EFFECT OF PERINATAL EXPOSURE TO PCB 153 AND PCB 118 ON THE SPECIFIC IMMUNE RESPONSE IN LAMBS

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Introduction

Several studies have indicated that organochlorines (OCs) may have an impact on the immune system, and it is a general acceptance that the immune system is particularly sensitive to OC exposure[1,2]. Most studies have been done in laboratory animals. However, *in vivo* studies of harbour seals (*Phoca vitulina*) fed contaminated Baltic fish, showed that OCs affects antibody response and cell-mediated (lymphocyte proliferation) immunity[3,4,5,6]. In polar bears, studies have indicated that both serum immunoglobulin G (IgG) level, antibody response following immunization and cellular immunity (antigen and mitogen induced lymphocyte proliferation) may be impaired by OCs in the Svalbard region as well[7,8,9]. In free-ranging species, OC immunotoxic effects on mitogen-induced lymphocyte response and IgG concentration, have been suggested in bottlenose dolphins (*Tursiops truncatus*)[10], striped dolphins (*Stenella coeruleoalba*), harbour seals[11]and in the St. Lawrence beluga whale (*Delphinapterus leucas*)[12,13,14].

The present study is a part of a larger study on "Endocrine disrupters: Risk assessment for food quality and animal health" with focus on effects on reproductive endpoints, immune function and behaviour in the offspring. To determine the immuno toxicity of PCB 153 and PCB 118 exposure *in utero* on the specific neonatal immunity, we conducted an experimental study in lambs with measurement of specific cell mediated immunity and antibody formation following immunization.

Materials and Methods

Experimental design: The effect of PCB exposure of sixty-two lambs was studied from birth until sacrifice at 60 days of age. Their mothers were dosed orally with PCBs dissolved in corn oil during pregnancy. Consequently, the offspring were exposed to PCB in utero and through mother's milk.

Forty-four adult female sheep of the Norwegian Dala breed were used. The ewes were allocated into 3 groups using block randomization. One group was exposed to PCB 153 (14 ewes), a second group received PCB 118 (16 ewes) and the third group served as a control (14 ewes). The animals in the experimental groups were orally administered either PCB 153 (98 µg/kg body weight/day), PCB 118 (49 µg/kg body weight/day), or corn oil 3 times a week (Monday, Wednesday, Friday) throughout gestation until delivery that took place 146 days later. The animals were kept indoors, and the groups were held in separate pens in order to avoid contamination through the ingestion of PCB-contaminated feces. The sheep received standard hay and concentrate feeding. The ewes delivered indoors and the mothers and lambs remained together until 60 days postpartum.

All lambs were immunized at week 2 of age by subcutaneous injection with 0.5 ml Diphtheria vaccine (Difterivaccine SSI, Lot 329901A; Statens Serum Institut, Copenhagen, Denmark) and by intracutaneous injection with 0.1 ml of a live, attenuated *Mycobacterium bovis* (BCG) (Bacille Calmette Guerin vaccine, Lot 203073H, Statens Serum Institut, Copenhagen, Denmark).

Serum from all lambs was collected at weeks 2, 4 and 6. In order to assess neonatal immunity following immunization the levels of antibodies to diphtheria toxoid was measured. Heparinized (15 IU/ml) blood was collected at week 6 to measure the lymphocyte proliferation and the IFN-γ production upon stimulation.

Adipose tissue samples were collected, following euthanasia of the lambs, at the age of 6o days. The samples were stored at -20°C until assayed.

Chemical analysis: Chemical analyses of PCBs 153 and 118 in adipose tissue were performed at the Laboratory of Environmental Toxicology, Norwegian School of Veterinary Science, Oslo as described elsewhere[15].

Immunological analyses

Serology: Specific antibodies against diphtheria toxoid (No.AS2/78b; The National Health Institute, Norway) were assayed by a hemagglutination test[16]. Sheep RBC were coated with diphtheria toxoid (900 Lf/ml) and used in the assay. The lowest serum dilution tested was 1:8. Sera that showed no hemagglutination at this dilution were entered as 4 ($\log_2 = 2$) in the statistical analyses. Lambs with an increased antibody titer of ≥ 2 were considered as significant responders.

Skin test using mitogens and antigen: The skin test was applied laterally on the thorax as described for dogs[17]. The same region of thorax was used for all lambs. The skin tests were performed by using a 1 ml, 27G, U-100 Insulin Myjector (Terumo, Europe N.V., Leuven, Belgium) for the intradermal application of 0.1 ml of pokeweed mitogen (PWM, L8777, Sigma-Aldrich, Inc., St. Louis, MO) (50 µg PWM/ml in PBS solutions) as mitogen, Bovine tuberculin purified protein derivative (PPD, PL 3326/4006, Veterinary Laboratories Agency, New Haw, Addlestone, Surrey, UK) (2.500 iu. PPD /0.1 ml) as antigen, and 0.1 ml 0.9% NaCl was used as a negative control. The skin reaction was measured as the diameter (mm) using a perspex template 24 hour after the injections.

Lymphocyte proliferation*:* Mitogen- and antigen-induced lymphocyte proliferation was assayed using purified lymphocyte culture[18], modified for sheep blood. Heparinized (15 IU/ml) blood was centrifuged 900 x g in 10 min. The buffy-coat was collected and mixed with 6-7 ml Hanks buffered saline solution (HBSS) (DAA laboratories, Austria), and was then placed on top of 3 ml Lymfoprep® (Axis-Shield PoC AS, Oslo, Norway) (1.077 g/ml) in siliconized vials. Each gradient was centrifuged 700 x g in 30 min and the lymphocyte layer was transferred to a siliconized vial and washed 700 x g in 15 min with 10 ml and washed a second time in 10 min. The supernatant was carefully removed and the remaining cells (ca. 0.2 ml) was added 9.8 ml HBSS and the cells were counted. The cells were centrifuged 700 x g in 10 min, and resuspended to 1 x 10^6 mononucleated cells/ml with RPMI 1640 cell culture medium (RPMI 1640, Gibco Div of Invitrogen, Paisley, UK) containing 10 % fetal calf serum (FCS), 2mM glutamine, 0.15 % bicarbonate, 1 % antibiotics (penicillin, streptomycin), and 100 µl was immediately dispensed into each flat-bottomed well in 96 wells micro titration plates (Nunc, Roskilde, Denmark). The following mitogens and antigen (10 µl/well) and final concentration were used: phytoheaemaglutinin (PHA) (10 µg/ml) (Wellcome, Beckenham, England); poke weed mitogen (PWM) (10 µg/ml) (Gibco, Renfrewshire, Scotland); concanavalin A (ConA) (20 µg/ml) (Pharmacia) and PPD (20 µg/ml) (Bovine tuberculin purified protein derivative PPD, 2500IU/0.1 ml, BNB30/01, Veterinary Laboratories Agency, New Haw, Addlestone, Surrey, UK). The RPMI 1640 medium was used as diluent and for controls. All assays were done in triplicate cultures. Plates were incubated at 37°C in a humidified atmosphere of 5% CO_2, 95% air for four days.
Lymphocyte proliferation was evaluated as the incorporation of 5-bromo-2'-deoxyuridine (BrdU), a thymidine analogue, added for another 16 hours of incubation, and further detected with a monoclonal antibody and a colorimetric enzymatic reaction (Cell Proliferation ELISA BrdU (colorimetric), Roch Diagnostics GmbH, Mannheim, Germany) as per manufacturer's instructions using an ELISA plate reader (Titertek Multiskan Plus MK2, Labsystems, Finland) at 450 nm with a reference wavelength of 650nm. The lymphocyte response was expressed as the difference of the mean OD value of stimulated cultures and control cultures.

Interferon gamma: IFN-γ production in whole blood cultures following stimulation with PPD was assessed using an ELISA for bovine IFN-γ (Bovigam, CSL, Victoria, Australia). Whole blood cultures (heparinised), in 180 µl volumes in duplicate in 96-well plates (Falcon), were stimulated with 20µl PPD (purified protein derivate of *M.a. bovis,* (VESO/National Veterinary Institute, Oslo, Norway) in a final concentration of 10 µg PPD ml^{-1} and 40 µg PPD ml^{-1} or with medium alone. After incubation for 24 h in a humidified atmosphere of 5% CO_2 at 37°C, plasma was harvested and stored at -20°C until assayed.
Quantification of produced IFN-γ was measured in duplicate using an ELISA test for bovine IFN-γ (CSL, Vic., Australia). Plasma samples were diluted 1:2 and 1:4. Positive and negative bovine controls were assayed in dublicate in serial wells. Results were expressed as equivalents of recombinant bovine IFN-γ (rBoIFN-γ, Serotec, lot 160905) by means of a standard curve ranging from 0,075 to 9,38 ng ml^{-1}. Results from control wells were subtracted from stimulated wells. Mean of the calculated amounts in duplicate samples was used.

Statistical analysis: Dependent variables were assessed for normality by the Shapiro-Wilks method. Group means of log PAG were compared by Student's *t*-test or the Wilcoxon rank sum test. Anova tests were also used. P-values less than 0.05 were considered statistically significant

Results and Discussion
Due to erroneous handling of animals single events of cross contamination occurred resulting in a mixed exposure scenario rather than the planned exposure to single compounds. The group planned to represent a control group has to be considered as low contaminated. Group 1 had high level of PCB 153 and moderate level of PCB 118, Group 2 had moderate level of both PCB 118 and PCB 153, and Group 3 (control) had low level of both PCB 118 and PCB 153. The mean PCB 153 concentrations in adipose tissue at 60 days of age in Group 1 (the PCB 153 group) was 51900 ng/g wet weight (range 45000 - 58700), in Group 2 (the PCB 118 group) 7420 ng/g (range 6240 - 8610) and in Group 3 (the control group) 892 ng/g (range 778 - 1010). The mean PCB 118 concentrations was 1310 ng/g (range 1150 - 1480), 8760 ng/g (range 7230 - 10300) and 47 ng/g (range 41 - 54) in Group 1, Group 2 and Group 3, respectively.

A significant lower delayed type hypersensitivity response to intradermal injection with PPD was found in lambs exposed to high doses of PCB 153 (Group 1) compared with the other groups. The general capability to induce lymphocyte proliferation after stimulation with mitogen (PWM) was also assayed with the skin test reaction showing no group difference (Table). Thus the PCB exposure did not induce a general suppression the lymphocyte proliferation in the skin. These results reflect an impaired ability of lambs, sensitized to BCG-vaccination, to induce specific CD4+ T lymphocyte response upon antigen stimulation when exposed to PCB 153. The findings that only the lymphocyte response to PPD and not the mitogen induced lymphocyte response in the skin was suppressed indicates that the lambs were not sufficiently sensitized to BCG during the immunization. In guinea pigs fed a PCB (Clophen A 60®) dietary concentration of 50 ppm the cell mediated immune response after tuberculin injection in the skin was significantly reduced when compared to the control group[19,20], and similar effects from DDT and Aroclor 1254® were found in rabbits fed 150 and 170 ppm, respectively[21] . In dogs fed mink whale blubber rich in OC contaminants both delayed type hypersensitivity T-lymphocyte intradermal response induced by mitogens and antigen (Keyhole Limpet Hemocyanin) were suppressed compared to the control group[17].
Significant reduced ability to produce interferon-gamma (IFN-γ) following PPD stimulation of whole blood cultures from lambs in Group 1 demonstrates a similar effect in vitro as shown in the skin test (Table). IFN-γ is a cytokine produced by activated CD4+ T lymphocyte (and NK cells) and is considered a protective response by lymphocytes in immunity to mycobacterial infections. In small ruminant the CD4+ T lymphocytes are believed to be the main IFN-γ producing cells in recall responses after vaccination with live strains of *M. a. paratuberculosis*[22]. Although the cytokine production was lower in Group 1 compared with the other groups, there were no such difference regarding mitogen and antigen induced lymphocyte proliferation in the present study (Table). In goat kids, perinatal exposed to PCB 153, decreased lymphocyte proliferation after stimulation with PHA and Con A could be demonstrated 2 to 8 weeks postnatally[23]. In polar bears high level of PCBs were associated with decreased lymphocyte proliferation[9].
The production of specific antibodies to diphtheria toxoid was significant lower two weeks following immunization in lambs exposed to PCB 153 (Group 1) compared with the other groups (Table). In a goat kid experiment such effect of PCB 153 exposure on the antibody response to diphtheria toxoid could not be demonstrated when assayed 4-6 weeks after the immunization[24]. The effect on the antibody production in the present study may reflect an impairment of a primary antibody response in that either the IgM response is depressed or the increase of antibody titre is delayed. In piglets exposed to a PCB/OC pesticide seal blubber mixture an impaired antibody response to *Mycoplasma hyopneumoniae* was demonstrated although an increased lymphocyte response to PHA was found[25]. In laboratory animals fed PCB/OC contaminated beluga whale blubber a decrease in the CD8+ T cell population (spleen), reduced specific antibody response to sheep red blood (SRBC) cells and reduced phagocytosis from peritoneal macrophages was found[26]. In polar bears relying on contaminated seal blubber, it was suggested that PCBs and OC pesticides may have a suppressive role in antibody response against influenza virus and reovirus[8]. In the present study an effect of PCB 153 on both specific immune response to PPD and diphtheria toxoid could be demonstrated.

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Table. Effect of PCB 153 and PCB 118 on specific immune response in lambs; Group 1 ("PCB 153 – group") (n=21), Group 2 ("PCB 118 – group") (n=23) and Group 3 (control) (n=18) (for further information see text). The skin reaction (mean diameter in mm of the group) to intradermal injection with PPD (Bovine tuberculin purified protein derivative) and PWM (Pokeweed mitogen) was measured in the Skin test. The cytokine response (mean IFN-γ ng ml^{-1} of the group) to PPD (10 μg PPD/ ml and 40 μg PPD/ ml) was measured with the interferon-gamma test (IFN), and the in vitro lymphocyte response (mean OD value of the group) following PHA (phytoheaemaglutinin), PWM, Con A (Concanavalin A) and PPD stimulation was assayed with the lymphocyte proliferation test (LP). The antibody response (mean $\log_2$ titre of the group) to diphtheria toxoid (DT) was measured 2 weeks following immunization.

Immunological test	Antigen/mitogen	Group 1	Group 2	Group 3	Significans
Skin test	PPD	25.7 (1.33)*	31.6 (1.39)	26.1 (1.42)	p<0.01
	PWM	12.4 (0.56)	13.0 (0.38)	13.3 (0.16)	ns
IFN	PPD 10	2.85 (0.916)	6.41 (1.626)	5.70 (1.787)	p<0.1
	PPD 40	3.19 (0.869)	7.81 (1.709)	6.09 (1.899)	p<0.05
LP	PHA	1.36 (0.114)	1.50 (0.100)	1.33 (0.120)	ns
	Con A	1.96 (0.124)	2.07 (0.120)	1.72 (0.142)	ns
	PWM	1.98 (0.119)	1.89 (0.109)	1.85 (1.111)	ns
	PPD	0.64 (0.088)	0.75 (0.116)	0.73 (0.111)	ns
Serology	DT	5.3 (0.37)	6.2 (0.27)	6.6 (0.25)	p<0.05

* (Standard error of the mean)