Arch Toxicol (1997) 71: 563–574

ORGAN TOXICITY AND MECHANISMS

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Impaired cellular immune response in rats exposed perinatally to Baltic Sea herring oil or 2,3,7,8-TCDD

Received: 7 January 1997 / Accepted: 15 April 1997

Abstract While the immunotoxicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been well established, the effects of complex environmental mixtures of polyhalogenated aromatic hydrocarbons (PHAHs) are poorly understood. Many PHAHs, including the polychlorinated-biphenyls (PCBs), -dibenzofurans (PCDFs), and dibenzo-*p*-dioxins (PCDDs), possess 'dioxin-like' activities, and accumulate in the aquatic food chain. Organisms occupying high trophic levels may therefore be exposed to concentrations which may present an immunotoxic risk. In this study, pregnant PVG rats were administered a daily oral dose of 1 ml of the following during pregnancy and lactation: (1) oil extracted from herring caught in the relatively uncontaminated Atlantic Ocean; (2) oil extracted from herring caught in the contaminated Baltic Sea; or (3) the Atlantic herring oil extract spiked with 2,3,7,8-TCDD. The daily intakes of aryl hydrocarbon (Ah)-receptor dependent toxic equivalents (TEQ) for mothers were 0.3 in the Atlantic group, 2.1 in the Baltic group, and 134 ng/kg body wt. in the 2,3,7,8-TCDD positive control group. Immune function and host resistance to rat cytomegalovirus (RCMV) were assessed in offspring aged 11, 25, 46 or 59 days. Rat pups in the positive control TCDD-spiked group exhibited immunosuppression characterized by reduced thymus weight and cellularity, reduced thymocyte and splenocyte proliferative responses to T-dependent mitogens in vitro, reduced virus-associated natural killer (NK) cell and specific antibody responses. While less pronounced, a similar pattern of effects was observed in the rat pups exposed only to the Baltic Sea herring oil. These immunotoxic effects were transient in both exposure groups, with a time-related recovery in immune function possibly due to the half-life of TCDD in rats and the waning exposure levels in the rapidly growing pups. We previously demonstrated that the same Baltic Sea herring led to impaired natural killer cell and T-lymphocyte function in harbour seals during the course of a long-term captive feeding study. The collective results of these studies in rats and seals indicate the immunotoxic potential of environmental mixtures at current levels in the aquatic environment, and suggest that the developing immune system of young mammals may be at particular risk.

Key words Food chain · Host resistance · Immunotoxicology · Polychlorinated biphenyls (PCBs) 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)

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Introduction

The immunotoxic potential of organochlorine chemicals has been well established in studies on laboratory animals, with polychlorinated biphenyls (PCBs), polychlorinated dibenzo-*p*-dioxins (PCDDs), and polychlorinated dibenzofurans (PCDFs) of particular concern (Vos et al. 1989). 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 (Osterhaus and Vedder 1988; Dietz et al. 1989), pollution-induced immunotoxicity could not be ruled out as a contributing factor. We subsequently demonstrated that sub-adult harbour seals fed herring from the contaminated Baltic Sea had impaired natural killer (NK) cell activity (Ross et al. 1996b) and T-lymphocyte responses in vitro (De Swart et al. 1994, 1995) and in vivo (Ross et al. 1995). We then speculated that contaminants played a role in the 1988 mass mortality (De Swart et al. 1996; Ross et al. 1996a).

Following our study using harbour seals, several questions remained unanswered as a result of legal, ethical, and methodological constraints in carrying out immunotoxicological 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 identical 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 4.5 months on the respective diets (Ross et al. 1996c). 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 could not be detected using our functional assays. Plasma thyroxine levels were significantly lower in the Baltic group, supporting the idea of a biological effect of PHAH exposure.

The results of several studies indicated the relative insensitivity of the adult rat compared to other species, to the immunotoxic effects of low levels of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds (Smialowicz et al. 1994). 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 (Vos and Moore 1974; Smialowicz et al. 1989; Faith and Moore 1977). Maternal exposure to two doses of 1 μg/kg body wt. of TCDD during gestation and three doses of 1 μg/kg body wt. during lactation resulted in thymus atrophy and reduced phytohaemagglutinin (PHA)-induced spleen cell stimulation in 25-day-old male rat pups (Vos and Moore 1974). In another study, a combined pre- and postnatal exposure to four doses of 5 μg/kg body wt. resulted in more profound and long-lasting effects than those observed in rats exposed only post-natally (Faith and Moore 1977).

Since species of wildlife 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 2,3,7,8-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 intervals in the offspring of these rats, and evaluated these in the context of host resistance to RCMV infection.

Materials and 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, Ymuiden, The Netherlands). The lipid fraction was mechanically removed, centrifuged once and the supernatant extracted. The resulting oil was mixed with 0.02% butyl-hydroxy-toluene (BHT) as anti-oxidant and aliquoted (30 ml) into 50-ml brown glass bottles. The 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 oils were analysed for congener-specific planar 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 by De Boer et al. (1995). Concentrations of all 2,3,7,8 chlorine-substituted PCDD ($n = 7$) and PCDF ($n = 10$) congeners were determined using methods described elsewhere (Liem et al. 1990). Values of 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 PCDDs and PCDFs (Van Zorge et al. 1989).

Study design

Pregnant rats were divided into three groups and given relatively uncontaminated Atlantic herring oil or Baltic Sea herring oil or Atlantic herring 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 (total of 41 exposure days). Immune function was assessed in four female pups from each nest at different time intervals 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 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 responses.

Rat study

Animals were housed and cared for under the supervision of the Animal Ethics Committee of the National Institute 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, Mich., USA) per ml Atlantic herring oil. Rats received an ad libitum supply of water and

standard irradiated rat pellet food (no. 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, mean body weights per sex determined, 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 delayed-type hypersensitivity (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 killed 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, N.Y., USA), 10% heat inactivated fetal calf serum (PAA, Linz, Austria), 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM glutamine. Cell suspensions were prepared as described elsewhere (Ross et al. 1996c), counted by Coulter counter and adjusted to the required concentration.

Cell suspensions of both thymus and spleen were analysed for CD4 and CD8 T-lymphocyte subpopulations by means of surface markers. Using a double-staining method, CD4 cells were labelled using fluorescein isothiocyanate (FITC) labelled ER-2 (Serotec, Oxford, UK) and CD8 cells labelled with biotinylated OX8 (Serotec) monoclonal antibodies as described previously (Ross et al. 1996c). A fluorescence-activated cell scanner (FACS; Becton Dickinson, Rutherford, N.J., USA) was used to measure triplicate samples of 10 000 cells. Analysis of mononuclear cell populations was carried out using gates on the basis of forward and side scatter characteristics.

Mitogen-induced lymphocyte stimulations were undertaken using thymus and spleen cell suspensions as described previously (Vos et al. 1984a). Briefly, 2×10^6 thymus cells or 8×10^5 spleen cells were stimulated with the following mitogens: concanavalin A (Con A; final concentration 2 μg/ml; Janssen Chimica, Beerse, Belgium), phytohaemagglutinin (PHA; final concentration 1:60; Wellcome Foundation, Darford, UK) or pokeweed mitogen (PWM; final concentration 1:60; Gibco) and 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 h 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 (De Jong et al. 1980). Natural killer cell activity was measured as the ability of 2×10^6 spleen cells to lyse 1×10^4 ^{51}Cr-labelled YAC-1 target cells in a 4-h co-incubation in 96-well cell culture plates. The value was calculated as (radioactivity counts in the supernatant minus spontaneous release by YAC)/(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 (ELISA) as described elsewhere (Vos et al. 1982). 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, Mo., USA) as described elsewhere (Vos et al. 1984b). These males, plus four non-immunized animals from each group, were tested for DTH reactivity to ovalbumin at age 46 days. For this purpose, rats were anaesthesized and a solution of 10 μg ovalbumin in 25 μl saline or a control injection of 25 μl saline was injected intradermally into each ear. Increase in ear skin thickness was measured at 24 and 48 h following injection using a digital micrometer (Mitutuyo, Tokyo, Japan). Aspecific swelling induced by ovalbumin in non-immunized rats was subtracted from the mean values obtained in immunized rats.

Host resistance to RCMV

The two remaining female pups per nest were infected intraperitoneally with 1×10^5 plaque forming units RCMV (obtained from C. Bruggeman, University of Limburg, The Netherlands) in saline at 34 days of age, and necropsies carried out at 46 and 59 days (12 and 25 days respectively 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 with Ficoll (Pharmacia LKB, Uppsala, Sweden) 1.077 g/ml density gradient isolation prior to culture and adjusted to 5×10^6/ml for both mitogen and RCMV stimulations.

RCMV-specific stimulations consisted of a co-incubation of 3×10^3 para-formaldehyde-fixed RCMV-infected rat embryo cells (REC) and 5×10^5 spleen cells in 150 μl/well in 96-well round-bottomed cell culture plates, using methods described previously (Ross et al. 1996c). Plates were incubated at 37 °C in a 5% CO_2 humidified incubator and [^{3}H]thymidine incorporation measured between 72 and 96 h. For the assessment of virus titres, salivary glands during both necropsies were removed aseptically and placed in Eagle basal medium (Gibco) containing 2% fetal calf serum. A 1:10 (w/v) suspension was frozen at −86 °C until the determination of RCMV titres as described elsewhere (Garssen et al. 1995). RCMV-specific total immunoglobulin titres were determined using an indirect ELISA similar to methods described elsewhere (Groen et al. 1989) with slight modifications. 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 giving 50% reduction of the maximum absorbance signal at 450 nm.

Plasma thyroid hormone measurement

Total plasma thyroxine (TT4) levels were determined using a chemiluminescence immunoassay (Amersham, Little Chalfont, UK) as previously described (Murk et al. 1994).

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-labelled KC-600) dynamics in pregnant and nursing rats (Takagi et al. 1986), a conservative estimate for TEQ dose in our rat pups was calculated using preliminary measurement of a transfer of 3.2 and 4.9% of the 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

Table 1 Breeding study: nest characteristics (mean ± SEM) 1 day following birth

	ANOVA	Atlantic	Baltic	TCDD
No. successful nests		16/17	15/16	11/11
No. pups per nest	NS	10.3 ± 0.47	9.4 ± 0.66	9.0 ± 0.65
No. female pups per nest	NS	4.67 ± 0.60	5.00 ± 0.80	5.38 ± 0.46
Weight of female pups (g)	NS	4.78 ± 0.09	4.59 ± 0.22	4.31 ± 0.11
Weight of male pups (g)	**	5.15 ± 0.07	5.18 ± 0.14	4.32 ± 0.09^{++}
Weight of mothers (g)	NS	186 ± 4.27	192 ± 2.67	191 ± 4.63

ANOVA NS, not significant; ** $P < 0.01$
Differences from Atlantic group by *t*-test, $^{++}P < 0.01$

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 delayed-type hypersensitivity test, a repeated measures analysis of variance with grouping factors was carried out. Significance levels are indicated by *P*-values of <0.01 or <0.05 for univariate ANOVA and for independent *t*-tests respectively. 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 1 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 2 Estimated intake of TCDD toxic equivalents (TEQs) per rat mother expressed as ng TEQ/kg body weight unless otherwise indicated (*TCDD* 2,3,7,8-tetrachlorodibenzo-*p*-dioxin)

	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	2143
Cumulative intake at pup age 11 days	6.62	57.2	3616
Cumulative intake at weaning	10.1	86.9	5490

Table 3 Estimated cumulative intake of 2,3,7,8-TCDD TEQs for each rat pup using PCB transfer data of Takagi et al. (1996) expressed as absolute daily dose (ng) or on a body weight basis in ng TEQ/kg body weight (*PCB* Polychlorinated biphenyl)

	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	1440
Cumulative dose at age 25 days (ng)	0.09	0.83	52.1
Cumulative intake at age 25 days (ng/kg)	1.83	17.3	1363

Intake of contaminants

Rat mothers in the Baltic group received 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 decline in TEQ levels which was associated with increase in body weight (Table 3).

Gross health parameters

Rat pups born to mothers in the Baltic Sea group exhibited no gross indications of toxicity, with organ weights being similar to those of 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 these pups, indicating the existence of normal growth patterns following birth.

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 pattern of reduced CD4+/CD8+ ratios in the thymus, particularly in the TCDD group, until the fourth necropsy. This was not the case in the spleen, where no alterations in CD4+/CD8+ ratios were detected (Fig. 1). 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 as indicated by the

Table 4 Gross health parameters. Data are of mean ± SEM, (RCMV Rat cytomegalovirus)

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 (ratio from 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 (× 1000):				
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 $^{+}P < 0.05$; $^{++}P < 0.01$

difference between titre on day 46 compared to day 25 ($P < 0.05$; results not shown) in the TCDD group of rats.

Mitogen-induced thymus and spleen cell proliferation

While there were no notable differences in gross health or immune parameters between rat pups of the groups treated with Atlantic and Baltic herring oil, there were 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 lymphocyte responses (Figs. 2 and 3). 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 (Fig. 3). While the effects on the Baltic group of rats were not always significant, there appeared to be a gradient of Atlantic > Baltic > TCDD for mitogen-induced stimulations of both thymus and

Table 5 Thymus and spleen cellularity (mean ± SEM)

	ANOVA	Atlantic	Baltic	TCDD
No. thymus cells ($\times 10^6$)				
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 $^{++}$
No. spleen cells ($\times 10^6$)				
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 $^{++}P < 0.01$

Table 6 Flow cytometry analyses of thymus and spleen cell subpopulations (mean ± SEM)

	ANOVA	Atlantic	Baltic	TCDD
No. CD4 in thymus ($\times 10^6$):				
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^{++}
No. CD8 in thymus ($\times 10^6$):				
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^{+}
No. CD4+/CD8+ in thymus ($\times 10^6$):				
11 days	**	99.6 ± 13.4	67.2 ± 7.44^{+}	47.0 ± 3.84^{++}
25 days	NS	187 ± 11.9	226 ± 19.4	106 ± 8.22^{++}
46 days (+RCMV)	NS	333 ± 19.6	344 ± 21.1	309 ± 18.9
59 days (+RCMV)	*	409 ± 20.5	333 ± 13.3^{++}	320 ± 27.5^{+}
No. CD4 in spleen ($\times 10^6$):				
11days	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
No. CD8 in spleen ($\times 10^6$):				
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 $^{+}P < 0.05$; $^{++}P < 0.01$

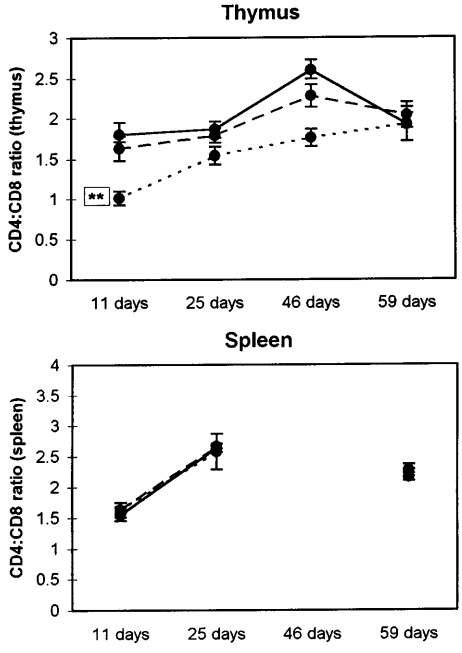

Fig. 1 CD4/CD8 ratios in thymus (*above*) and spleen (*below*) mononuclear cells before and after rat cytomegalovirus (RCMV) infection in rat pups of the Atlantic (*solid line*), Baltic (*long dash*), and TCDD (*short dash*) groups. Data represent mean ± SEM of eight pups. CD4 and CD8 analyses were not determined for spleen cell suspensions in pups aged 46 days

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, with immunized rats having greater swellings than non-immunized rats 24 h after intradermal injection. A gradient of Atlantic > Baltic > TCDD was observed at the peak swelling time of 24 h, though there were no significant differences (Fig. 4). 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 the differences were not significant (Table 7).

Natural killer cell activity

Basal NK cell activity was virtually undetectable in the youngest rats, and was higher in the TCDD pups at age 25 days than the other two groups of rats (Fig. 5).

Table 7 Immunoglobulin titres and specific antibody responses. Data are of mean ± SEM (*p.i.* Post infection *DTH* delayed-type hypersensitivity)

	ANOVA	Atlantic	Baltic	TCDD
Total IgM titre ($\times 10^3$):				
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 ($\times 10^3$):				
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
Ovalbumin-specific antibody titre in DTH males:				
IgM	NS	57 ± 19	44 ± 13	45 ± 13
IgG ($\times 10^3$)	NS	19.6 ± 7.9	23.5 ± 2.6	31.7 ± 7.8
RCMV-specific IgG titre:				
46 days (12 days p.i.)	NS	184 ± 13	230 ± 31	203 ± 18
59 days (25 days p.i.)	**	529 ± 88	241 ± 25^{++}	324 ± 54^{+}

ANOVA NS, not significant; ** $P < 0.01$
t-test $^{+}P < 0.05$; $^{++}P < 0.01$

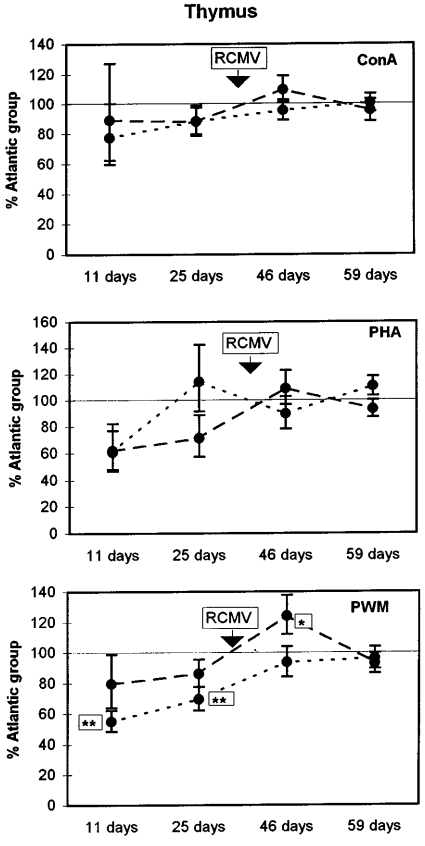

Fig. 2 Mitogen-induced proliferative responses of thymus cells expressed as a percentage of the Atlantic group in Baltic (*long dash*) and TCDD (*short dash*) groups ($n = 8$) before and after RCMV infection. Data were natural log-transformed and means expressed as a percentage of the Atlantic group in order to correct for age-related differences. *Error bars* representing 66% confidence interval of this ratio were calculated as the anti-log transformation of the differences between the groups on the log scale ± SEM of these differences. (*ConA* concanavalin A, *PHA* phytohaemagglutinin, *PWM* pokeweed mitogen)

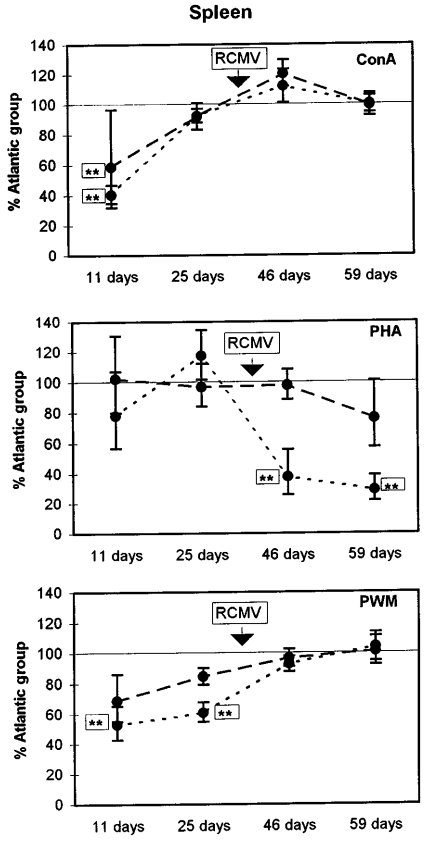

Fig. 3 Mitogen-induced proliferative responses of spleen cells expressed as a percentage of the Atlantic group in Baltic (*long dash*) and TCDD (*short dash*) groups before and after RCMV infection. Data were corrected for numbers of cells per organ, natural log-transformed and means expressed as a percentage of the Atlantic group in order to correct for age-related differences. Further details are as given in the legend to Fig. 2

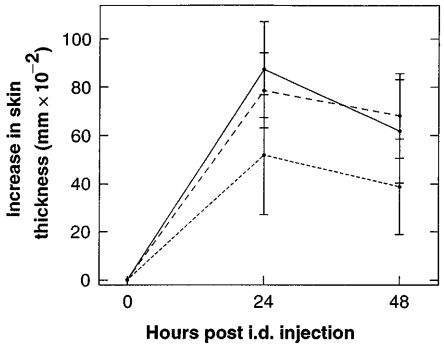

Fig. 4 Delayed-type hypersensitivity responses to ovalbumin in male pups of the Atlantic (*solid line*), Baltic (*long dash*) and TCDD (*short dash*) groups. Data represent the mean absolute increase ± SEM of $n = 8$ pups following subtraction of the mean responses of non-immunized animals to antigen (*i.d.* intradermal)

However, when corrected to incorporate virus infection, the RCMV-associated increase in NK cell activity was significantly lower in both Baltic and TCDD rat pups than in the Atlantic group (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 at age 46 days, but there was no significant effect in the Baltic group (Fig. 6). Total RCMV-specific immunoglobulin titres did not differ among groups at 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).

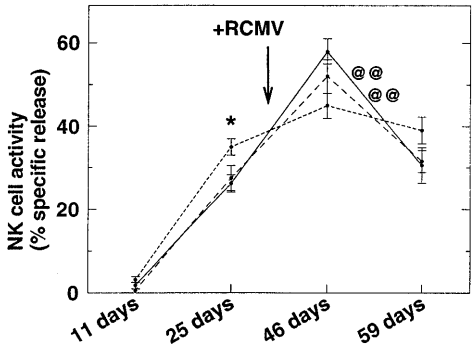

Fig. 5 Natural killer (NK) cell activity in Atlantic (*solid line*), Baltic (*long dash*) and TCDD (*short dash*) groups, expressed as natural cytotoxic activity of spleen cells using ^{51}Cr-labelled YAC-1 tumour cells as targets. Data represent mean ± SEM of $n = 8$ pups. Differences are indicated as: (i) for basal or spontaneous activity ($*P < 0.05$); and (ii) for virus-associated increase following RCMV infection at 37 days (calculated as specific release at 46 days/specific release at 25 days; $@@P < 0.01$)

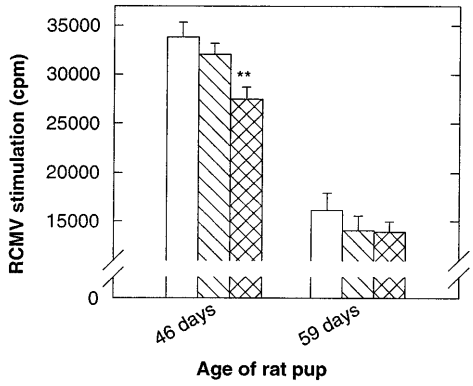

Fig. 6 RCMV-induced stimulation in vitro of spleen cells isolated from rats infected for 12 days (46-day-old rats) or 25 days (59-day-old rats), in Atlantic (*open column*), Baltic (*single hatched column*), or TCDD (*double hatched column*) groups. *Bars* represent mean gross counts per minute for $n = 8$ rats ± SEM of wells co-incubated with RCMV-infected rat embryo cells following 4 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 11- and 25-day-old TCDD group (Table 9).

Discussion

The immune systems of the rats in our study were clearly in a developmental phase, evidenced by the increasing numbers of thymus and spleen cell subpopulations, increasing NK cell activity and mitogen-induced lymphocyte proliferation with age. The plasma IgM and IgG levels in rat pups with time reflected the patterns of maternal transfer via milk (IgG) consistent with the endothelio-chorial placentation of rats and endogenous production by the pups (IgM and IgG). Because of the age-related immunological changes, the evaluation of an effect of contaminants was restricted to a comparison among the treatment groups of a given age. Exposure to the contaminants did not lead to any effects on maternal body weight, number of successful nests, or number of pups per nest in any of the treatment groups.

Rat pups of the positive control 2,3,7,8-TCDD group had an estimated cumulative intake of 1.4 μg/kg body weight by 25 days of age, a dose which led to significant immunotoxic effects. While this exposure level did result in some gross pathological effects (reflected by body, spleen and liver weights) at certain points during the study, growth was not affected, suggesting that the animals developed normally following birth. The thymus proved to be most notably affected, with reduced weight (absolute and body weight adjusted) in the younger animals, and reductions of thymocyte numbers and function at various points. In addition, the proliferative responses of splenocytes were reduced at various time points. The TCDD-related suppression of the immune

Table 8 RCMV titres in the salivary glands of rat pups. Data are of mean ± SEM

	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

Table 9 Plasma thyroxine levels (nmol/l). Data are of mean ± SEM

	ANOVA	Atlantic	Baltic	TCDD
11-day-old pups	*	47.7 ± 2.0	45.3 ± 2.5	$38.4 \pm 2.8^{+}$
25-day-old pups	**	29.0 ± 1.0	28.5 ± 1.1	$15.7 \pm 1.2^{++}$
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 $^{+}P < 0.05$; $^{++}P < 0.01$

function parameters led to a diminished capacity of the rats to mount specific cellular and antibody responses following RCMV infection. The lack of effect on RCMV titres in the TCDD group of rats may have reflected the transient nature of the immunosuppression, with an immunological recovery overcoming any effects on virus titres. Alternatively, host resistance to this virus may involve other features of the immune system which were not measured.

Rat pups exposed to the Baltic Sea herring contaminants had an estimated cumulative intake of only 17 ng/kg body weight at age 25 days. While no gross pathological effects were observed in this group, there were significant effects on immune function. Though less pronounced than in the TCDD group, the Baltic rats showed transient reductions in thymus cellularity and splenocyte functionality. Virus-associated effects were also observed, with RCMV-associated increase in NK cell activity and IgG titres being significantly lower than in the Atlantic group.

The immunological effects of contaminants in both the TCDD and Baltic groups indicate an effect at the level of the thymus or thymocyte precursors. The thymus is a sensitive target for 2,3,7,8-TCDD in rats (De Heer et al. 1994) and other laboratory animals, with thymus atrophy being attributed to reduced maturation of the thymocytes (Blaylock et al. 1992; Greenlee et al. 1985) and reduced seeding of the thymus by bone marrow progenitors (Fine et al. 1990). In addition to noticeable effects on the actual thymus, populations of mature circulating T-lymphocytes were also impaired.

The impaired virus-associated increase in NK cell activity following RCMV infection in the two exposure groups was consistent with observations of other studies (Selgrade et al. 1992; Yang et al. 1994). In the first of these studies, the authors concluded that virus-associated increases in NK cell activity correlated better with mortality following virus infection than basal, or spontaneous, NK activity in exposed animals. The lower RCMV-specific antibody titres observed at 25 days post-infection in the two exposure groups suggest an effect of contaminants on the humoral response, possibly reflecting an effect on B-cells, T-helper cells, or their precursors. In addition, the impaired RCMV-specific T-cell responses may indicate that activity of cytotoxic T-lymphocytes was affected in TCDD-exposed rats (Ross et al. 1996c).

Suppression of spontaneous NK cell activity, T-cell function in vitro, antibody responses to ovalbumin, and delayed type hypersensitivity responses in vivo were previously demonstrated to occur during a long-term captive feeding study of harbour seals fed the same Atlantic and Baltic herring used in this study (for reviews see De Swart et al. 1996; Ross et al. 1996a). The concordance of immune effects between the perinatally exposed rat pups in this study and juvenile seals exposed to the Baltic Sea herring contaminants provides the basis for a comparison of the two species. While there were inevitable differences in the study designs and the observed effects, both rats and harbour seals had impaired T-cell and NK cell responses following dietary exposure to contaminants in the Baltic herring. Since the application of certain invasive techniques or a host resistance model would not have been appropriate in the seal study, additional information has been generated in this parallel rat model. The impaired T-cell immunity in the harbour seals fed Baltic Sea herring is speculated possibly to have reflected toxicity at the level of the thymus, and the seals may have also had diminished specific immune responses following virus infection.

The estimated dose of TEQs to which the pregnant rats and their pups were exposed in the current work was relatively low. The 11-day-old rat pups of the group exposed to Baltic herring oil exhibited immunological alterations when their mothers had received a daily dose of only 2 ng/kg and a total cumulative TEQ dose of 57 ng/kg body wt., at which point we estimated the cumulative pup exposure to be 20 ng/kg body wt. While the effects on the thymus and on cellular immunity are consistent with the known effects of 2,3,7,8-TCDD (Vos and Luster 1989), most studies have utilized dosages far exceeding those used here, and relied primarily upon acute exposures administered on a once only or limited basis. Earlier studies demonstrating the developing im-

mune system of rats to be relatively sensitive to the immunotoxic effects of 2,3,7,8-TCDD used multiple maternal doses of 1 or 5 μg/kg body wt. (Vos and Moore 1974; Faith and Moore 1977). Pre- and postnatal exposure was shown to result in more profound effects than postnatal exposure alone (Faith and Moore 1977), 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. We exposed the TCDD group of female rats in this study to a daily dosage (28 ng per rat; 134 ng/kg body wt.), which would lead to a cumulative exposure of approx. 5 μg/kg body wt. at weaning, a dosage which has been demonstrated to be immunotoxic in laboratory rats (De Heer et al. 1994; Ross et al. 1996c). Therefore this group served as a positive control in the study. The partial recovery observed in our rat pups is likely to have 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 (Rose et al. 1976) implies that the rapidly growing pups in our study would have diminishing body burdens during nursing and after weaning.

In another feeding study, a reduction in 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 4 months (Cleland et al. 1989). Owing to relative sensitivity of mice to the immunotoxic actions of TCDD (Smialowicz et al. 1994), the different contaminant characteristics in the diets and the choice of immune function tests used, a comparison is difficult of 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.

A possible synergistic or antagonistic effect of different PHAH congeners cannot be ruled out to explain 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 (Ross et al. 1996c). However, what appears to be a dose-related pattern of immunotoxic effects among the three groups (Atlantic < Baltic < TCDD) may suggest a similar mechanism of immunotoxic action in the Baltic and TCDD groups. Immunotoxicity by organochlorines has been shown to be largely mediated by the Ah receptor (Safe 1990; Nagarkatti et al. 1984). The TCDD group of rat pups was exposed to the Ah-prototype immunotoxicant, 2,3,7,8-TCDD (a very 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 PCBs comprised the majority of total TEQ in the Baltic Sea herring lipid (Ross et al. 1996a,c), PCBs would have been largely responsible for an Ah-dependent immunotoxicity in rat pups of the Baltic group (the total contribution of all PCDDs to the TEQ in Baltic herring was <10%, whereas PCBs contributed nearly 60%).

We did not detect any changes in plasma thyroxine levels in the Baltic group of rats, even though prenatally exposed rats have been shown to be sensitive to the effects of PCBs (Morse et al. 1993). While an earlier effect may conceivably have disappeared by the time of first necropsy at 11 days, our results also suggest that immunotoxicity may occur irrespective of thyroid alterations in animals exposed perinatally to a complex mixture of contaminants. Thyroid hormone levels are considered a reliable indicator of exposure to certain PHAH compounds and some of their metabolites (Brouwer et al. 1986; Brouwer and Van den Berg 1983); the lower thyroxine levels in the TCDD group at the time of the first two necropsies support this suggestion. In our previous study of adult rats, a reduction in plasma thyroid hormone levels was observed while, other than differences in RCMV loads in the salivary glands, immune alterations were undetectable (Ross et al. 1996c).

The immunological alterations observed in the Baltic rat pups were largely transient and were less pronounced than in the TCDD group. However, even subtle alterations in immune status can affect host resistance, leading to increased susceptibility to infection (Luster et al. 1993). The fact that a lipid extract of herring caught from the Baltic Sea could lead to immunological alterations, however small, should be of concern, particularly in a species which has been shown to be relatively insensitive to the effects of TCDD.

The concordance in immune effects between perinatally exposed rats in this study and juvenile seals (De Swart et al. 1996; Ross et al. 1996a) provides a basis for comparison of 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 may be more sensitive to the effects of immunotoxic environmental contaminants than was indicated by our previous studies of juvenile seals. Whereas the exposure to immunotoxic contaminants in the rat pups ceased at weaning, predators at the top of the food chain would be exposed to an ongoing source of TCDD-like contaminants in their diets. Thus 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, immunotoxicity. The described results suggest chronic exposure to low levels of environmental contaminants presents a 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.

Acknowledgements The authors gratefully acknowledge the contributions of the following people to these studies: Jan Assink, Sjors Groenemeyer, Ben van Middelaar, Djien Liem, Marcel Menkhorst, Coen Moolenbeek, Nico Schmidt, Herman Naring, Aldert Piersma and Elze de Ruiter.

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