NK cell activity was measured as the ability of 2 x 10^6 spleen cells to lyse 1 x 10^4 ^{51}Cr-labelled YAC-1 target cells in a four-hour coincubation in 96-well cell culture plates, and was calculated as the (radioactive counts in the supernatant minus the spontaneous release by YAC) divided by (the maximal release by YAC cells minus the spontaneous release by YAC cells).

Total plasma IgG and IgM levels were determined using enzyme-linked immunosorbent assays (ELISAs) as described elsewhere (271), and titres were defined as the plasma dilution at which the maximum absorbance signal obtained from pooled plasma samples from the given necropsy day at 450 nm was reduced by 50%.

Delayed-type hypersensitivity responses

Eight males aged 21 days from each group were immunized subcutaneously in the neck using a 0.1 ml emulsion of Freund's Complete Adjuvant (FCA) and 100 µg ovalbumin (grade II; Sigma Chemicals, St. Louis, USA) as described elsewhere (277). These males, plus four non-immunized animals from each group, were tested for DTH reactivity to ovalbumin at age 46 days. For this, rats were anesthesized and a 10 µg ovalbumin in 25 µl saline solution or a control injection of 25 µl saline was injected intradermally into each ear. Increase in ear thickness was measured at 24 and 48 hours following this injection using a digital micrometer (Mitutuyo, Tokyo, Japan), and aspecific swelling induced by ovalbumin in non-immunized rats subtracted from mean values obtained from immunized rats.

Host resistance to RCMV

The two remaining female pups per nest were infected intraperitoneally with 1 x 10^5 plaque forming units (PFU) RCMV (obtained from C. Bruggeman, University of Limburg, The Netherlands) in saline at 34 days of age, and necropsies carried out at age 46 and 59 days (at 12 and 25 days following infection). In addition to carrying out the same tests of immune function as described above, the specific spleen cell responses to RCMV *in vitro* and virus titres in salivary glands were assessed. Spleen cell suspensions were further purified for mononuclear cells by Ficoll (Pharmacia LKB, Uppsala, Sweden) 1.077 g/ml density gradient isolation prior to culture and adjusted for both mitogen and RCMV stimulations to 5 x 10^6/ml.

RCMV-specific stimulations consisted of a coincubation of 3 x 10^3 paraformaldehyde-fixed RCMV-infected rat embryo cells (REC) and 5 x 10^5 spleen cells in 150 µl per well in 96-well round-bottomed cell culture plates, using methods described previously (206). Plates were incubated at 37°C in a 5% CO_2 humidified incubator and ^{3}H-thymidine incorporation measured between 72 and 96 hours.

For the assessment of virus titres, salivary glands during both necropsies were removed aseptically and placed in Basal medium Eagle (GIBCO) containing 2% FCS. A 1:10 weight:volume suspension was frozen at -86°C until the determination of RCMV titres as described elsewhere (91).

RCMV-specific total immunoglobulin titres were determined using an indirect ELISA with slight modifications to methods described elsewhere (101). Briefly, RCMV cell lysate prepared from rat embryo fibroblasts was coated onto 96-well flat-bottomed microtitre plates, and horseradish peroxidase (HRPO)-labelled goat anti-rat IgG (Cappel Organon, Turnhout, Belgium) was used as conjugate. Titres were expressed as the plasma dilution at which the maximum absorbance signal at 450 nm was reduced by 50%.

Plasma thyroid hormone measurement

Total plasma thyroxine (TT4) levels were determined using a chemiluminescence immunoassay (Amersham, Little Chalfont, UK) as previously described (175).

Estimation of contaminant intake by pups

Since nests were standardized to seven pups immediately following birth, the theoretical dosage of TEQs for pups aged 11 and 25 days was calculated on the basis of the cumulative intake of TEQs by the mothers between day 6 of gestation and the two first respective necropsy days. Based on PCB (U-^{14}C KC-600) dynamics in pregnant and nursing rats (239), a conservative estimate for TEQ dose in our rat pups was calculated using this author's measurement of a transfer of 3.2% and 4.9% of total maternal dose to each rat pup by age 11 and 25 days, respectively, but assuming no loss by mothers via faeces and urine and no metabolic breakdown of contaminants in the pups.

Statistical analysis

Among group differences were tested using univariate analysis of variance (ANOVA) for each parameter measured on a given necropsy day. If a significant difference was detected, independent *t*-tests were carried out to determine which group was significantly different from the control Atlantic group. For the DTH test, a repeated measures analysis of variance with grouping factors was carried out. Significance levels are indicated by **$p<0.01$ or *$p<0.05$ for univariate ANOVA, and ++$p<0.01$ or +$p<0.05$ for independent *t*-tests in tables. Because of the non-normal

distribution of virus titres following RCMV infection, a Wilcoxon's signed rank test was used.

Results

Breeding experiment

Of the 45 females bred, 44 were pregnant and carried to full term. At one day following birth, total pups per nest, female pup numbers and female weights were not significantly different among the three treatment groups of eight nests, although males were significantly smaller in the TCDD group (Table 1). Following the loss of two nests and the standardization of nest size to four female and three male pups, eight randomly-selected nests per treatment group were maintained for the duration of the study. Mothers showed no differences in body weights.

Table 1: Breeding study: nest characteristics one day following birth:

	ANOVA	Atlantic	Baltic	TCDD
# successful nests		16/17	15/16	11/11
# pups per nest	ns	10.3 ± 0.47	9.4 ± 0.66	9.0 ± 0.65
# female pups per nest	ns	4.67 ± 0.60	5.00 ± 0.80	5.38 ± 0.46
Weight of female pups	ns	4.78 ± 0.09	4.59 ± 0.22	4.31 ± 0.11
Weight of male pups	**	5.15 ± 0.07	5.18 ± 0.14	4.32 ± 0.09 ++
Weight of mothers	ns	186 ± 4.27	192 ± 2.67	191 ± 4.63

ANOVA: ns, not significant; *, $p<0.05$; **, $p<0.01$; *t*-test: different from Atlantic group; +, $p<0.05$; ++, $p<0.01$.

Intake of contaminants

Rat mothers in the Baltic group received a 8.5 times higher daily dosage of TEQs than mother rats in the Atlantic group, while those in the TCDD positive control group received a 63 times higher daily dosage than those of the Baltic group (Table 2). Rat pups born to these mothers were exposed via the placenta during pregnancy for 16 days, and via milk until necropsy at 11 days of age or weaning at age 24 days. The intake of aryl hydrocarbon (*Ah*)-dependent contaminants by rat pups was estimated, with the older pups having a body weight growth-associated decline in TEQ levels (Table 3).

Gross health parameters

Rat pups born to mothers in the Baltic Sea group exhibited no gross indications

Table 2: Estimated intake of TCDD toxic equivalents (TEQs) per rat mother expressed as ng TEQ/kg body weight unless otherwise indicated:

	Atlantic	Baltic	TCDD
Daily dose (ng TEQ)	0.05	0.44	27.7
Daily intake	0.25	2.12	134
Cumulative intake at birth	3.92	33.9	2 143
Cumulative intake at pup age 11 days	6.62	57.2	3 616
Cumulative intake at weaning	10.1	86.9	5 490

Table 3: Estimated cumulative intake of 2,3,7,8-TCDD TEQs for each rat pup using PCB transfer data of Takagi *et al.* (239) expressed as absolute daily dose (ng) or on a body weight basis in ng TEQ/kg body weight:

	Atlantic	Baltic	TCDD
Cumulative dose at age 11 days (ng)	0.04	0.35	22.2
Cumulative intake at age 11 days (ng/kg)	2.03	19.9	1 440
Cumulative dose at 25 days (ng)	0.09	0.83	52.1
Cumulative intake at age 25 days (ng/kg)	1.83	17.3	1 363

of toxicity, with organ weights being similar to the Atlantic group (Table 4). On the other hand, rat pups born to mothers in the TCDD positive control group had a number of significant effects, including reduced body weights at the time of all necropsies, reduced liver weights at the time of the second and last necropsies, and reduced thymus weights at the time of the first three immune function necropsies. However, there was no significant effect of TCDD on the growth rates of pups.

Of the gross immunological parameters, thymocyte numbers (Table 5) and thymus subpopulations (Table 6) were most affected by contaminants, while spleen cell subpopulations showed more resiliency. Of particular note was a consistent pattern of reduced $CD4^+$:$CD8^+$ ratios in the thymus, particularly in the TCDD group, until the fourth necropsy (Figure 1a). This was not the case in the spleen, where no alterations in $CD4^+$:$CD8^+$ ratios were detected (Figure 1b).

Table 4: Gross health parameters:

Parameter/age of pup	ANOVA	Atlantic	Baltic	TCDD
Body weight (g)				
11 days	**	19.8 ± 0.54	18.2 ± 1.16	15.4 ± 0.59 ++
25 days	**	48.7 ± 1.24	48.0 ± 1.36	38.2 ± 1.23 ++
46 days (+RCMV)	**	112.5 ± 2.16	109.6 ± 2.88	97.1 ± 3.66 ++
59 days (+RCMV)	**	140.1 ± 1.61	137.8 ± 2.7	118.6 ± 2.6 ++
Growth (since birth)				
11 days	ns	4.2 ± 0.18	4.3 ± 0.41	3.6 ± 0.20
25 days	ns	10.4 ± 0.45	10.8 ± 0.83	8.9 ± 0.35
46 days (+RCMV)	ns	23.4 ± 0.8	24.3 ± 1.4	22.7 ± 1.0
59 days (+RCMV)	ns	29.4 ± 0.74	30.7 ± 2.0	27.7 ± 1.0
thymus weight (mg)				
11 days	**	56.9 ± 3.52	47.6 ± 4.31	34.5 ± 2.00 ++
25 days	**	147 ± 5.40	140 ± 5.38	87.7 ± 10.4 ++
46 days (+RCMV)	**	276 ± 12.1	289 ± 10.7	241 ± 4.86 +
59 days (+RCMV)	ns	248 ± 9.3	250 ± 5.77	233 ± 7.20
thymus:body weight ratio (x1000)				
11 days	**	2.86 ± 0.13	2.70 ± 0.09	2.23 ± 0.08 ++
25 days	**	2.82 ± 0.14	2.92 ± 0.08	2.29 ± 0.27 +
46 days (+RCMV)	ns	2.45 ± 0.06	2.63 ± 0.06	2.50 ± 0.08
59 days (+RCMV)	ns	1.77 ± 0.06	1.81 ± 0.03	1.98 ± 0.08
spleen weight (mg)				
11 days	ns	101 ± 6.38	94.7 ± 9.21	89.9 ± 4.75
25 days	*	232 ± 9.30	225 ± 7.38	190 ± 22.2 ++
46 days (+RCMV)	ns	363 ± 11.02	363 ± 8.10	353 ± 12.4
59 days (+RCMV)	ns	359 ± 5.87	344 ± 7.78	325 ± 14.1
liver weight (mg)				
11 days	ns	585 ± 18.4	547 ± 34.8	535 ± 17.0
25 days	*	2252 ± 74.6	2277 ± 53.3	2000 ± 223 ++
46 days (+RCMV)	ns	5360 ± 99.9	5370 ± 200	5092 ± 196
59 days (+RCMV)	**	6552 ± 179	6570 ± 265	5640 ± 82.6++

ANOVA: ns not significant; * $p<0.05$; ** $p<0.01$; *t*-test: + $p<0.05$; ++ $p<0.01$).

There were no significant differences in total IgM and IgG titres among the three groups during the course of the experiment (Table 7). However, total IgM titres had a significantly greater increase immediately following RCMV infection (difference between titre on day 46 compared to day 25; $p<0.05$; results not shown) in the TCDD group of rats.

Table 5: Thymus and spleen cellularity:

	ANOVA	Atlantic	Baltic	TCDD
# thymus cells (x 10^6)				
11 days	**	121 ± 15.8	87.4 ± 7.2	57.0 ± 4.2 ++
25 days	**	226 ± 14.2	272 ± 23.2	130 ± 14.6 ++
46 days (+RCMV)	ns	403 ± 24.3	416 ± 25.4	372 ± 22.0
59 days (+RCMV)	*	508 ± 23.4	418 ± 17.0 ++	393 ± 33.8 ++
# spleen cells (x 10^6)				
11 days	ns	46.4 ± 2.62	47.2 ± 5.02	45.5 ± 4.02
25 days	ns	145 ± 10.5	147.4 ± 11.1	120.1 ± 15.0
46 days (+RCMV)	ns	280 ± 16.7	279 ± 22.7	277 ± 19.9
59 days (+RCMV)	ns	248 ± 10.2	231 ± 8.57	231 ± 10.9

ANOVA: ns not significant; * $p<0.05$; ** $p<0.01$; *t*-test: + $p<0.05$; ++ $p<0.01$.

Table 6: Flow cytometry analyses of thymus and spleen cell subpopulations:

	ANOVA	Atlantic	Baltic	TCDD
#CD4 in thymus (10^6):				
11 days	*	7.54 ± 1.14	5.88 ± 0.52	2.88 ± 0.28 +
25 days	**	19.7 ± 1.44	23.0 ± 1.92	10.6 ± 1.06++
46 days (+RCMV)	*	41.7 ± 2.68	41.5 ± 2.94	32.2 ± 2.44 +
59 days (+RCMV)	*	54.9 ± 2.47	48.2 ± 2.73	40.4 ± 4.04++
#CD8 in thymus (10^6):				
11 days	**	8.90 ± 0.90	8.82 ± 0.86	5.04 ± 0.30 +
25 days	**	10.8 ± 0.98	13.1 ± 1.16	7.08 ± 0.60 +
46 days (+RCMV)	ns	16.5 ± 1.60	18.5 ± 1.45	18.6 ± 1.42
59 days (+RCMV)	*	29.1 ± 1.44	24.3 ± 1.82	21.9 ± 2.32 +
#CD4+CD8+ in thymus (10^6):				
11 days				
25 days	**	99.6 ± 13.4	67.2 ± 7.44 +	47.0 ± 3.84++
46 days (+RCMV)	ns	187 ± 11.9	226 ± 19.4	106 ± 8.22++
59 days (+RCMV)	ns	333 ± 19.6	344 ± 21.1	309 ± 18.9
	*	409 ± 20.5	333 ± 13.3 ++	320 ± 27.5 +
#CD4 in spleen (10^6):				
11 days	ns	2.10 ± 0.24	2.08 ± 0.28	1.44 ± 0.20
25 days	**	16.1 ± 1.10	16.2 ± 0.52	11.72 ± 0.96++
46 days (+RCMV)	nd	nd	nd	nd
59 days (+RCMV)	ns	82.4 ± 3.32	78.6 ± 3.18	77.2 ± 5.13
#CD8 in spleen (10^6):				
11 days	ns	1.32 ± 0.10	1.28 ± 0.18	0.94 ± 0.08
25 days	**	6.06 ± 0.36	5.70 ± 0.24	4.54 ± 0.32++
46 days (+RCMV)	nd	nd	nd	nd
59 days (+RCMV)	ns	37.0 ± 1.14	36.5 ± 1.49	33.9 ± 1.17

ANOVA: ns not significant; nd, not determined; * $p<0.05$; ** $p<0.01$; *t*-test: + $p<0.05$; ++ $p<0.01$.

Table 7: Immunoglobulin titres and specific antibody responses:

	ANOVA	Atlantic	Baltic	TCDD
Total IgM titre ($x10^3$):				
11 days	ns	2.2 ± 0.3	1.8 ± 0.2	1.5 ± 0.2
25 days	ns	10.1 ± 1.1	9.8 ± 1.0	8.7 ± 0.9
46 days (+RCMV)	ns	27.8 ± 3.6	30.2 ± 5.1	35.7 ± 3.4
59 days (+RCMV)	ns	26.5 ± 2.3	25.1 ± 2.4	21.7 ± 2.2
Total IgG titre ($x10^3$):				
11 days	ns	518 ± 79	452 ± 54	357 ± 50
25 days	ns	492 ± 102	632 ± 137	633 ± 75
46 days (+RCMV)	ns	399 ± 64	300 ± 57	369 ± 71
59 days (+RCMV)	ns	329 ± 61	289 ± 47	399 ± 72
Ova-specific antibody titre in DTH males:				
IgM	ns	57 ± 19	44 ± 13	45 ± 13
IgG ($x10^3$)	ns	19.6 ± 7.9	23.5 ± 2.6	31.7 ± 7.8
RCMV-specific IgG titre:				
46 days				
59 days	ns	184 ± 13	230 ± 31	203 ± 18
	**	529 ± 88	241 ± 25++	324 ± 54 +

ANOVA: ns not significant; * p<0.05; ** p<0.01; *t*-test; + p0<.05; ++ p<0.01.

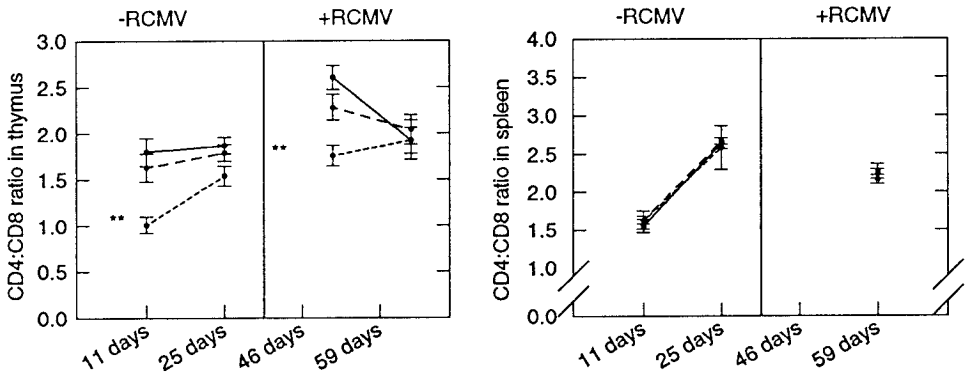

Figure 1: CD4:CD8 ratios in a) thymus (left) and b) spleen (right) mononuclear cells before and after RCMV infection in rat pups of the Atlantic (solid line), Baltic (long dash), and TCDD (short dash) groups. Dots represent mean ± SE of eight pups.

Mitogen-induced thymus and spleen cell proliferation

While there were no notable differences in gross health or immune parameters between rat pups of the Atlantic and Baltic groups, there were clear indications of cellular immunosuppression in the Baltic group as exemplified by functional tests. The

young rat pups of the TCDD group had lower Con A, PHA and PWM-induced thymus cell proliferative responses, and lower Con A- and PWM-induced spleen

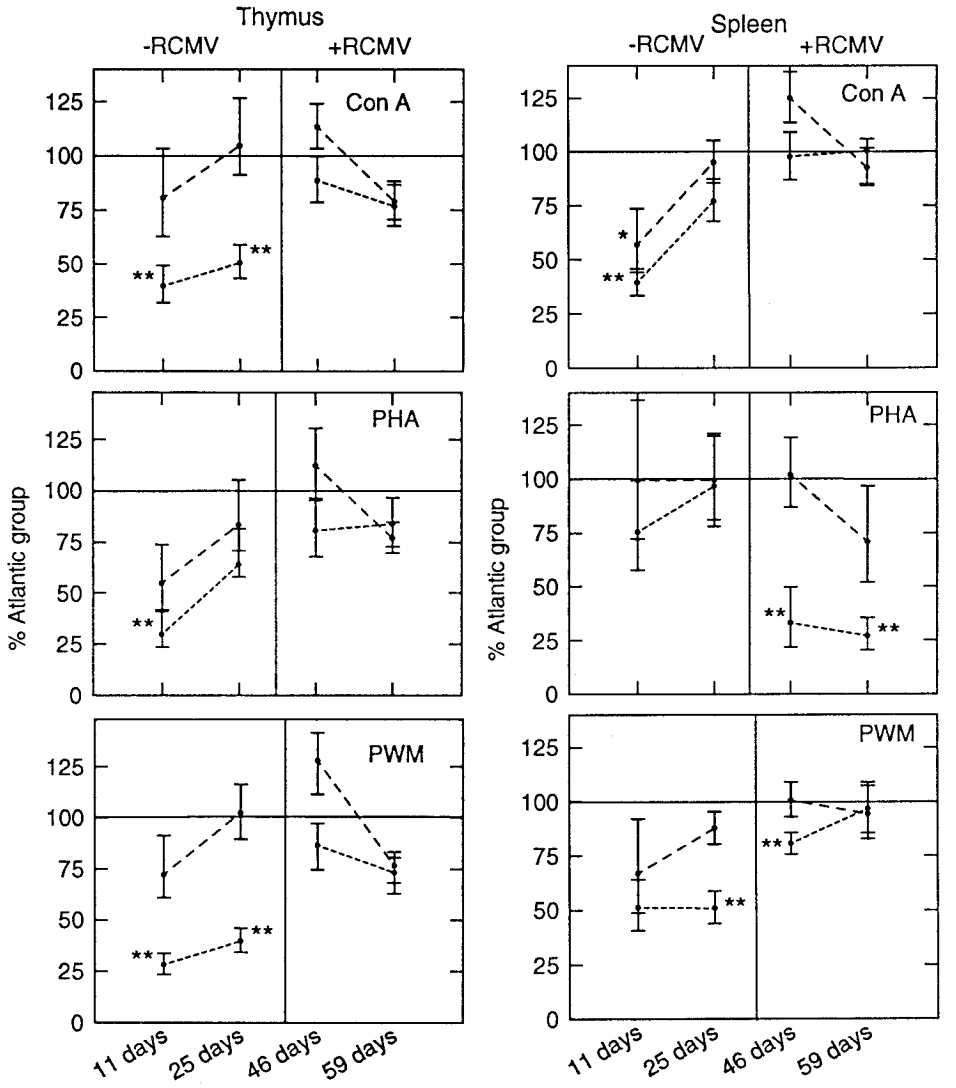

Figure 2: Mitogen-induced proliferative responses of thymus (left) and spleen cells (right) expressed as a % of the Atlantic group in Baltic (long dash) and TCDD (short dash) groups before and after RCMV infection. Data are corrected on an organ basis and natural log-transformed, and vertical error bars represent the 66% confidence interval of the ratio of means, and was calculated as the anti-log transformation of the differences between the groups on the log scale plus or minus the standard errors of these differences (n=8 per data point).

lymphocyte responses (Figure 2). Effects were most notable in 11 day-old rat pups of both Baltic and TCDD groups, when Con A-induced spleen lymphocyte stimulation was significantly lower. There was a consistent gradient of Atlantic > Baltic > TCDD for mitogen-induced stimulations of both thymus and spleen cells in the first two immune function necropsies. Following RCMV infection in the older rat pups, proliferation to mitogens by thymus cells appeared to have recovered, while responses of spleen lymphocytes to PHA were lower in the TCDD group.

Delayed-type hypersensitivity responses

Ovalbumin-specific DTH responses were observed, since immunized rats had greater swellings than non-immunized rats 24 hours after intradermal injection. A gradient of Atlantic > Baltic > TCDD was observed at the peak swelling time of 24 hours, though there were no significant differences (Figure 3). Specific IgG responses to ovalbumin revealed an inverse pattern to that observed for many of the other immune function parameters (TCDD > Baltic > Atlantic) and to that observed for IgM, although differences were not significant (Table 7).

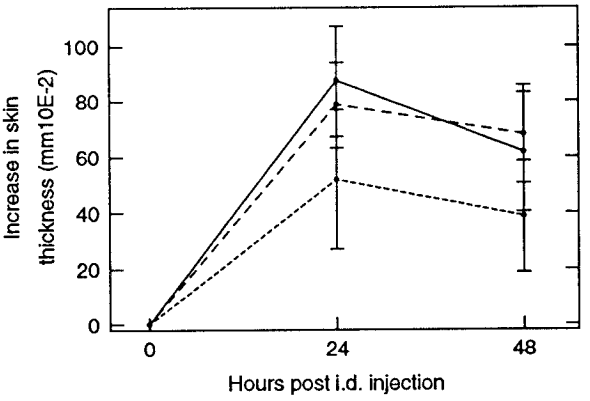

Figure 3: Delayed-type hypersensitivity responses to ovalbumin in male pups of the Atlantic (solid line), Baltic (long dash) and TCDD (short dash) groups. Data points represent the mean absolute increase ± SE of eight pups after subtraction of the mean responses of non-immunized animals to antigen.

Natural killer cell activity

Basal NK cell activity was virtually undetectable in the 11-day-old rats, and was higher in the TCDD pups aged 25 and 59 days (Figure 4). However, the RCMV-associated increase in NK cell activity was significantly lower in both Baltic and TCDD rat pups than in the Atlantic group (the ratio of NK cell activity in 46-day-old:25-day-old pups).

Host resistance to RCMV

Proliferative responses of spleen lymphocytes to RCMV-infected stimulator cells were significantly lower in the TCDD group than the Atlantic group in pups aged 46 days, and a gradient was apparent for Atlantic > Baltic > TCDD groups (Figure 5).

Total RCMV-specific immunoglobulin titres did not differ among groups 12 days following infection, but were significantly lower in both the Baltic and TCDD groups at 25 days post-infection (Table 7). Virus titres were not significantly different among groups on both necropsy days after either 12 or 25 days of infection (Table 8).

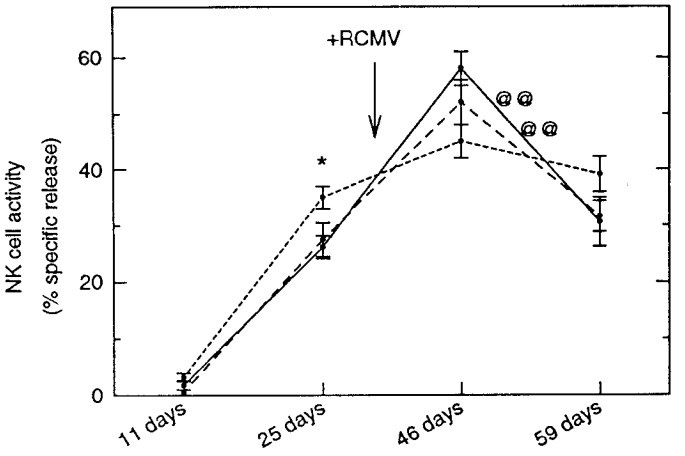

Figure 4: Natural killer cell activity in Atlantic, Baltic and TCDD groups, expressed as the natural cytotoxic activity of spleen cells using ^{51}Cr-labelled YAC-1 tumour cells as targets. Data points represent the mean ± SE of eight pups. Differences are indicated for basal activity (**p<0.01; *p<0.05) and for virus-associated increase following RCMV infection at age 37 days (specific release at age 46 days / specific release at age 25 days; @@p<0.01).

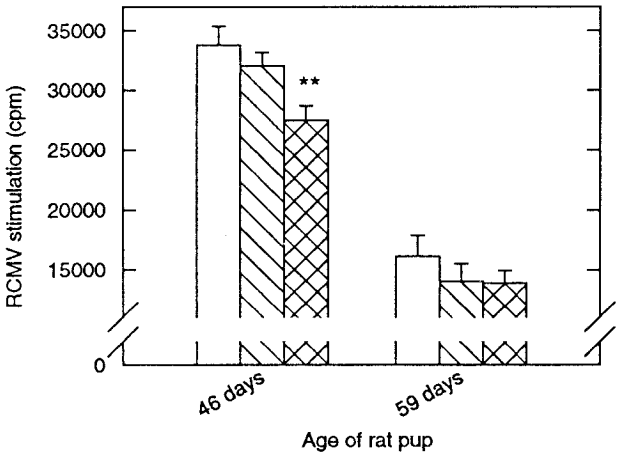

Figure 5: RCMV-induced stimulation of spleen cells isolated from rats infected for 12 days (46-day-old rats) or 25 days (59-day-old rats), *in vitro*, in Atlantic (no fill), Baltic (single hatch), or TCDD (double hatch) groups. Bars represent mean gross counts per minute for eight rats ± SE of wells coincubated with RCMV-infected REC following four days of incubation.

Plasma thyroid hormone

Total thyroxine levels in plasma did not differ between Atlantic and Baltic groups at any age, but were significantly lower in the 12 and 25 day-old TCDD group (Table 9).

Table 8: RCMV titres in the salivary glands of rat pups:

	ANOVA	Atlantic	Baltic	TCDD
46 day-old pups (12 days RCMV)	ns	5.51 ± 0.30	5.69 ± 0.33	5.03 ± 0.36
59 day-old pups (25 days RCMV)	ns	5.84 ± 0.28	6.23 ± 0.14	5.42 ± 0.24

ANOVA: ns not significant; * p<0.05; ** p<0.01; *t*-test: + p<0.05; ++ p<0.01.

Table 9: Plasma thyroxine levels:

	ANOVA	Atlantic	Baltic	TCDD	
11 day-old pups	*	47.7 ± 2.0	45.3 ± 2.5	38.4 ± 2.8	+
25 day-old pups	**	29.0 ± 1.0	28.5 ± 1.1	15.7 ± 1.2	++
46 day-old pups	ns	33.9 ± 2.9	28.7 ± 2.5	32.3 ± 3.0	
59 day-old pups	ns	32.6 ± 2.9	35.0 ± 1.5	39.0 ± 2.7	

ANOVA: ns not significant; * p<0.05; ** p<0.01; *t*-test: + p<0.05; ++ p<0.01.

Discussion

Perinatal exposure to low levels of lipophilic environmental contaminants in Baltic herring resulted in a suppression of cellular immune responses in pups of exposed mothers. These effects were most severe in the youngest rat pups, and immune function responses of rats in the Baltic group fell consistently between a negative control group (Atlantic herring oil) and a positive control group (Atlantic herring oil spiked with TCDD).

The immune systems of the rats in our study were clearly in a developmental phase, as evidenced by the increasing numbers of thymus and spleen cell sub-populations, and the increasing NK cell activity and mitogen-induced lymphocyte proliferation with age. The plasma IgM and IgG levels in rat pups over time reflected the patterns of maternal transfer via milk (IgG) consistent with the endotheliochorial placentation of rats, and the endogenous production by the pups (IgM and IgG). Because of the age-related immunological changes, the evaluation of an effect of con-

taminants was restricted to a comparison among the treatment groups of a given age.

The thymus was particularly affected by contaminants, with reduced numbers and functionality of thymocytes being evident in the younger pups of the TCDD group. Effects upon developing thymocytes appeared to have systemic repercussions, with spleen T-lymphocyte proliferative responses being affected to differing degrees throughout the study. However, a direct effect on peripheral lymphocytes cannot be excluded. There was a trend towards recovery in many functional parameters with age, but numbers of thymocytes and thymus subpopulations, PHA-induced spleen lymphocyte proliferative responses, and thymus CD4:CD8 ratios were lower in the older TCDD pups, and to a lesser extent, the Baltic pups.

The results of these *in vitro* non-specific tests of immune function were generally corroborated by specific *in vivo* responses to protein or viral antigen. Dose-related DTH and IgM responses to ovalbumin were observed (Atlantic > Baltic > TCDD). This pattern was more pronounced in our harbour seal study, where ovalbumin-specific DTH and antibody responses were significantly lower in the Baltic group than in the Atlantic group (204). Following infection with RCMV, the virus-associated increase in NK cell activity was impaired in both the Baltic and TCDD groups of rats, consistent with observations in other studies (220,291). The lower RCMV-specific antibody titres 25 days post-infection in both Baltic and TCDD groups suggest an effect of contaminants on the humoral response, which may reflect a direct action on B-cells, or more likely, an effect at the T-helper cell level. In addition, the impaired RCMV-specific T-cell responses may indicate that CTL activity was affected in TCDD-exposed rats (206). RCMV titres in the salivary glands of immunosuppressed rat pups were not elevated, suggesting that the age-related recovery in immune function that we observed led to an ability of the rats to control the virus infection. This contrasts the results of our previous study of adult rats, where higher RCMV titres in the Baltic herring-fed group indicated an immunosuppression that we were otherwise unable to detect using functional tests.

The estimated dose of TEQs to which the pregnant rats and their pups were exposed here were low by comparison to those used in other studies. The 11-day-old Baltic rat pups exhibited immunological dysfunction when their mothers had received a daily dose of only 2 ng/kg and a total cumulative TEQ dose of 57 ng/kgbw, at which point we estimated the cumulative pup exposure to be 20 ng/kgbw. While the thymus atrophy and related impairment of cellular immunity is consistent with the known effects of 2,3,7,8-TCDD (273), most studies have utilized dosages far exceeding those used here, and relied primarily on acute exposures administered on a one-time or limited basis. Earlier studies which demonstrated the developing immune system of rats to be relatively sensitive to the immunotoxic effects of 2,3,7,8-TCDD used multiple maternal doses of 5 μg/kgbw (80,274). Pre- and postnatal exposure was shown to result in more profound effects than postnatal exposure alone (80), while the

long lasting effects may have reflected the high initial dosage and resulting maintenance of immunotoxic levels of TCDD in rat pups of those studies. The partial recovery observed in our rat pups likely reflected the removal of the contaminant source (milk) at weaning, the metabolic loss of 2,3,7,8-TCDD in the rat pups, and the rapid growth of pups during this period. The estimated 24-day half-life of 2,3,7,8-TCDD in rats (203) would mean that the rapidly growing pups in our study would have diminishing body burdens during nursing and after weaning from their mothers.

A possible synergistic or antagonistic effect of different PHAH congeners cannot be ruled out in the observed immunotoxicity in the Baltic group, nor can a contribution of non-*Ah*-receptor contaminants. More detailed analyses of contaminant residues in the herring are presented elsewhere (206). However, the consistent dose-related pattern of effects on immune function among the three groups (Atlantic < Baltic < TCDD) appears to suggest a similar mechanism of action of immunotoxicity in the Baltic and TCDD groups, which would implicate the TCDD-like contaminants in the complex Baltic oil contaminant mixture in the observed immunosuppression. Immunotoxicity by organochlorines has been shown to be largely mediated by the *Ah*-receptor (176,212). The TCDD group was exposed to the *Ah*-prototype immunotoxicant, 2,3,7,8-TCDD (a small amount of other organochlorines were present in the relatively uncontaminated Atlantic herring oil), while the Baltic group was exposed to a complex mixture of lipophilic PHAH compounds. Since they made up the majority of the total TEQ in the Baltic Sea herring oil (206), PCBs would have been largely responsible for an *Ah*-dependent immunotoxicity in rat pups of the Baltic group.

The lack of changes in plasma thyroxine levels in the Baltic group may reflect a recovery from lower levels by the time of the first necropy day at age 11 days, since prenatally-exposed rats have been shown to be sensitive to the effects of PCBs. Conversely, our results may suggest that immune dysfunction occurs irrespective of thyroid alterations in animals exposed perinatally to a complex mixture of contaminants. Thyroid hormone levels are considered a sensitive indicator of exposure to certain PHAH compounds and some of their metabolites (30,32), and the lower thyroxine levels in the TCDD group at the time of the first two necropsies support this. In our previous study of adult rats (206), a reduction in plasma thyroid hormone levels was observed while, other than differences in RCMV loads in the salivary glands, immune alterations were undetectable.

A reduction IgM, IgG and IgA plaque-forming responses to sheep red blood cells was observed in *Ah*-responsive C57Bl/6 adult mice fed salmon from the relatively contaminated Lake Ontario for four months (49). Owing to relative sensitivity of mice to the immunotoxic actions of TCDD (229), the different contaminant characteristics in the diets and the choice of immune function tests used, it is difficult to compare these results to those obtained in our studies. However, this study also supports the

idea that anthropogenic contaminants in the aquatic food chain are immunotoxic to mammals.

The impaired cellular immune responses in rat pups exposed to contaminants from Baltic Sea herring via placental transfer and via milk was in line with the effects observed in juvenile harbour seals fed Baltic herring (60,62). As in the seals, we observed impaired T-lymphocyte responses in rats exposed to Baltic Sea herring contaminants. Since the basis for this impairment in rat pups appeared to be predominantly related to changes in the thymus or thymocyte precursors, the target of immunotoxic action in the seals fed Baltic herring may also reside in the thymus. The thymus is a sensitive target for 2,3,7,8-TCDD in rats (56) and other laboratory animals, with thymus atrophy and associated alterations in systemic T-cell function being attributed to a reduced maturation of thymocytes (19,100) and a reduced seeding of the thymus by bone marrow progenitors (84).

The concordance between immune effects in perinatally-exposed rat pups in this study and in juvenile seals (60,62,204,205) provides a basis for comparison between the two species. Extrapolation of our observations of an effect on both non-antigen- and RCMV-directed immune responses in rat pups suggests that seals exposed perinatally are more vulnerable to the effects of immunotoxic environmental contaminants than our previous studies may suggest. Whereas the exposure to immunotoxic contaminants in the rat pups ceased at weaning, humans and top predators would be exposed to an ongoing source of TCDD-like contaminants in their diets. As such, seals inhabiting contaminated environments would be exposed perinatally and subsequently via their diet, and may therefore be expected to suffer from prolonged, if not permanent, immunosuppressive effects.

Results here suggest that chronic exposure to low levels of environmental contaminants may present a significant risk to the developing immune system. Since both the Atlantic and Baltic herring used in this study were destined for human consumption, such results are not only relevant to seals and other aquatic wildlife, but also to human health concerns.

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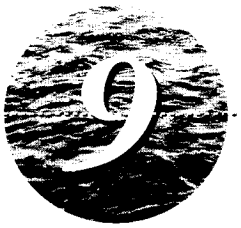

Summarizing Discussion

Contaminant-related immunotoxicity in harbour seals: implications for free-ranging populations

Abstract

Persistent, lipophilic polyhalogenated aromatic hydrocarbons (PHAHs) accumulate readily in the aquatic food chain and are found in high concentrations in seals and other marine mammals. While epidemiological studies have identified a number of bioeffects of these compounds in free-ranging pinniped populations, little is known of the potential immunotoxic impact of these contaminants on seals. The phocid distemper virus-1 (PDV-1) epizootic in 1988 resulted in the deaths of about 20,000 harbour (*Phoca vitulina*) and several hundred grey (*Halichoerus grypus*) seals in Europe. While the event was attributed to the virus isolated from affected animals, a contaminant-induced immunosuppression was hypothesized to have contributed to the severity and extent of the event. We addressed this issue by carrying out a semi-field study in which captive harbour seals were fed herring from either the relatively uncontaminated Atlantic Ocean or the contaminated Baltic Sea for 2½ years. Prior to this, immune function tests were developed and applied to a study of free-ranging harbour seals on Sable Island, Canada, and the newborn pup was demonstrated to be relatively immunocompetent compared to its terrestrial carnivorous counterparts. When these and other tests of immune function were used in the captive feeding study, a contaminant-related impairment of natural killer (NK) cell activity, *in vitro* T-lymphocyte function, antigen-specific *in vitro* lymphocyte proliferative responses and *in vivo* delayed-type hypersensitivity (DTH) responses was observed.

In an attempt to expand on these results, we carried out two similar studies in laboratory rats. In the first of these, adult PVG rats fed freeze-dried Atlantic and Baltic Sea herring for 4½ months did not ehibit immunosuppression, although rat cytomegalovirus (RCMV) titres were higher in the salivary glands of the Baltic group following infection. In the second of these studies, pregnant and subsequently lactating PVG rats were chronically exposed to oil extracted from the two herring batches, and immune function was assessed in their rat pups. Impaired cellular immunity was evidenced by reduced T-lymphocyte function and reduced RCMV-specific antibody responses and RCMV-associated NK cell responses in the Baltic group. However, virus titres in the salivary glands were not affected, possibly reflecting the age-related decline in body burdens of PHAH and associated recovery of immune function.

Together with the pattern of TCDD toxic equivalents of different PHAHs in the herring, these data indicate that present levels of PCBs in the aquatic food chain are immunotoxic, and that free-ranging populations of seals and other marine mammals inhabiting polluted areas of Europe and North America may therefore be at risk to an increased severity of infectious disease. Since the Baltic herring used in our studies was destined for human consumption, questions related to human health risks may arise for certain consumer groups.

Environmental contaminants and virus epizootics in marine mammals

Many of the polyhalogenated aromatic hydrocarbons (PHAHs) readily bioaccumulate in wildlife species occupying high trophic levels as a consequence of their chemical characteristics and relatively non-metabolizable nature. Classes of particular biological concern include the polychlorinated biphenyls (PCBs), the polychlorinated dibenzo-*p*-dioxins (PCDDs), and the polychlorinated dibenzofurans (PCDFs). The widespread contamination of the environment with these compounds, coupled with laboratory evidence implicating them in toxic effects at relatively low levels, highlight the risk that these contaminants may present to free-ranging animals. Such chemicals have long been implicated in a number of population-level effects in raptors, piscivorous birds and seals, including reproductive and developmental toxicities (111,197,248) and skeletal malformations (17,87,171). While PHAHs are also well established immunotoxicants in laboratory animals (273), little attention was paid to this aspect of their potential toxicity to wildlife species until recent virus-induced mass mortalities occurred among marine mammals. However, earlier studies did establish a link between premature pupping in California sea lions (*Zalophus californianus*) and environmental contaminants (63). Recently, decreased lymphocyte proliferative responses to T-dependent mitogens were found to be correlated with serum PCB and dichlorodiphenyl-trichloro-ethane (DDT) levels in five bottlenose dolphins (*Tursiops truncatus*) sampled along the west coast of Florida, USA (147). Epidemiological studies established that striped dolphins (*Stenella coeruleoalba*) that died during the course of the dolphin morbillivirus epizootic in the Meditteranean Sea in 1990-91 had significantly higher concentrations of organochlorine concentrations that those that survived (5). Similarly, harbour seals that died during the 1988 phocid distemper virus-1 (PDV-1) epizootic in Europe had higher levels of organochlorines than those that survived (104). Establishing a causal link between environmental contaminant levels and the outcome of virus infection in these two latter cases proved to be impossible, since decreased blubber stores may have had a concentrating effect on organochlorine levels in the blubber of dead animals. However, the extremely high levels of contaminants found in such studies provide grounds for speculation about their potential immunotoxicity in free-ranging populations of marine mammals.

In 1988, an epizootic of then unknown etiology led to the deaths of approximately 20,000 harbour seals (*Phoca vitulina*) and several hundred grey seals (*Halichoerus grypus*) in Europe (73). The plethora of disease symptoms in affected animals, including fever, cutaneous lesions, gastrointestinal dysfunction, nervous disorders, and respiratory distress (266) cast doubt on the likelihood of a single disease entity. This prompted speculation about the involvement of an immunosuppressive agent or a role for pollution. A previously unidentified morbillivirus was eventually identified as the causal agent (189), being subsequently characterized and named

phocid distemper virus-1, or PDV-1 (162,189,190). While an identical, or very similar, virus was later shown to have been enzootic among North American seal populations (113,210), serological analysis of archival samples established that European harbour and grey seal populations had been seronegative prior to 1988 (188). Different hypotheses attempted to explain the catastrophic nature of the PDV-1 epizootic in Europe in 1988, and population density, migratory movements, algal blooms, climatological factors and environmental pollution were listed as possible co-factors (73,150).

Susceptibility to virus infections involves multiple and often interacting factors, with numerous contributing elements. Such a relationship, for example, has been observed in measles epidemics in humans, in which nutritional, demographic and sociological factors influence the outcome of the event to differing degrees (51). In the case of animals occupying high trophic levels, elevated concentrations of immunotoxic xenobiotics accumulated through the food chain represent an additional factor to be considered.

Immunotoxicological studies in the harbour seal

Subsequent to the PDV-1-induced epizootic in seals in 1988, we addressed the specific role that pollution-induced immunotoxicity may have played in the event. A semi-field experiment was carried out, in which immune function was compared in two groups of harbour seals that were fed herring originating from either a relatively uncontaminated area or a contaminated coastal area.

An assessment of the role of contaminants in this study was facilitated by limiting all additional variables which may have affected immune function. The functioning of the immune system results from the dynamic interaction between its components and external antigens. This is influenced by numerous external and intrinsic factors, including age, sex, season, stress and reproductive status (21,44,129). Seals in our study had been caught as recently-weaned pups in a relatively uncontaminated area, and were allowed an acclimation period of one year prior to the commencement of the feeding study. Herring destined for human consumption was obtained from either the relatively clean Atlantic Ocean or the contaminated Baltic Sea, and was fed to the two groups of seals. The estimated daily intakes of 2,3,7,8-TCDD toxic equivalents (TEQs) by the Baltic group of seals were 10 times higher than those of the Atlantic group of seals (62), and led to a blubber concentration of 286 ± 17 ng TEQ/kg lipid in the Baltic seals compared to 90 ± 6 ng/kg lipid in the Atlantic seals (204). Blood samplings for immunological studies were carried out periodically with minimal capture stress and were consistently completed within four hours. Samples were processed together in a double-blind manner. During the course of the feeding experiment, seals of both groups remained healthy and exhibited normal

growth patterns.

While the immune systems of different mammalian species are structurally and functionally similar, fundamental differences do exist, precluding a detailed characterization of immune function in less studied species, such as the harbour seal. In initial studies, we demonstrated that immune function tests could be successfully adapted for application to studies in seals and yield biologically relevant information (208,209). These studies represented the first major investigation of the developing immune system of the harbour seal. The most striking finding was the demonstration that newborn harbour seals are immunocompetent compared to their carnivorous terrestrial counterparts. It was hypothesized that this reflects an adaptation to its birth into a relatively hostile environment and a short period of maternal care. The importance of colostral intake was illustrated by showing the efficient transfer of PDV-1 neutralizing antibodies to pups, which may be expected to provide temporary protection against this seal pathogen. Subsequent studies provided additional information on the potential of immune function tests in seals (58) which we applied in the feeding experiment.

During the course of the feeding experiment, blood was sampled from both groups of seals every 6-9 weeks and different parameters of immune function were assessed. An early indication of a contaminant-related effect upon immune function was observed within four to six months of the start of the feeding experiment, when the natural cytotoxic activity of peripheral blood mononuclear cells (PBMC) against the YAC-1 tumour target cell proved to be reduced in the Baltic group (62,205). We subsequently showed that the functional characteristics of these cells in harbour seals were similar to those of natural killer (NK) cells described for other mammals, and concluded that the system detected NK cell activity (205). This was based upon the demonstration of the tumour cell-directed cytotoxicity, the interleukin 2 (IL-2) responsiveness of effector cells, and the inhibiting effect exerted by anti-asialo antibodies. All of these findings are consistent with the functional characteristics of NK cells described for other mammals. Mitogen-induced T-lymphocyte proliferative responses began to decline somewhat later (six to ten months) (60,62). This was the first indication of impaired T-lymphocyte responses: only responses induced by the mitogens concanavalin A (Con A), phytohaemagglutinin (PHA) and pokeweed mitogen (PWM) proved to be reduced. Responses to the B-cell mitogen lipopolysaccharide (LPS) were unaffected. While the results of these non-specific tests of immune function indicated that contaminants in the Baltic herring were immunotoxic, further evidence was provided when impaired mixed lymphocyte reactions (MLR) and antigen-specific lymphocyte proliferative responses were observed in the Baltic group (60). Since the MLR reflects the capacity of T-lymphocytes to respond to non-self lymphocytes, it may be considered a good parameter for T-cell function. The impairment of *in vitro* antigen-specific proliferative responses to rabies

virus and tetanus toxoid antigens confirmed that specific immune responses were impaired in the Baltic group of seals. Finally, impaired delayed type hypersensitivity (DTH) and serum antibody responses to the protein ovalbumin *in vivo* provided evidence that the immune system as a whole was less capable of responding normally to a foreign substance in the Baltic group (204). The DTH swelling was characterized by the infiltration of mononuclear cells in the skin and a peak in skin thickness 24 hours after intradermal injection, as in DTH reactions observed in other animals.

The impaired immune responses observed in seals fed the Baltic Sea herring are consistent with the effects observed in studies of laboratory animals exposed to TCDD-like compounds (273). Recent studies have found one-time doses of TCDD to impair NK cell responsiveness during virus infection in SPF rats, but not basal or spontaneous NK cell activity (220,291). Differences in species used, contaminant profiles to which study animals were exposed, or the non-SPF status of the seals in our study, may explain why the spontaneous NK cell activity of the Baltic group of seals was reduced. The impaired T-lymphocyte function in seals of the Baltic group may reflect an immunotoxic effect in the thymus, since this lymphoid organ has been demonstrated to be highly sensitive to the actions of TCDD and related compounds in laboratory animals (56).

Impaired DTH responses have also been observed in other immunotoxicological studies (158,275). While this *in vivo* test of immune function provides an indication of the memory-based ability of the immune system to mount an overall response to a foreign antigen, results of this test are considered to largely reflect T-helper cell function. The correlations between *in vivo* DTH reactions and the *in vitro* T-cell dependent mitogen-induced lymphocyte proliferative responses support the concept of a mediating role for T-lymphocytes in the DTH responses of seals in our study (204), and provide additional evidence of an immunotoxic effect which targeted T-lymphocytes or their precursors.

Our studies provided evidence that contaminants accumulated in the Baltic Sea food chain were immunotoxic to harbour seals. However, it remains difficult to translate the observed immunosuppression in the Baltic group of seals to an increase in susceptibility to infectious disease. Challenging seals in our study with a live virus would clearly not be ethically or legally acceptable. In addition, recreating a situation which would encompass the multiple variables involved in the PDV-1 epizootic in a controlled situation is virtually impossible.

Chronic feeding studies in the rat: mimicking the seal study

In an attempt to expand on our observations in seals, we conducted two feeding studies in laboratory rats. The wider availability of specific reagents for the determination of lymphocyte subpopulations and immunoglobulins represent a major

advantage of studies using rats. In addition, the rat has been widely used in toxicological studies and extra information can be obtained by an evaluation of lymphoid tissue characteristics and by applying host resistance tests (260,263).

In the first of these studies, daily doses of the Baltic herring contaminants that were similar to those used in the seal study did not appreciably alter immune function in adult PVG rats fed freeze-dried herring from the Baltic Sea for 4½ months (206). However, rat cytomegalovirus (RCMV) titres were higher in the salivary glands of rats in the Baltic group, suggesting that contaminants may have affected the outcome of this virus infection without our being able to identify an immunological basis for this observation. Since immune function parameters were clearly impaired in the seals, we concluded that the harbour seal may be more sensitive than the rat to the immunotoxic effects of the contaminants in the Baltic Sea herring. While similar diets were used in both the seal and rat studies, differences in lifespan, the half-life of TCDD-like PHAHs in the two species, and nutritional requirements may also have contributed to the observed differences in species sensitivity to the immunotoxic contaminants in the Baltic Sea herring. The relative insensitivity of the adult rat to the effects of TCDD-like compounds has been shown in other studies (229), and perinatal exposure has been suggested to be a prerequisite to low level TCDD-induced immunotoxicity in rats (274). The multiple and often overlapping effector functions involved in the immunological control of virus infections render it difficult to explain such a finding, although it is conceivable that our functional tests did not comprehensively reflect the defence mechanisms against RCMV infection.

For these reasons, we carried out a second experiment in which pregnant female PVG rats were dosed with the same Atlantic and Baltic Sea herring contaminants mixtures, and immune function was assessed in their pups (207). In order to eliminate any possible dietary influence on immune function other than lipophilic contaminant levels, oil was extracted from the two herring batches and orally administered on a daily basis. A positive control group was exposed to Atlantic herring oil spiked with TCDD. Exposure began on day 6 of gestation and continued until the pups were weaned. Rat pups exposed perinatally to the Baltic herring contaminant mixture had impaired cellular immune responses, with this being most pronounced at an early age. Effects on non-specific immune function parameters were characterized by impaired mitogen-induced T-lymphocyte proliferative responses and thymus-related effects, suggesting that developing thymocytes or their precursors were targetted. RCMV-directed immune responses including virus-associated NK responsiveness and specific antibody responses were impaired in both the Baltic and TCDD groups, while RCMV-specific T-lymphocyte proliferative responses were affected in the TCDD group. Functional immune responses in the youngest rat pups of the Baltic group fell consistently between the negative control group (Atlantic) and the positive control group (TCDD), but these differences became less apparent with time. Following

infection, RCMV titres were similar in the salivary glands of all groups of experimentally-infected rats at the time of necropsy, likely reflecting the observed recovery of immune function with time. The 24-day half-life of TCDD in rats (203) would lead to rapidly diminishing contaminant burdens in the growing pups, essentially resulting in a removal of the source of immunotoxicity in the study animals. The reversibility of TCDD-induced thymus atrophy has been observed previously (262).

Our observations of elevated RCMV titres in the salivary glands of adult rats but not rat pups suggest that the outcome of this virus infection may be affected by the contaminants in the Baltic herring, but also underline the complexity of the immune response. Results of other studies have demonstrated that a chemical-induced decrease in host resistance in laboratory animals is pathogen-dependent (260). However, predictive relationships between immunosuppression and susceptibility to infection are difficult to establish (159). A selective impairment of immune function parameters as a result of genetic deficiencies (18,221), medication use (64) or exposure to PHAHs (279) can affect the outcome of virus infections. Immune alterations or host resistance effects were detected in rats exposed either in their diet as adults or as pups exposed perinatally, suggesting that the contaminants in the Baltic Sea herring were immunotoxic in these laboratory animals.

The more pronounced immunosuppression observed in the harbour seal study as compared to the rat studies may be related to differences in the experimental design, although it would appear that seals are more sensitive than rats to the immunotoxic action of contaminants in Baltic Sea herring. These observations are consistent with the demonstrated relative insensitivity of rats to the immunotoxic effects of TCDD (229,275). The young rat pups exposed perinatally exhibited immune alterations which were similar to those in the seals, providing a basis for comparison of these two species. The reductions in thymus weights, thymus and spleen T-cell numbers and T-cell function observed in perinatally-exposed rats, suggest that the impaired T-lymphocyte responses in the harbour seals of the Baltic group is related to an effect on thymus function or its thymocyte precursor cells. The observation that rat pups exposed perinatally are more sensitive to the immunotoxic actions of the Baltic Sea herring contaminants than adult rats, provides a basis to speculate that harbour seals exposed perinatally are at more risk than our studies of captive juveniles may suggest.

Free-ranging seal populations at risk?

PHAH-induced immunotoxicity has been shown to be largely mediated by the aryl hydrocarbon (*Ah*)-receptor (141,222). While it is not possible to rule out a contribution of non-*Ah*-mediated compounds to the observed immunotoxicity in the Baltic group of seals, the TCDD-like PHAHs represent highly immunotoxic contaminants found at high concentrations in the Baltic Sea herring used in this study.

The dose-related pattern of impaired immune functions observed among groups of perinatally-exposed rat pups lends support to the concept of an *Ah*-receptor mediated basis for Baltic herring-induced immunotoxicity.

While 2,3,7,8-TCDD remains the most potent *Ah*-related immunotoxicant among the 410 possible PCB, PCDD and PCDF congeners, chemical residue analysis of Baltic Sea herring suggested that the dioxins presented a limited immunotoxic risk in the diet of the harbour seals (Figure 1). In fact, three PCB congeners (IUPAC numbers 126, 118 and 156), together with one PCDF congener, 2,3,4,7,8-PeCDF, accounted for 80% of the total TEQ profile in the Baltic Sea herring, while the contribution of all PCDDs was less than 10%. The PHAH profile in the blubber of harbour seals fed this fish for two years suggested that PCBs accounted for the great majority of the toxicity, with PCDDs and PCDFs contributing only slightly to the total TEQ. Such data confirm the results of others, who have found the TCDD-like PCBs (135,243) and PCDFs (183) to represent a greater toxic potential than PCDDs in marine mammals. The diminishing contribution of PCDDs to the total TEQ profile from the herring to the seals in our studies suggests that harbour seals may be able to preferentially metabolize the planar PCDDs. Similar observations of the limited potential for bioaccumulation of toxic PCDDs in seals (16,242) further illustrate the threat that PCBs pose to seals in the marine environment.

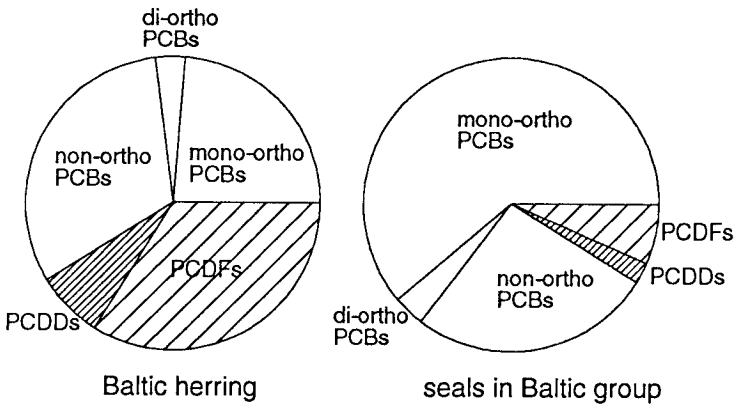

Figure 1: The relative contributions of PCBs, PCDDs and PCDFs to the total TCDD toxic equivalents (TEQs) in Baltic Sea herring lipid and blubber of harbour seals fed this herring for two years.

Taken together, our results demonstrate that current levels of persistent lipophilic contaminants present an immunotoxic risk to seals inhabiting many coastal

areas of North America and Europe. Since the harbour seals fed Baltic herring in our study accumulated levels of PHAHs which are lower than those currently found in many marine mammal populations, we conclude that contaminant-induced immunotoxicity represents a real threat to aquatic wildlife. In retrospect, contaminants may well have affected the severity and extent of the 1988 PDV epizootic in Europe, as well as other recent virus epizootics. While the immunotoxicity observed in our Baltic group of seals probably resulted from a combined action of different chemicals in the herring, the mono-ortho PCBs are of particular concern.

The results obtained in these studies may also be of relevance for human health, since the Baltic Sea herring used was destined for human consumption. While direct interspecies comparisons are complicated by differences in, among others, the capacity to metabolize or accumulate immunotoxic PHAHs, length of nursing periods, feeding habits, and general health factors, they can serve to identify potential problems associated with the intake of dietary contaminants. Although some information is available on the intake and accumulation of PHAHs for humans, little is known about the effects of these. Certain human consumer groups, including fisherman in industrialized nations (69,122,237) and native Inuit in northern Canada (65,67), have been found to have high daily intakes of TCDD-like PHAHs. In the latter case, an association was observed, and a causative link postulated, between PCB intake and an increased frequency of infectious disease in breast-fed babies (66). This suggests that food chain-accumulated PHAHs may be immunotoxic to certain human consumer groups even in remote areas previously considered to be pristine. The observed immunotoxicity in laboratory rats and seals in our study occurred at daily intake levels that are in the same order of magnitude as those estimated for breast-fed Inuit infants (66,68), whose mothers consume large quantities of fish and marine mammal products (Table 1).

Table 1: Estimated daily intakes of dietary TCDD toxic equivalents accumulated in the aquatic food chain by animals in our studies and in breast-fed Canadian Inuit infants.

study	exposure route	daily intake TEQ/kg	ref.
harbour seals	Atlantic herring	0.3 - 0.6	(Chapters 3,7)
harbour seals	Baltic herring	1.2 - 5.6	(Chapters 3,7)
adult PVG rats	Baltic herring	1.6	(Chapter 7)
young PVG rats	Baltic herring oil	0.9	(Chapter 8)
Inuit infants	human breast milk	0.7	(69,70)

Although PCB levels in the aquatic food chain declined following regulatory controls, they have largely stabilized since the 1980s (122,155,182). The continued cycling of persistent PHAH compounds suggests that the contamination of the aquatic food chain will remain of ecological concern into the 21st century. Since perinatally-exposed animals exhibit more pronounced PHAH-induced immunosuppression and the lifespan of seals allows for long-lasting accumulation, free-ranging seals are likely to be more vulnerable to the immunotoxic effects of persistent PHAHs than our study seals. Such immunotoxicity may therefore predispose free-ranging seals in certain areas to an increased severity of infectious disease outbreaks in the decades to come.

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Nederlandse samenvatting

Toen in 1988 duizenden gewone en grijze zeehonden stierven langs de kusten van noordwest Europa werden verschillende mogelijke oorzaken overwogen, waaronder veranderingen in het klimaat, algenbloei, te hoge populatiedichtheid, een infectieziekte en milieuvervuiling. Met de ontdekking van het zeehondenziektevirus, ofwel phocine distemper virus (PDV), als primaire oorzaak van de sterfte, was een mogelijke rol van chronische blootstelling aan vervuilende stoffen als additionele factor nog steeds niet uit te sluiten. Zeehonden bevinden zich aan de top van de aquatische voedselketen, en worden via hun voedsel veelal blootgesteld aan hoge concentraties van anthropogene vetoplosbare organische stoffen, die zich ophopen in hun speklaag. Enkele bijzonder gevreesde groepen van stoffen zijn de gepolychloreerde biphenylverbindingen (PCB's), -dibenzofuranen (PCDF's) en -dibenzo-*p*-dioxines (PCDD's). Uit dierproeven bleek dat relatief lage concentraties van deze verbindingen toxisch kunnen zijn voor het immuunsysteem, wat in een aantal gevallen een verminderde weerstand tegen infecties met virussen, bacteriën en parasieten tot gevolg had. Zouden de thans in het milieu voorkomende complexe mengsels van zulke immunotoxische chemicaliën het vermogen van de zeehond om een aanval van een tot nog toe onbekend virus af te slaan hebben aangetast?

Al zal het waarschijnlijk nooit mogelijk zijn om alle factoren te identificeren die een rol hebben gespeeld in de PDV uitbraak onder zeehonden in 1988, een nauwgezette analyse van een aantal van deze factoren kan misschien toch meer duidelijkheid verschaffen. In dit proefschrift wordt een onderzoek beschreven naar de schadelijke gevolgen van vervuiling van de aquatische voedselketen op het afweersysteem van de zeehond. De gegevens gepresenteerd in hoofdstuk 1 geven aan dat PDV of een nauw hieraan verwant virus al vóór 1988 bij Canadese gewone en grijze zeehonden voorkwam zonder bij deze dieren abnormale sterfte te veroorzaken. Deze bevinding leidde tot de speculatie dat de lagere concentraties aan vervuilende stoffen in de Canadese wateren in vergelijking met die in de Europese wateren géén negatieve invloed hadden op het functioneren van het immuunsysteem van de zeehond. Dit zou mogelijk kunnen verklaren waarom het ziektebeeld bij de Europese zeehonden zoveel ernstiger was verlopen. Hoe aantrekkelijk deze verklaring ook moge lijken, een minstens zo plausibele verklaring kan worden gevonden in het feit dat het virus mogelijk al langere tijd enzoötisch was in de Canadese wateren, terwijl het nieuw was voor de Europese zeehonden. De Canadese zeehondenpopulatie was in dat geval meer resistent tegen de infectie als gevolg van een reeds bestaande immuniteit.

Om na te gaan of vervuiling inderdaad een negatief effect heeft gehad op het functioneren van het immuunsysteem van de Europese zeehonden, en zodoende de resistentie tegen een nieuw virus nadelig heeft beïnvloed, was het nodig om een functionele studie van het immuunsysteem van zeehonden uit te voeren. Omdat aan het

begin van deze studies weinig bekend was over het immuunsysteem van zeehonden, moesten nieuwe methoden ontwikkeld worden, of voor andere diersoorten bestaande methoden worden aangepast voor gebruik bij deze species. In hoofdstuk 2 wordt een studie beschreven naar zowel passief als actief verkregen immuniteit in de pasgeboren zeehond. Deze studie werd uitgevoerd bij een in het wild levende populatie van gezonde gewone zeehonden in het relatief onvervuilde leefgebied van Sable Island, voor de oostkust van Canada. Niet alleen werden de methoden met succes toegepast bij een zoogdier dat nog niet eerder in dit opzicht werd bestudeerd, maar de resultaten bleken ook biologisch relevant te zijn. De pasgeboren zeehond bleek een beter ontwikkeld immuunsysteem te hebben dan pasgeboren honden en katten, wat mogelijk verband houdt met de korte periode van verzorging door de moeder en de onvriendelijke omgeving waarin het dier wordt geboren. Het dier is echter nog in hoge mate afhankelijk van de tijdelijke bescherming door specifieke antistoffen in colostrum en moedermelk, wat mede blijkt uit de efficiënte overdracht van PDV specifieke antistoffen na de geboorte.

Naast andere methodologische ontwikkelingen op het gebied van zeehonden immunologie hebben deze studies mede de basis gevormd voor een meer uitgebreid onderzoek naar het functioneren van het immuunsysteem van de zeehond en de invloed van milieuvervuiling hierop. Pasgespeende zeehondenpups werden gevangen op de relatief schone kust van noordoost Schotland en naar de zeehondencrèche in Pieterburen gebracht. Nadat de dieren gedurende een jaar met relatief schone haring afkomstig van de Atlantische Oceaan waren gevoed, werden ze verdeeld over twee groepen van elk elf dieren. De ene groep werd vervolgens met hetzelfde dieet gevoed, terwijl de andere groep haring kreeg afkomstig van de sterk vervuilde Oostzee. In de hieropvolgende 2½ jaar werd het functioneren van het immuunsysteem van beide groepen zeehonden nauwlettend bestudeerd in het kader van het promotieonderzoek van zowel Rik de Swart (promotie Erasmus Universiteit 1995) als van de auteur van dit proefschrift. Een uitgebreid arsenaal aan immuunfunctiestudies werd uitgevoerd, terwijl tevens aandacht werd besteed aan verschillen in klinische chemie, haematologie en concentraties van contaminanten in bloed en spek.

Naarmate het experiment vorderde werd duidelijk dat milieuvervuilende stoffen een negatieve invloed hadden op het functioneren van het immuunsysteem van de zeehonden. Een eerste aanwijzing hiervoor was een lagere "natural killer" (NK) cel activiteit van witte bloedcellen afkomstig van zeehonden in de Oostzeegroep. Omdat NK cellen niet eerder bij zeehonden waren beschreven, werd dit celtype eerst gekarakteriseerd aan de hand van criteria die bij andere diersoorten voor deze cellen werden gehanteerd. In een serie experimenten werd aangetoond dat de cytotoxische activiteit van deze cellen sterk leek op die van soortgelijke cellen van andere zoogdieren. De NK cellen worden als belangrijk onderdeel van de eerstelijns verdediging tegen infecties en tumorcellen beschouwd, omdat ze niet afhankelijk zijn van een eerder contact of immunologisch geheugen en snel

worden geactiveerd in de eerste fase van een infectie.

Vervolgens werd een verlaagde *in vitro* mitogeen geïnduceerde proliferatieve T lymfocyten respons bij de Oostzeegroep waargenomen. Deze waarneming werd versterkt door bij deze dieren tevens verminderde *in vitro* en *in vivo* antigeen specifieke responsen aan te tonen: *in vitro* "mixed lymphocyte responses" en proliferatieve lymfocyten responsen tegen rabiesvirus antigeen en tetanus toxoid na immunizatie bleken lager te zijn in de Oostzeegroep. Aanwijzingen voor deze verminderde cellulaire immuunrespons werden ook *in vivo* gevonden door aan te tonen dat dieren uit de Oostzeegroep minder in staat waren om een vertraagd type overgevoeligheidsreactie en een antistof respons tegen ovalbumine te ontwikkelen. Dit bevestigde dat deze dieren inderdaad minder in staat waren om een specifieke immuunrespons te ontwikkelen als gevolg van chronische blootstelling aan milieuvervuilende stoffen via het visdieët.

Alles tezamen maken de resultaten van deze studie waarschijnlijk dat zeehonden die in de vervuilde kustgebieden van Europa en Noord-Amerika leven waarschijnlijk een verminderde immuunfunctie hebben. Daarom lijkt de speculatie gerechtvaardigd dat milieuvervuilende stoffen hebben bijgedragen aan de ernst van de uitbraak van zeehondenziekte in 1988. Dit kan hebben geleid tot een snellere verspreiding van het virus door de zeehondenpopulatie, en het kan tevens een hoger sterftecijfer tot gevolg hebben gehad. Ethische, wettelijke en praktische overwegingen hebben de mogelijkheden beperkt om verdere gedetailleerde immunologische studies in zeehonden in gevangenschap uit te voeren. Daarom werd een tweetal parallelle studies opgezet, waarin laboratoriumratten werden blootgesteld aan dezelfde mengsels van vervuilende stoffen als waaraan de zeehonden waren blootgesteld. Een uitgebreide studie naar het functioneren van het immuunsysteem van deze ratten werd uitgevoerd.

In de eerste studie werden volwassen ratten gedurende 4½ maand gevoerd met een mengsel van gevriesdroogde Oostzee of Atlantische haring en voor de rat noodzakelijke voedselsupplementen. Ze werden vervolgens geïnfecteerd met het rattecytomegalovirus (RCMV) en 12 dagen later geëuthanaseerd. Aantallen cellen en een totaalbeeld van specifieke en niet specifieke immuunfunctie parameters werden bepaald met milt en thymuscellen afkomstig van deze dieren. Tevens werden de gewichten van deze organen bepaald. Ondanks dat de ratten en de zeehonden ongeveer dezelfde dagelijkse opname van milieuvervuilende stoffen op basis van lichaamsgewicht hadden, werden bij de ratten geen duidelijke immuunfunctiestoornissen waargenomen. Hogere RCMV-specifieke antistof concentraties gevonden in de speekselklieren van ratten uit de Oostzeegroep suggereerden echter dat deze stoffen mogelijk wel een invloed op het beloop van de infectie hadden gehad.

In de tweede rattenstudie werden zwangere ratten gedurende de dracht en de lactatie

dagelijks blootgesteld aan olie, die was geëxtraheerd uit de twee voorraden haring waarmee de zeehonden waren gevoerd. Een derde groep, toegevoegd als positieve controle, werd blootgesteld aan visolie van de Atlantische haring waaraan 2,3,7,8-TCDD was toegevoegd. Immuunfunctie parameters werden vervolgens gemeten bij vrouwelijke rattebabies van 11, 25, 46 en 59 dagen oud. De metingen op de laatste twee tijdstippen werden gedaan bij dieren die tevens met RCMV waren geïnfecteerd. Een dosis-afhankelijk patroon van immunosuppressie werd waargenomen in de babies van alle drie groepen: de laagste responsen werden gevonden in de rattebabies uit de 2,3,7,8-TCDD groep, terwijl de hoogste responsen werden gevonden in de dieren uit de Atlantische groep. De jongste babyratten uit de Oostzeegroep vertoonden afgenomen T lymfocytenfuncties en andere tekenen van immunotoxiciteit. Deze verschijnselen verdwenen grotendeels in de loop van de tijd. Er bleken geen verschillen te zijn in RCMV-specifieke antistof concentraties tussen de drie groepen aan het eind van het experiment, hetgeen mogelijk ook het gevolg is van het herstel van immuunfuncties in zowel de Oostzee als de 2,3,7,8-TCDD groep. Deze resultaten geven aan dat perinatale blootstelling mogelijk een groter risico vormt dan blootstelling tijdens latere leeftijd, alhoewel dit gedeeltelijk veroorzaakt kan zijn door de farmacokinetiek van overdracht van organische chloorverbindingen van moederrat naar haar nageslacht.

De resultaten van de twee rattestudies gaven aan dat de milieuvervuilende stoffen in de Oostzeeharing inderdaad immunotoxisch waren, en vooral bepaalde aspecten van de celgemedieerde immuniteit aantastte. Zeehonden lijken gevoeliger te zijn voor effecten van de immunotoxische stoffen in de Oostzeeharing dan ratten. De duidelijke effecten die in de perinataal blootgestelde ratten werden gevonden suggereren dat zeehonden die geboren worden in vervuilde gebieden een duidelijkere immuunsuppressie kunnen vertonen dan de zeehonden in het voedingsexperiment. Bovendien geven de rattestudies aan dat de waargenomen suppressie van de cellulaire immuniteit in de zeehonden mogelijk zijn oorzaak heeft in een verminderde functie van de thymus, dan wel het resultaat is van verslechterde T helper of cytotoxische T cel responsen. Omdat de olie geëxtraheerd uit de Oostzeeharing effecten te zien gaf in de babyratten die in vele opzichten overeen kwamen met de effecten van de hele vis in de zeehondenstudie, is het waarschijnlijk dat vetoplosbare contaminanten in de Oostzeeharing de oorzaak van de immunotoxische effecten in de zeehonden zijn geweest.

De veranderingen immuunfunctie van zowel zeehonden als ratten blootgesteld aan de Oostzeeharing tonen aan dat de gehaltes aan de milieuvervuilende stoffen in de voedselketen in de Oostzee op dit moment zo hoog zijn dat ze een verlaagde weerstand tegen infecties met virussen en bacteriën bij de zeehond tot gevolg kunnen hebben. Wanneer hierbij wordt bedacht dat de vis gebruikt in deze experimenten oorspronkelijk was bestemd voor menselijke consumptie, rijst ontegenzeggelijk de vraag naar de mogelijke immunotoxische effecten van visconsumptie voor de mens.

Abbreviations

Ah-receptor	Aryl hydrocarbon receptor
ANOVA	analysis of variance
BHT	butyl-hydroxytoluene
BSA	bovine serum albumin
CD	cluster of differentiation
CDV	canine distemper virus
CM	culture medium
Con A	concanavalin A
cpm	counts per minute
CTL	cytotoxic T-lymphocyte
DDA	dimethyldioctadecylammonium bromide
DDT	dichlorodiphenyl-trichloro-ethane
DMV	dolphin morbillivirus
DRE	dioxin regulatory element
DTH	delayed-type hypersensitivity
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EROD	ethoxyresorufin-0-deethylase
FBS	fetal bovine serum
FCA	Freund's Complete Adjuvant
FCS	fetal calf serum
HCB	hexachlorobenzene
HCH	hexachlorohexane
H&E	haematoxylin and eosin
HPLC	high pressure liquid chromatography
HRPO	horseradish peroxidase
IFN	interferon
Ig	immunoglobulin
IL	interleukin
IU	International Units
IUPAC	International Union of Pure and Applied Chemistry
KLH	keyhole limpet haemocyanin
LCMV	lymphocytic choriomeningitis
LPS	lipopolysaccharide
MCMV	mouse cytomegalovirus
MHC	major histocompatibility complex
MLR	mixed lymphocyte reaction
MV	measles virus
NK cell	natural killer cell

Ova	ovalbumin
PAH	polycyclic aromatic hydrocarbons
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCB	polychlorinated biphenyl
PCDD	polychlorinated dibenzo-*p*-dioxin
PCDF	polychlorinated dibenzofuran
PDV	phocid distemper virus
PFU	plaque forming unit
PHA	phytohaemagglutinin
PHAH	polyhalogenated aromatic hydrocarbon
PMV	porpoise morbillivirus
PPRV	peste des petits ruminants
PWM	pokeweed mitogen
RCMV	rat cytomegalovirus
REC	rat embryo cells
RFFIT	rapid focus fluorescence inhibition test
rhINF	recombinant human gamma interferon
rhIL-2	recombinant human interleukin-2
RPV	rinderpest virus
RV	rabies virus
SD	standard deviation
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SE	standard error
SPF	specific pathogen free
TCDD	2,3,7,8-tetrachlorodibenzo-*p*-dioxin
TEF	toxic equivalent factor
TEQ	toxic equivalent
TMB	tetramethylbenzadine
TBTO	bis(tri-n-butyltin)oxide
TT	tetanus toxoid
VN	virus neutralization
WBC	white blood cell

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Curriculum vitae

Peter Stuart Ross was born in Sherbrooke, Québec, Canada, on April 17th 1963. Following his graduation from Fisher Park High School in Ottawa, he pursued studies in Biology at Trent University in Peterborough, Ontario. He obtained his Bachelor of Science (Honours) in 1985. He subsequently worked for two years as the National Coordinator of Canadian Student Pugwash, a non-profit organization concerned with the social and ethical implications of science and technology. During this period, he organized an international conference entitled "Resolving Global Problems into the 21st Century: How can Science Help?" in Ottawa. He returned to scientific research in 1988 with the Canadian Wildlife Service (D.V. Weseloh) and studied the effects of toxic chemical pollution on double-crested cormorants (*Phalacrocorax phalacrocorax*) on the five Great Lakes. Between 1988 and 1990, he pursued his Master of Science at Dalhousie University in Halifax, Nova Scotia, publishing a thesis entitled "Immunocompetence of free-ranging harbour seal (*Phoca vitulina*) mothers and their pups over the course of lactation". He continued directly into Ph.D. studies jointly between Dalhousie University and the Bedford Institute of Oceanography (W.D. Bowen) in Canada, and the National Institute of Public Health and Environmental Protection and the Seal Rehabiliation and Research Centre in The Netherlands (A.D.M.E. Osterhaus). Both Rik De Swart and Peter Ross worked together as Research Biologists for the Seal Rehabilitation and Research Centre on a long-term immunotoxicological study with captive harbour seals, culminating in the completion of their doctorate degrees in 1995 (Erasmus University Rotterdam and the University of Utrecht, respectively). Peter Ross will be returning to Canada, where he will carry out a post-doctoral project on seal immunotoxicology at the Institute of Ocean Sciences (R.F. Addison) on Vancouver Island, British Columbia.

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