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ORIGINAL INVESTIGATION

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

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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 $4\frac{1}{2}$ months. Daily contaminant intakes of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) toxic equivalents (TEQ) 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 spleen CD_4^+/CD_8^+ cellularity, natural killer cell activity, or mitogen-induced proliferative responses of thymus or spleen cells could be detected. However, total thymocyte numbers and thymus CD_4^+/CD_8^+ ratios were reduced in the Baltic group. 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 and changes in thymus cellularity suggest that the dietary exposure to low levels of contaminants may have been immunotoxic at a level which our immune function test could not otherwise detect. While the herring diet per se appeared to have an effect on several immune function parameters, lower plasma thyroid hormone levels in the Baltic Sea group of rats confirmed that exposure to the environmental mixture of contaminants led to adverse PHAH-related health effects.

Key words Contaminants · Immunotoxicology · Host resistance · TCDD· PCB · Food chain · Rats · Seals · RCMV

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Introduction

The immunotoxic potential of the polyhalogenated aromatic hydrocarbons (PHAH) including the polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs) has been well established in laboratory animals (Vos and Luster 1989). However, exposure regimes in such studies usually use a 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 (Tanabe et al. 1994). 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 (Bosveld et al. 1994), reproductive impairment (Helle et al. 1976; Reijnders et al. 1986; Fox et al. 1991), embryotoxicity (Tillitt et al. 1992); skeletal malformations (Fox et al. 1991; Bergman et al. 1992; Mortensen et al. 1992), vitamin A deficiencies (Brouwer et al. 1989) and thyroid hormone deficiencies (Brouwer et al. 1989). While direct cause-and-effect relationships are difficult to identify, evidence from these semi-field and epidemiological studies have tended primarily to 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 (Vos and Luster 1989). 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 (Silkworth et al. 1982, 1986; Nagarkatti et al. 1984). 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 (Safe 1990, 1992).

The occurrence of virus-induced mass mortalities among marine mammal populations in recent years (Dietz et al. 1989; Osterhaus et al. 1995) 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 (Ross et al. 1996), mitogen- and antigen-induced T-lymphocyte proliferative responses (De Swart et al. 1994, 1995), and in vivo antibody and delayed-type hypersensitivity responses to ovalbumin (Ross et al. 1995). 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 (Ross et al. 1995). 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 (Osterhaus et al. 1988). 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 (Vos and Luster 1989). Laboratory rats are predominantly used in toxicological studies, and their use in immunotoxicology can provide 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 (Van Loveren et al. 1989; Vos et al. 1991). 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 (Thigpen et al. 1975), endotoxin (Vos et al. 1978) and influenza virus (House et al. 1990; Yang et al. 1994). While exposure to bis(tri-*n*-butyltin)oxide led to elevated virus titres in the salivary glands of rats infected with rat cytomegalovirus (RCMV) (Garssen et al. 1995), 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 (Bruggeman et al. 1985).

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 or 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 (Brouwer et al. 1983, 1986a; Lans et al. 1990).

Materials and 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 (De Swart et al. 1994). 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 $-20^{\circ}C$ 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 the custom-designed supplement consisting of 0.25% standard vitamin mix, 0.40% choline Cl 50%, 5% cellulose, 10% corn starch and 5.14% cerelose. The Baltic herring consisted of 54% freeze-dried Baltic herring powder, 0.25% standard vitamin mix, 0.40% choline C1 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 (De Swart et al. 1994). While the Atlantic group served as the experimental control in these feeding studies, a parallel group was fed standard irradiated rat pellets (#1210 SP, Hope Farms) and provided an indication of the effect of diet on general health and immune function parameters.

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 (Van der Velde et al. 1993). 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 (De Boer et al. 1995). Concentrations of all 2,3,7,8-substituted PCDD ($n = 7$) and PCDF ($n = 10$) congeners were determined as described elsewhere (Liem et al. 1990). Values of 2,3,7,8,-TCDD toxic equivalents (TEQ) were then determined for each of these congeners using recently described toxic equivalent factors (TEF) for PCBs (Ahlborg et al. 1994), and for PCDDs and PCDFs (Van Zorge et al. 1989). Dichlorodiphenyl-trichloro-ethane (DDT) and hexachlorobenzene (HCB) levels were determined as previously described (Boon et al. 1987). 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 strain 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 8 week-old specific-pathogen free (SPF) rats of both strains were housed in pairs in cages placed in negative pressure closed barrier isolators. Following 1 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 killed and thymus weights determined.

Subsequently, 24 recently weaned, female, specific pathogen free (SPF) PVG (Harlan-Olac) rats were housed in pairs in filter top cages for the long-term feeding study, Following 1 week of acclimation and ad libitum water and standard irradiated rat pellet diet (Hope Farms), rats were randomly divided into two 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 or the Baltic herring diet. 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 1×10^5 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 $-86^{\circ}C$. 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 $-86^{\circ}C$ (Garssen et al. 1995).

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 (FACScan; Becton Dickinson,

Rutherford, USA). Thymus and spleen cell suspensions were analyzed for cells with CD_4^+ and CD_8^+ surface markers using the appropriate monoclonal antibodies. Briefly, cells were incubated with 50 µl FITC-labelled ER2 (Serotec, Oxford, UK) and biotinylated OX-8 (Serotec), followed by R-phycoerythrin-conjugated streptavidin (Jackson Immunoresearch Laboratories, West Grove, USA). Samples were analyzed by FACScan after gating based on the forward and side scatter profiles of 10 000 total cells. Numbers of cell subpopulations in thymus and spleen were calculated as the number determined in total organ suspensions multiplied by the percentage determined positive by FACScan for each subset.

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 (Vos et al. 1984). Cells were adjusted to concentration and 4×10^5 were placed triplicate wells of a 96-well, round-bottom cell culture plate (Greiner, Nürtingen, Germany) along with the relevant mitogen solution. Cellular profileration was assessed by measuring the incorporation of ^{3}H-thymidine following 72 h of culture.

Natural killer cell activity

Natural killer cell activity was assessed as described elsewhere (De Jong et al. 1980). 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 4-h 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 (Vos et al. 1982), 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 (Garssen et al. 1995). 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 h in culture, monolayers were infected with RCMV at a multiplicity of infection of ten PFU per cell for 1 h at 37°C in phosphate buffered saline, and subsequently incubated with culture medium for a further 18 h. 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 1×10^7/ml and blocked with 0.2 M glycine as described elsewhere (Van Binnendijk et al. 1990). Non-RCMV infected control stimulator cells were prepared in the same manner.

RCMV-specific lymphocyte stimulations

RCMV-specific stimulations involved a coincubation of 1.5×10^5 effector cells (2×10^5 for the feeding study) with either 3×10^3 fixed RCMV-infected REC or 3×10^3 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 CD_4^+ and CD_8^+ 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 1×10^5 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 killed 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 (Garssen et al. 1995).

Plasma thyroid hormone

Plasma thyroxine (total T4; TT4) was determined using a chemiluminescent immunoassay (Amersham, Little Chalfont, UK) as described elsewhere (Murk et al. 1994).

Statistics

Results of each assay on each necropsy day were analyzed by independent two-sided *t*-tests between the Atlantic group (contaminant control) and the Baltic group. 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 (Fig 1). This was statistically significant at 1 μg/kg in PVG rats and 4 μg/kg 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 (Fig. 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. Peripheral blood mononuclear cells 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

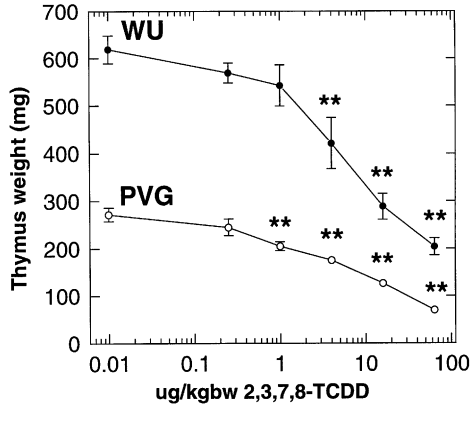

Fig. 1 Preliminary studies: absolute thymus weights in WU (*closed circles*) and PVG (*open circles*) rats 8 days following oral administration of different doses of 2,3,7,8-TCDD

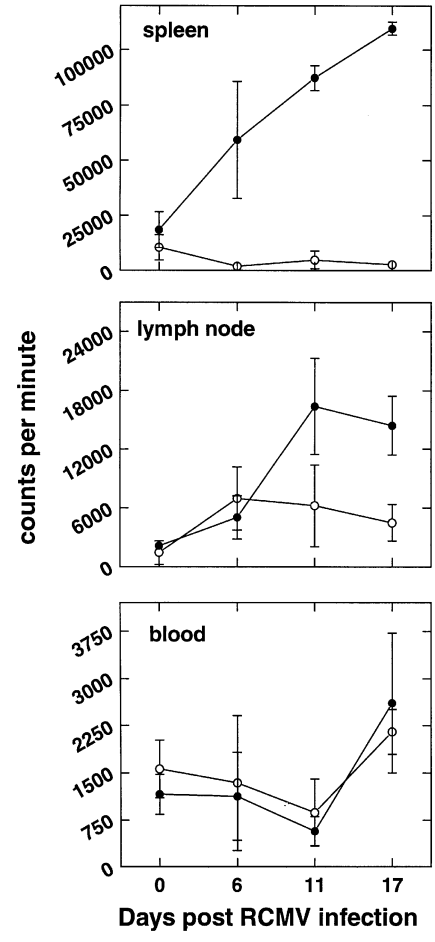

Fig. 2 Preliminary studies: RCMV-specific proliferative responses of rat lymphocytes isolated from three lymphoid compartments (spleen cells pooled posterior cervical lymph node cells, and pooled PBMC) following different in vivo infection incubation times. Rats were infected with RCMV for 6, 11 or 17 days prior to necropsy, or not at all (0 days), and cells from the identified compartment were coincubated with 3×10^3 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 circles* represent cultures with control (non-RCMV infected) stimulator cells. *Closed circles* 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

of both $CD_4^+CD_8^-$ and $CD_4^-CD_8^+$ phenotypes were detected following the in-vitro coincubation of spleen cells from infected rats with stimulator cells (Fig. 3a). Despite the expansion of both major T-lymphocyte subpopulations, there was a progressive shift towards CD_8^+ cell expansion in rats during the course of infection (Fig. 3a). This preferential CD_8^+ expansion is apparent in a comparison of CD_4^+/CD_8^+ ratios of blast cells in culture in the presence of RCMV-infected stimulator cells, as compared to CD_4^+ cells (see Fig. 3b).

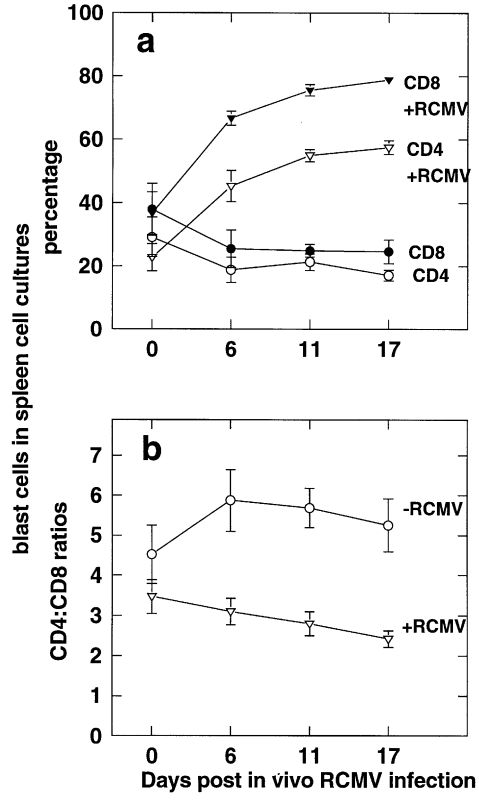

Fig. 3a, b Preliminary studies: following 6 days in culture, RCMV-induced expanded lymphocytes were labelled for flow cytometric analysis for the three groups of rats infected with RCMV for different lengths of time or not at all: **a** the percent of total CD8 (T-cytotoxic) cells (*closed symbols*) 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) 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 (*triangles*) or control non-RCMV-infected cells (*circles*)

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 8 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 4 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

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
β-HCH	<10	140
Dieldrin	154	340

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).

There were no differences in gross health parameters between rats of the Atlantic and the Baltic groups (Table 3).

Non-specific tests of immune function in rats

Rats of the Baltic group had reduced total thymocyte numbers and thymocyte CD_4^+/CD_8^+ ratios, but elevated numbers of $CD_4^+CD_8^+$, $CD_4^+CD_8^-$ and $CD_4^-CD_8^+$, as compared to those in the Atlantic group (Table 4). There were no significant differences in CD_4^+ or CD_8^+ subpopulation patterns in the spleens between the groups (Table 5).

NK cell activity was readily measurable in spleen cell suspensions of both groups (Table 6). No significant differences in natural killer cell activity between the groups were detected.

There were no significant differences in spleen or thymus cell proliferation induced by Con A, PHA or PWM between Atlantic and Baltic groups (Table 7).

No significant differences in total plasma IgM or IgG levels were observed (Table 8), 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 (Table 7). RCMV titres in the salivary glands of the Baltic rats were significantly higher than those in the Atlantic group of rats (Fig. 4).

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

Compound	Atlantic		Baltic	
	μg/kg	ng TEQ/kg	μg/kg	ng TEQ/kg
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.
β-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

	Atlantic	Baltic	*t*-Test
Body weight: start (g)	71.4 ± 4.7	70.9 ± 3.5	ns
Body weight: end (g)	192 ± 5.4	188 ± 3.4	ns
Growth (since birth)	2.75 ± 0.15	2.71 ± 0.15	ns
Thymus (mg)	143 ± 8.5	148 ± 4.6	ns
Thymus: body weight index (× 1000)	0.73 ± 0.03	0.79 ± 0.02	ns
Spleen (mg)	347 + 11.3	359 + 4.8	ns
Liver (g)	7.6 ± 0.27	8.1 ± 0.22	ns

ns, not significant

Table 4 Cellularity of the thymus ($\times 10^6$ cells)

	Atlantic	Baltic	*t*-Test
No. cells in thymus	192 ± 10.6	151 ± 5.3	**
No. CD_4^+/thymus	6.4 ± 0.52	11.3 ± 0.46	**
No. CD_8^+/thymus	3.2 ± 0.31	7.3 ± 0.34	**
No. $CD_4^+CD_8^+$ /thymus	37.7 ± 3.34	69.8 ± 2.8	**
Ratio CD_4^+/CD_8^+	2.0 ± 0.06	1.6 ± 0.04	**

** $p < 0.01$.

Table 5 Cellularity of the spleen ($\times 10^6$ cells)

	Atlantic	Baltic	*t*-Test
No. cells in spleen	324 + 34.5	325 ± 50.9	ns
No. CD_4^+/spleen	74.3 ± 9.22	64.5 ± 11.96	ns
No. CD_8^+/spleen	50.6 ± 6.59	45.5 ± 8.83	ns
Ratio CD_4^+/CD_8^+	1.5 ± 0.04	1.4 ± 0.05	ns

ns, not significant

Plasma thyroid hormone

Plasma TT4 levels were significantly lower in the Baltic Sea group, as compared to the Atlantic group (Table 9).

Table 6 Natural killer cell activity (% specific release ^{51}Cr from YAC target cells)

Effector : target ratio	Atlantic	Baltic	*t*-Test
100 : 1	44.5 ± 1.6	48.1 ± 1.4	ns
50 : 1	29.8 ± 1.6	31.9 ± 0.5	ns
25 : 1	19.3 ± 0.8	21.6 ± 0.8	ns

ns, not significant

Table 7 Proliferative responses of lymphocytes (stimulation index as calculated by counts per minute of cultures containing mitogen or antigen divided by cultures containing no test mitogen or antigen) isolated from thymus or spleen and coincubated with mitogens or RCMV-infected stimulator cells

Cell type and test	Atlantic	Baltic	*t*-Test
Thymus			
ConA	6.1 ± 0.7	5.2 ± 0.4	ns
PHA	2.1 ± 0.5	1.9 ± 0.3	ns
PWM	1.2 ± 0.1	1.5 ± 0.1	ns
Spleen			
ConA	24.2 ± 4.1	34.5 ± 7.2	ns
PHA	0.6 ± 0.0	1.0 ± 0.1	ns
PWM	2.8 ± 0.2	4.1 ± 0.6	ns
Spleen + RCMV stimulators	7.6 ± 0.5	9.3 ± 0.4	ns

ns, not significant; * $p < 0.05$; ** $p < 0.01$

Effect of diet

Rats readily consumed the two herring diets and showed no overt signs of diet-related health problems. Since these two diets were designed so as to limit the difference in dietary intake to the contaminant profiles in the herring while assuring the nutritional requirements of the rats, the Atlantic group served as an appropriate control. An additional group fed standard rat pellets for the duration of the feeding study

Table 8 Total plasma immunoglobulin titres

	Atlantic	Baltic	*t*-Test
IgG titre ($\times 10^3$)	109 ± 59	168 ± 52	ns
IgM titre ($\times 10^3$)	9.3 ± 1.0	9.0 ± 0.6	ns

ns, not significant

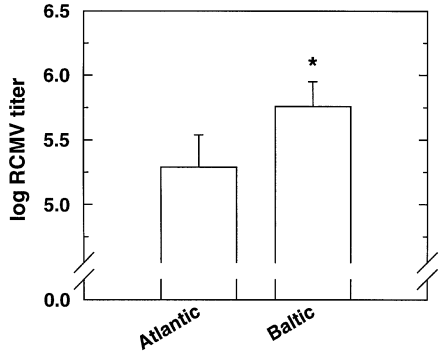

Fig. 4 Feeding experiment: RCMV titres in the salivary glands of rats fed Atlantic or Baltic diets for 130 days, and following 13 days of infection with RCMV. *Bars* represent mean $\pm$ SE of eight animals. Significant differences are indicated following Wilcoxon's signed rank tests (*$p < 0.05$; **$p < 0.01$)

Table 9 Plasma thyroid hormone levels

	Atlantic	Baltic	*t*-Test
Plasma TT4 (nmol/l)	37.9 ± 1.3	32.2 ± 1.5	*

*$p < 0.05$

demonstrated that fish, per se, had an effect on weight gain, lymphocyte proliferative responses to mitogens and RCMV antigen, but not on NK cell activity, thymus weights, CD_4^+/CD_8^+ ratios in thymus or spleen, or plasma thyroid hormone concentrations (results not shown).

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. Natural killer cell activity, and mitogen- and RCMV- induced lymphocyte proliferative responses 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, reduced thymocyte numbers and thymus CD_4^+/CD_8^+ ratios may indicate quantitative changes which did not lead to a measurable effect on the immune function parameters tested. Unexpectedly low proliferative responses of lymphocytes to mitogens obtained from rats of both herring-fed groups, which we attribute to an effect of diet, may have masked any contaminant-related effect. 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. 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 function 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 $4\frac{1}{2}$-month feeding study was similar to that which led to immunosuppression in our previous harbour seal studies which lasted $2\frac{1}{2}$ years. In these latter semi-field studies, we observed decreases in NK cell activity (Ross et al. 1996) and T-cell responses (De Swart et al. 1994, 1995; Ross et al. 1995) 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 (Vos et al. 1973; Smialowicz et al. 1994). 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.

The low lymphocyte proliferative responses observed in both groups of herring-fed rats when compared to rats fed pellet food confirm the importance of nutrition and diet in affecting immune function (Kelley et al. 1993), and highlight the need for caution in designing diets for such studies. The custom-prepared complements for the two herring diets in our studies were designed to meet the nutritional requirements of rats, and assured the comparability of these two groups.

Previous feeding studies using laboratory mice have demonstrated that contaminated fish can be immunotoxic. C57B1/6 mice fed a 33% diet of Coho salmon from Lake Ontario for 4 months had impaired serum antibody responses to SRBC, but no effects on peripheral lymphocyte subpopulation numbers or CTL activity were detected (Cleland et al. 1989). 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 CD_4^+ and CD_8^+ 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 CD_8^+ lymphocytes in vitro may indicate that this system reflects CTL activity in RCMV-infected rats, as has been demonstrated in other systems (Van Binnendijk et al. 1990). 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/kg (Clark et al. 1981; Nagarkatti et al. 1984), while others have observed no effect on CTL activity at doses as high as 3 μg/kg in mice (Hanson et al. 1994) or 30 μg/kg in rats (Rice et al. 1995).

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 (Brouwer et al. 1989; De Swart et al. 1994). Thyroid hormones are well established markers of exposure to PCDDs, PCBs, and some of their metabolites (Brouwer et al. 1986a; Lans et al. 1990). 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 (Barter et al. 1992) and competitive binding to the serum transport protein for T4, transthyretin (Brouwer et al. 1986b). In another chronic feeding study, *Ah* responsive C57Bl/6 mice fed a 33% diet of salmon from Lake Ontario for 4 months exhibited hepatomegaly, elevated ethoxyresorufin-*O*-deethylase (EROD) enzyme levels, decreased thyroxine and triiodo-L-thyronine (Cleland et al. 1987). A 2 month diet of Lake Ontario salmon also resulted in thyroid disorders and lower serum T4 levels in rats (Sonstegard et al. 1979).

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 and altered thymus cell profiles 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 (Garssen et al. 1995) 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 changes in thymus cellularity and higher RCMV loads in their salivary glands following infection suggest an immunotoxicity which may have been masked by other dietary influences or was otherwise undetectable in our functional tests. The observed alterations, 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 (Vos et al. 1974; Thomas et al. 1979), 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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