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Impaired cellular immune response in harbour seals (*Phoca vitulina*) feeding on environmentally contaminated herring

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SUMMARY

In a 2·5-year immunotoxicological study, two groups of captive harbour seals (*Phoca vitulina*) were fed herring from the heavily polluted Baltic Sea or from the relatively uncontaminated Atlantic Ocean. Blood samples were collected at regular intervals, and functional immunological parameters were monitored. T cell mitogen and mixed lymphocyte-induced proliferative responses of peripheral blood mononuclear cells (PBMC) obtained from seals fed Baltic herring were significantly reduced over the course of the experiment. Upon immunization with rabies virus antigen (RV) and tetanus toxoid (TT), specific proliferative responses of PBMC from the seals fed Baltic herring were also significantly reduced. Impairment of T cell-mediated immune responses became especially apparent during the second year on the respective diets, and correlated significantly to 2,3,7,8-tetrachloro-dibenzo-*p*-dioxin toxic equivalent levels in blubber biopsies taken from the seals after 2 years on the respective diets. Humoral immune responses, including lipopolysaccharide (LPS)-induced lymphoproliferative responses, *in vitro* immunoglobulin production by PBMC, as well as RV-, TT- and poliovirus-specific serum antibody responses following immunization, remained largely unaffected. We conclude that suppression of the cellular immune response in the seals fed Baltic herring was induced by the chronic exposure to immunotoxic environmental contaminants accumulated through the food chain. Since cellular immune responses are known to be of crucial importance in the clearance of morbillivirus infections, these results suggest that environmental pollution-related immunosuppression may have contributed to the severity and extent of recent morbillivirus-related mass mortalities among marine mammals.

Keywords immunotoxicology immunosuppression marine mammals *Phoca vitulina* phocine distemper virus

INTRODUCTION

Studies in laboratory animals have shown that the mammalian immune system can be adversely affected by a variety of chemical agents [1–3]. 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 [4–6], 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 (*P. sibirica*) in Siberia (Lake Baikal), and striped dolphins (*Stenella coeruleoalba*) in the Mediterranean Sea [7]. Recently, retrospective evidence has been presented of an involvement of a morbillivirus in an epizootic among bottlenose dolphins (*Tursiops truncatus*) along the

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Atlantic coast of the USA in 1987 [8]. 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 [9,10]. Toxicity of these chemicals is therefore largely dependent on their stereochemical resemblance to TCDD, the chemical with the highest affinity for this receptor. Based on this resemblance, the toxicity of a complex mixture of different dioxin-, dibenzofuran- and PCB-congeners can be expressed in TCDD toxic equivalents (TEQ) [11,12]. In all mammalian species studied thus far, TCDD-like chemicals induce thymus atrophy and impairment of T cell-mediated immune responses, especially following perinatal exposure, although species sensitivities differ markedly [1,13].

In order to assess the impact of ambient levels of environmental contaminants on immune function in seals, we conducted a prospective feeding study under semi-field conditions. During a period of 2·5 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 on Baltic herring were found in blubber biopsies taken from the seals after 2 years on their respective diets. Blood samples were collected at regular intervals, and functional immunological assays were carried out. Previously we reported impaired *in vitro* natural killer (NK) cell and lymphocyte functions and *in vivo* DTH responses in the seals fed Baltic herring [14,15]. Haematological studies showed increased leucocyte (neutrophils) and erythrocyte counts in these animals [14]. Here we report effects of the different diets on cellular and humoral immune responses of these animals.

MATERIALS AND 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 1 year. The seals were matched for weight and sex 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 2·5 years [14,15]. 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 ≈ 15 months old. At the end of the experiment all 22 seals were fed Atlantic herring for a period of 6 months, after which 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 tetanus toxoid (TT) adsorbed to aluminium phosphate at weeks 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 [14,15]. Estimated daily intakes of potentially immunotoxic xenobiotics analysed in the fish diets (PCBs, PCDDs, PCDFs, HCB, dieldrin, β-HCH and DDT) were three to more than 10 times higher in the seals feeding on Baltic herring. Estimated daily intakes of dioxin-like organochlorines were 29 ng and 288 ng TEQ per day for the seals feeding on Atlantic or Baltic herring, respectively [14].

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, and kept refrigerated during transport to the laboratory. All serological assays were carried out using heat-inactivated plasma (30 min, 56°C).

Mitogen- and antigen-induced proliferation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation as described previously [14,16]. All samples were coded before processing, and assays were carried out double blind. Isolated PBMC were stored overnight in RPMI 1640 medium containing 20% fetal bovine serum (FBS). The following day PBMC were counted in duplicate using a haemocytometer, and cell concentrations were adjusted to 2×10^6 PBMC/ml in RPMI 1640 medium supplemented with penicillin (100 U/ml), streptomycin (100 μg/ml), L-glutamine (2 mM), 2-mercaptoethanol (2-ME; 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 [16]. PBMC were stimulated with the mitogens concanavalin A (Con A; 5 μg/ml), pokeweed mitogen (PWM; 2·5 μg/ml), phytohaemagglutinin (PHA; 20 μg/ml) or lipopolysaccharide (LPS; 100 μg/ml), or with rabies virus antigen (RV; 15 μg/ml) or TT (20 LF/ml). PBMC were cultured in triplicate for 4 days (Con A, PWM, PHA), 5 days (LPS, RV) or 6 days (TT) before harvesting, and were pulsed with 0·5 μCi tritium-labelled thymidine (^{3}H-TdR) per well during the last 16 h 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 responses

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

In vitro *immunoglobulin production*

In vitro immunoglobulin production by PBMC was measured as previously described [16]. Briefly, PBMC were cultured in CM in 24-well plates (2×10^6 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 ELISAs as previously described [16]. Briefly, plates were coated with the respective antigens and blocked with bovine serum albumin (BSA). After incubation with serial dilutions of seal plasma (in duplicate per sample), bound antibodies were detected using horseradish peroxidase (HRP)-labelled protein A. Previously, we have shown that protein A predominantly binds phocine IgG [17]. 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 [18].

Statistical analysis

Longitudinal data were analysed using a repeated measures ANOVA model (BMDP module 5 V), 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 [15], and were natural log-transformed before correlation analyses. Proliferative responses measured after the first seven and 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 Fig. 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 ($P < 0{\cdot}01$). 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

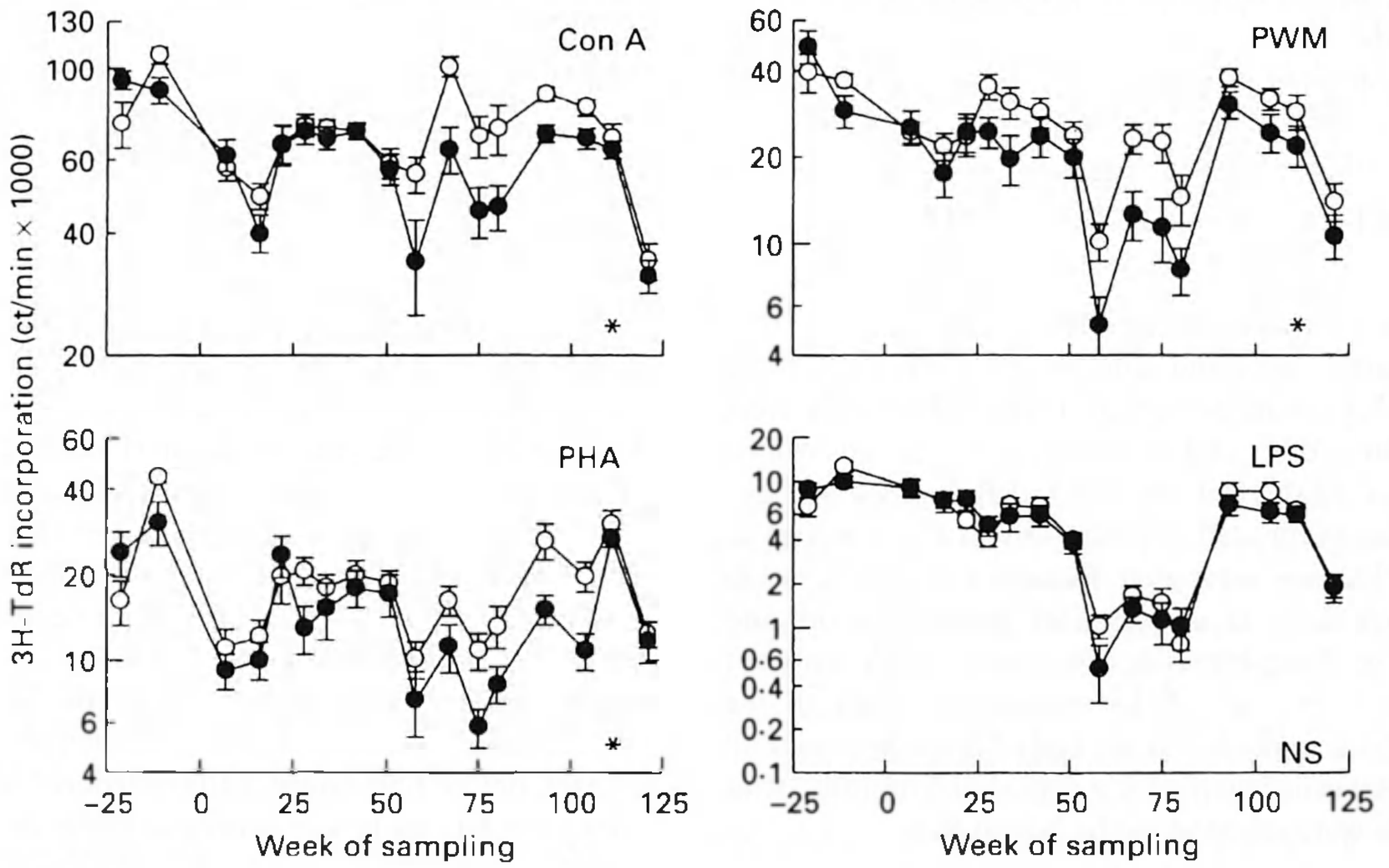

Fig. 1. Mitogen-induced proliferative responses of peripheral blood mononuclear cells (PBMC) obtained from harbour seals fed Atlantic herring (○) or Baltic herring (●), measured as ^{3}H-thymidine (^{3}H-TdR) incorporation after subtraction of controls. Data shown are mean ct/min ± s.e.m. of 11 animals per group (seven females and four males each). *Significant difference between the two groups over time (repeated measures ANOVA, $P < 0{\cdot}01$). NS, Not significant. Con A, Concanavalin A; PWM, pokeweed mitogen; PHA, phytohaemagglutinin; LPS, lipopolysaccharide.

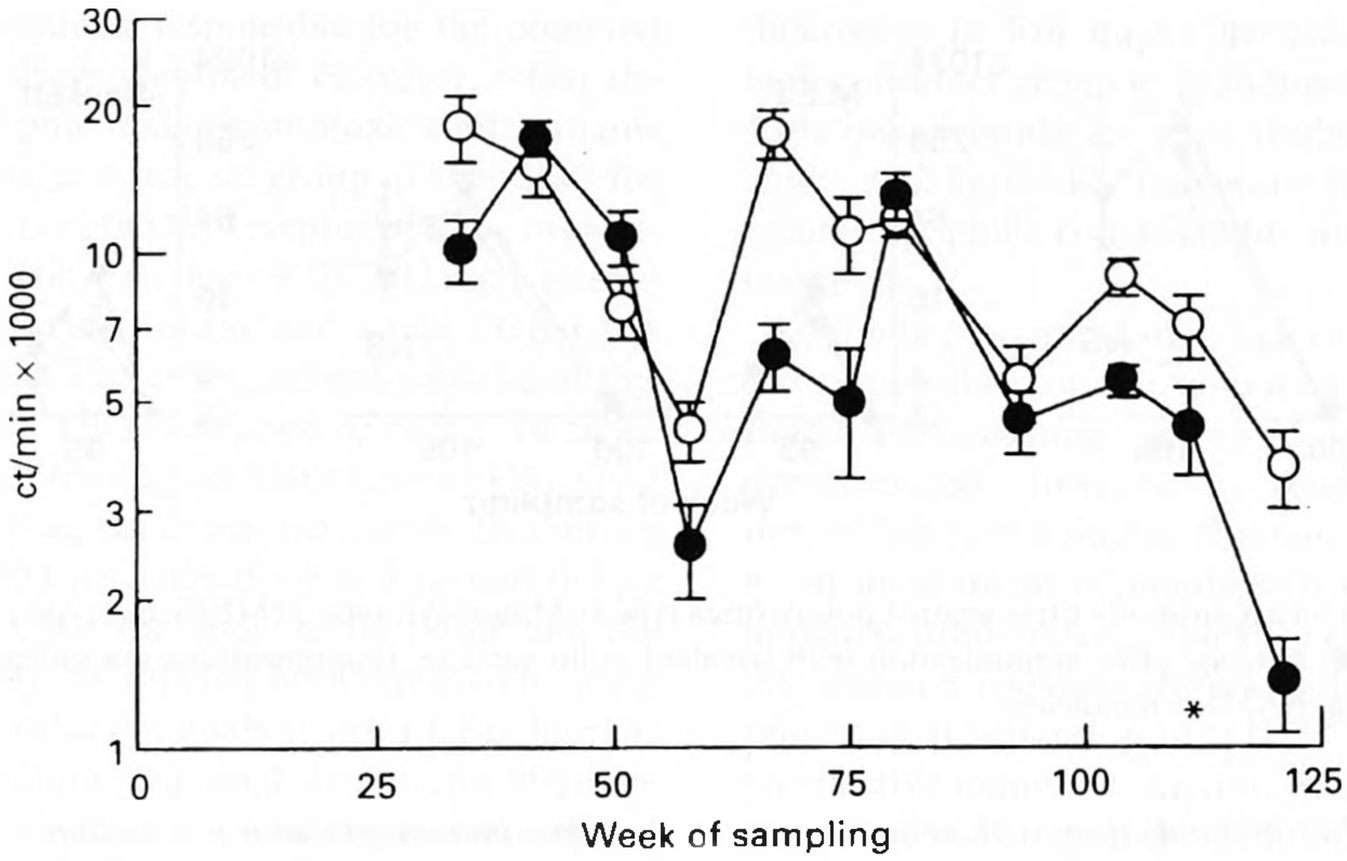

Fig. 2. Mixed lymphocyte responses (MLR) of peripheral blood mononuclear cells (PBMC) obtained from harbour seals fed Atlantic herring (○) or Baltic herring (●), measured as ^{3}H-thymidine (^{3}H-TdR) incorporation after subtraction of controls (mean ct/min ± s.e.m.). *Significant difference between the two groups over time (repeated measures ANOVA, $P < 0{\cdot}01$). NS, Not significant.

after 2 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 with these blubber contaminant levels (Con A, $r = -0{\cdot}72$, $P < 0{\cdot}01$; PWM, $r = -0{\cdot}44$, $P < 0{\cdot}05$; PHA, $r = -0{\cdot}56$, $P < 0{\cdot}01$). 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 was carried out using a lymphosarcoma cell line from harbour seal origin 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 Fig. 2, MLR responses of seals feeding on Baltic herring were significantly lower ($P < 0{\cdot}01$). Mean MLR responses from the last seven blood samplings correlated inversely with total TEQ levels in blubber biopsies ($r = -0{\cdot}55$, $P < 0{\cdot}01$).

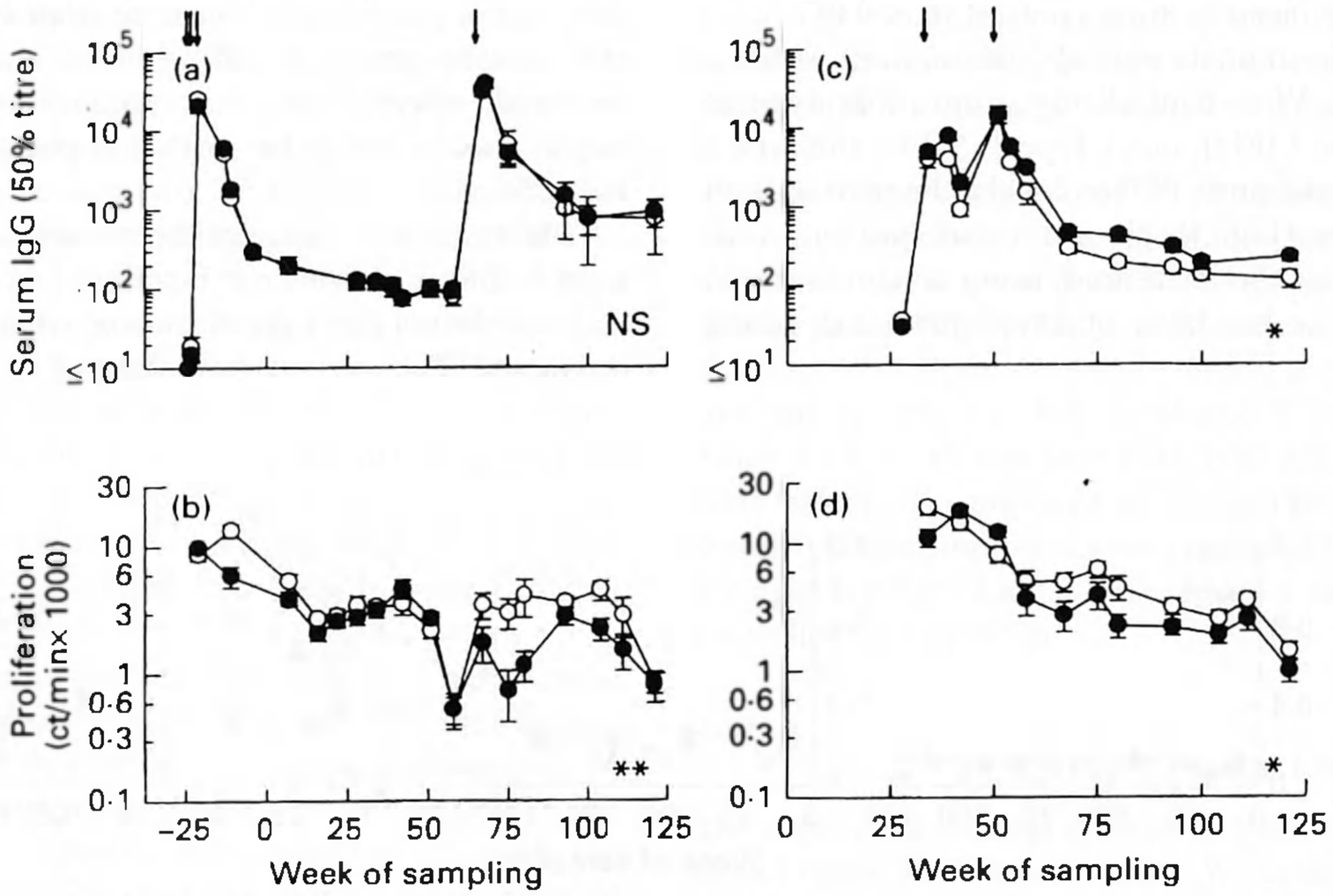

Fig. 3. Specific serum antibody titres (a,c) and proliferative responses (b,d) of seals fed Atlantic (○) or Baltic (●) herring, following immunization with the primary antigens, rabies virus antigen (RV, a,b) or tetanus toxoid (TT, c,d). Immunizations are indicated by arrows. Data shown are means ± s.e.m. Significant difference between the two groups over time (repeated measures ANOVA): $**P < 0{\cdot}01$; $*P < 0{\cdot}05$; NS, not significant.

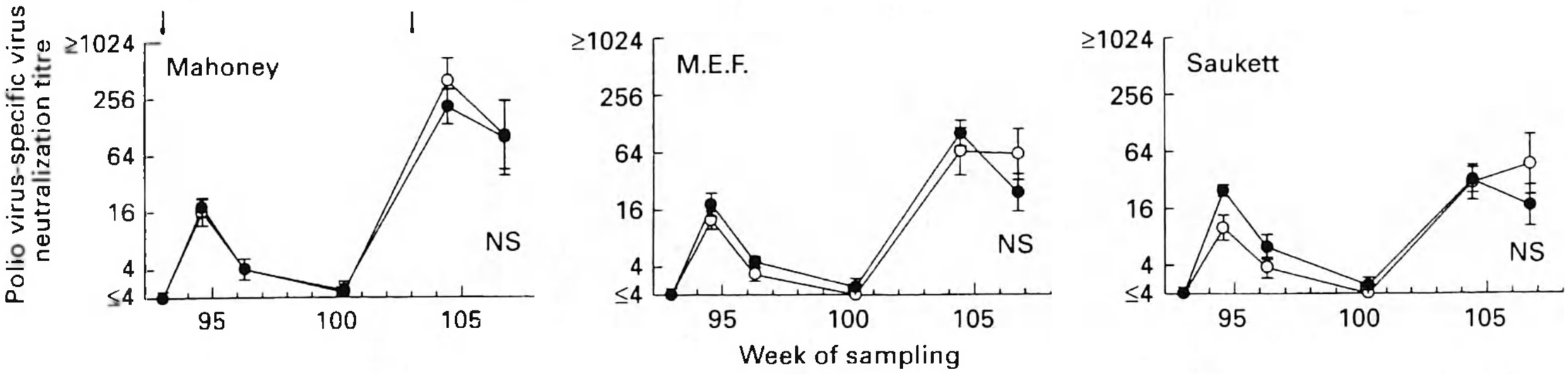

Fig. 4. Virus neutralizing serum antibody titres against polioviruses type 1 (Mahoney), type 2 (MEF) and type 3 (Saukett) in seals fed Atlantic (○) or Baltic (●) herring, after immunization with trivalent polio vaccine. Immunizations are indicated by arrows. Data shown are means ± s.e.m. NS, Not significant.

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 (Fig. 3a). However, proliferative responses of PBMC after *in vitro* stimulation with RV were significantly lower in seals feeding on Baltic herring (Fig. 3b, $P < 0{\cdot}01$). Mean RV-induced proliferative responses from the last seven blood samplings showed a significant inverse correlation with total TEQ levels in blubber biopsies ($r = -0{\cdot}45$, $P < 0{\cdot}05$).

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 (Fig. 3c, $P < 0{\cdot}05$), but *in vitro* proliferative responses of PBMC obtained from the same animals were significantly lower (Fig. 3d, $P < 0{\cdot}05$).

At a later stage, the animals were also immunized with the 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 (Fig. 4). No poliovirus-specific proliferative responses could be measured using a concentrated antigen preparation, as has been observed previously using human PBMC [19].

In vitro *immunoglobulin production*

In vitro non-specific immunoglobulin production was measured in culture supernatant of PBMC 6 days after stimulation with the B cell mitogens PWM and LPS, and shown in Fig. 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 study we have reported results obtained in studies on immune function in seals feeding on fish with different levels of naturally accumulated contaminants. These extend our previous observations, which indicated an impairment of *in vitro* NK cell activity and T cell mitogen-induced lymphoproliferative responses and *in vivo* DTH responses in seals fed on Baltic Sea herring [14,15]. 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 *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 'semi-field' 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

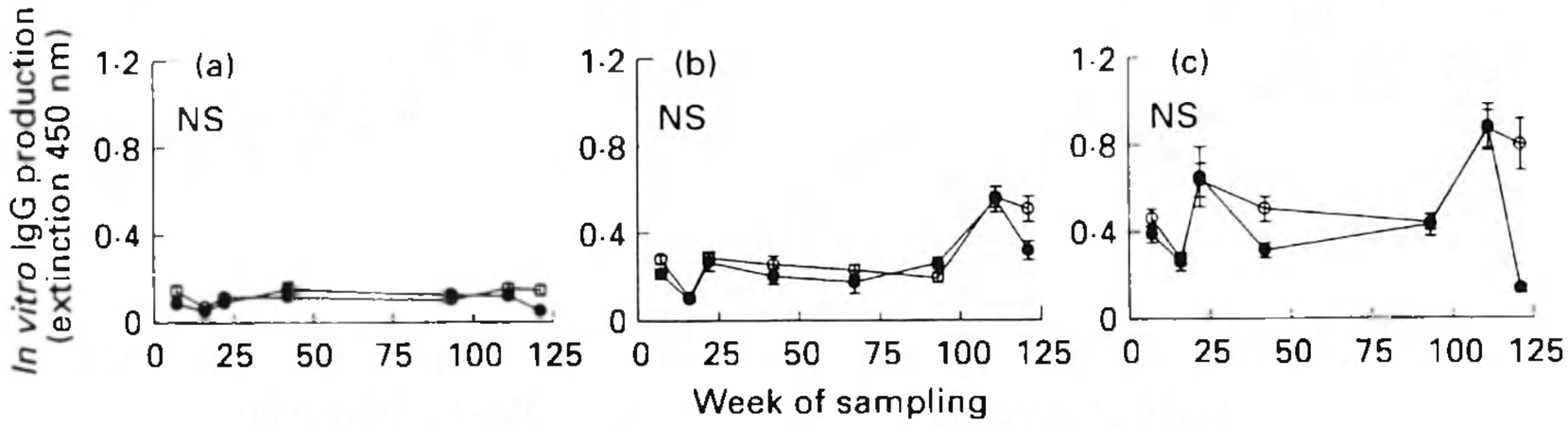

Fig. 5. *In vitro* total IgG production by peripheral blood mononuclear cells (PBMC) obtained from harbour seals fed Atlantic herring (○) or Baltic herring (●), after stimulation with pokeweed mitogen (PWM, b) or lipopolysaccharide (LPS, c) (a, medium control). Data shown are protein A binding antibodies in culture supernatants 6 days after stimulation, shown as means ± s.e.m. of optical densities at 450 nm. NS, Not significant.

chemical or group of chemicals responsible for the observed effects cannot be conclusively identified. However, when the estimated daily intakes of potential 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 [1,13]. The estimated daily intake of this group of contaminants in TEQ was approximately 10 times higher in the group of seals feeding on Baltic herring [14]. Over the course of the experiment, the estimated cumulative intake was 270 μg and 27 μg TEQ per animal, or $\approx$ 5 μg 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 lymphoproliferative responses [1,13]. 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 [1].

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 has been identified as potentially immunotoxic [2,20]. In addition, many contaminants are present in the natural environment which have an as yet unknown immunotoxic potential.

Contrary to our previous observations of reduced serum IgG responses following immunization with the primary antigen ovalbumin [15], we found no suppression of humoral immune responses. *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 [21]. One explanation for the apparent difference in results 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 [22,23], while dimethyldioctadecyl-ammonium bromide (DDA), the adjuvant to the ovalbumin immunizations, is an adjuvant which is particularly potent in inducing cell-mediated immune responses [24]. 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 to 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 [25], and indications of immunological differences were reported in the high fish consumption group [26]. 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. Perinatal exposure to immunotoxic chemicals, especially TCDD-like organochlorines, generally leads to more pronounced adverse effects than adult exposure [1]. Therefore, 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 with uncontaminated herring for 1 year. 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 (CTL) are thought to play an essential role in clearance of morbillivirus infections [27,28]. 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 [29], 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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