POTENTIAL EFFECTS OF BLOOD CONTAMINANTS ON IMMUNE RESPONSES IN HARBOUR SEALS (*PHOCA VITULINA*)

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Introduction

Concerning levels of polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), pesticides (hexachlorobenzene, HCB, dichloro-diphenyl-trichloroethane DDT), total mercury (T-Hg) and other trace elements have been previously described in the blood of free ranging harbour seals from the Wadden Sea[1-3]. These compounds display *in vivo* and *in vitro* immunotoxic properties and the reported levels in free-ranging harbour seals inhabiting the polluted North Sea are sufficiently high to suggest that population may be at risk of immunotoxicity[2,4-9]. *In vitro* exposure of leucocytes is increasingly used to understand effects and biochemical pathways of pollutants affecting the immune system of marine mammals[2,10-19]. This non-invasive method is convenient as leucocytes can be isolated from peripheral blood of free-ranging animals. However, a recurring question occurs about the potential effects of contaminants present in the blood of seals on leucocytes viability and proliferation, prior to any *in vitro* exposure. Seals can indeed display large ranges of metal and organic contaminant levels depending not only on the environmental contamination but also on their age, sex, diet, body condition and physiological status. A decreased lymphocyte response has been associated with increased PCB and DDT levels in peripheral blood of bottlenose dolphins[20]. The purpose of the present study was to determine if there is a relationship between chemical contaminant exposure and immune function in harbour seals.

Materials and methods

Samples. Blood samples were collected from 13 free-ranging harbour seals older than 2 years (adults), caught in seal-catch campaigns on Lorenzenplate, North Sea (Germany) in 2008 and 2010. Seals were physically restrained. Blood was drawn from the extradural venous sinus into sterile evacuated blood collection tubes (BD Vacutainer® Lithium Heparin tubes for lymphocyte isolations, Serum Tubes Monovette® for POPs analysis, S-Monovette® metal analysis tubes for metal analysis) and kept at room temperature for the *in vitro* cultures, and at -20°C for the pollutants and metal analysis. Serum was isolated by centrifugation at 1500g during 20 min at 21°C (Multifuge 3 S-R, Kendro).

Hematology profile. The hematology profiles were determined using a ScilVet ABC (Scil Animal Care Company GmbH, D-68519 Viernheim, Germany). Blood smears were dyed with Pappenheimer Dye, and the leucocyte subgroups were counted manually (40x magnification).

Isolation and culture of lymphocytes. The blood was diluted 1:2 with phosphate-buffered saline (PBS). Lymphocytes were separated on a Ficoll gradient (Pashing and Amersham), washed twice in PBS and suspended in culture medium containing 10% foetal calf serum (RPMI 1640 Lonza added with 0.33% L-glutamine, 1%non-essential amino-acids, 1% Na pyruvate and 1% penicillin-streptomycin). PBMCs were incubated without mitogen or in the presence of ConcavalinA (ConA, 5 $\mu g.ml^{-1}$), a T-cell-specific mitogen [21]. 2 x 10^5 cells were seeded in a final volume of 200 µl per well. Cells were then incubated for 72 h at 37 °C with 5% CO_2.

Viability of PBMCs. The viability and number of cells were determined before and after the incubation period with a Nucleocounter® (Chemometec). This integrated fluorescence microscope detects signals from the fluorescent dye, propidium iodide (PI), which binds to the nuclei of the cells with impaired plasma membranes.

First, the counting of non-viable cells was done, and then, the total number of cells was determined following a specific treatment, which permeated all plasma membranes. The number of viable cells was then obtained by subtraction of the number of dead cells from the total number of cells.

The cells were also stained with PI and Annexin V according to the manufacturer's recommendations (Annexin V : FITC Apoptosis Detection Kit I, BD Belgium) and analyzed by flow cytometry in order to determine the proportions of the different cell populations in early apoptosis, late apoptosis and necrosis, and non-apoptotic. The flow cytometric analysis was conducted with a FACSCanto™ II (Becton Dickinson Biosciences) flow cytometer.

Functional test. The actively proliferating lymphocytes were assayed with bromodeoxyuridine (BrdU) incorporation into newly synthesized DNA (BrdU Proliferation Assay, Calbiochem®). During the final 24 hours of culture, BrdU, a thymidine analog, was added to wells of the microtiter plate. After 72h of incubation, cells were fixed, permeabilized and the DNA denatured to enable antibody (Ab) binding to the incorporated BrdU. Detector anti-BrdU monoclonal Ab was pipetted into the wells and allowed to incubate for one hour, during which time it binds to any incorporated BrdU. Unbound Ab was then washed away and horseradish peroxidase-conjugated goat anti-mouse was added, which binds to the detector Ab. The horseradish peroxidase catalyzed the conversion of the substrate to a blue product which intensity was proportional to the amount of incorporated BrdU in the cells. The blue product was quantified by measuring the absorbance at 450 nm with a spectrophotometer (PowerWave X, Bio-Tek).

Trace elements. After being lyophilized, 200 mg of whole blood were digested in Teflon tubes with concentrated nitric acid, deionised water and H_2O_2 in a microwave oven (20 minutes between 0 and 600 Watt). After cooling, samples were diluted to 50ml with deionised water in a volumetric flask. Samples for Cd, Fe, Ni, Cu, Se, Pb, and Zn were analysed by Inductively Coupled Plasma-Mass Spectrometer (ICPMS, Elan DRC II). Samples for T-Hg were analysed by Direct Mercury Analyzer (DMA80, Milestones). Concentrations are expressed in $\mu g.g^{-1}$ dry weight (dw). Parallel to samples, a set of certified control material samples (DOLT-3 liver, National Research Council Canada and Seronorm™ Trace Element Whole Blood L-3, SERO AS) were repeated throughout each set of analyses to ensure method's accuracy and precision.

Organic contaminants. In all samples, 36 PCBs, 21 HO-PCBs, 10 PBDEs, 2 MeO-PBDEs, chlordanes and total DDT were targeted. The method for serum analysis was slightly adapted from the methods described previously[22]. A volume of serum (~ 1.5 ml) was spiked with internal standards (PCB 143, BDE 77 and 4'-HO-CB 159), diluted 1:1 with Milli Q water, mixed with formic acid, sonicated for 20 min and extracted using solid-phase extraction (SPE) cartridges (6 ml/500 mg Oasis HLB, Waters). Elution was done by 10 ml of MeOH:dichloromethane (DCM, 1:1). After evaporation to near dryness, the analytes were reconstituted in 500 µl hexane:DCM (1:1) and fractionated on silica SPE cartridges (3ml/500 mg, Varian) topped with 200 mg acid silica (25%, w/w). A first fraction containing PCBs and PBDEs was eluted with 5 ml hexane, while the phenolic compounds were eluted in a second fraction with 6 ml DCM (1:1, v/v). Both fractions were evaporated to dryness. The first fraction was reconstituted in 100 µl iso-octane, while the second fraction was derivatized for 30 min with diazomethane to form MeO-PCBs and MeO-PBDEs.
For the analysis of PBDEs and MeO-derivatives, a GC-MS operated in electron capture negative ionisation (ECNI) mode was equipped with a 30 m x 0.25 mm x 0.25 µm DB-5 capillary column (J&W Scientific). The MS was used in the SIM mode with two ions monitored for each MeO-PCB congener in specific windows, while ions m/z = 79 and 81 were monitored for MeO-PBDEs and for PBDEs during the entire run. For the PCB analysis, a GC-MS operated in electron impact ionisation (EI) mode was equipped with a 25 m x 0.22 mm x 0.25 µm HT-8 capillary column (SGE). The MS was used in the SIM mode with two ions monitored for each PCB homologue group in specific windows.

Quality Assurance/Quality Control (QA/QC). Multi-level calibration curves ($r^2 > 0.999$) in the linear response interval of the detector were created for the quantification. QC was performed by regular analyses of procedural blanks, by random injection of standards, spiked samples and solvent blanks. The quality control scheme is also assessed through regular participation to interlaboratory comparison exercises organized by AMAP (POPs in serum). Obtained values were deviating with < 20% from consensus values. For analytes detected in procedural blanks, the mean procedural blank value was used for subtraction.

After blank subtraction, the limit of quantification (LOQ) was set at 3 times the standard deviation of the procedural blank. For analytes not detected in procedural blanks (HO-PCBs and HO-PBDEs), LOQs were calculated for a signal-to-noise ratio = 10.

Statistical analysis. Principal Component Analysis (PCA) were conducted using Statistica 9.1. Differences between possible groups were detected using one-way ANOVA. The level of statistical significance was defined at $p < 0.05$.

Results and discussion:

Hematology. Granulocytes constituted the majority of the leucocyte population in whole blood (64.8%), followed by lymphocytes (29%), and monocytes (6.3%). These mean blood values remained within previously reported normal ranges for harbour seals[23,24].

Leucocytes were successfully isolated from whole blood samples of 13 seals, in which concentrations of trace elements and organic pollutants were measured. Lymphocyte proliferation in presence of ConA was demonstrated by flow cytometry and BrdU test analysis. Despite a strong inter-individual variability, lymphocytes showed strong stimulation responses (Figure 1).

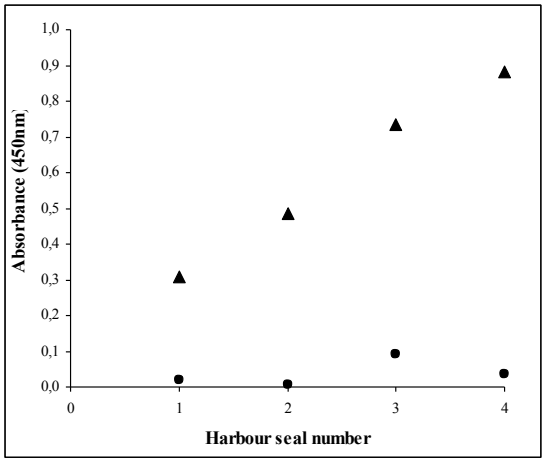

Figure 1. BrdU proliferation assay of harbour seal lymphocytes after 72h of incubation. The absorbance value at 450nm of ConA-stimulated lymphocytes of 4 individuals (harbour seal numbers 1-4) are represented by triangles (▲), and of control, non-stimulated cells, by circles (●).

Contaminants. T-Hg levels ranged from 0.66 to 1.36 $\mu g.g^{-1}$ dw in the blood of analysed seals. Other non-essential elements such as Cd and Pb remained low (< 0.0058 and 0.026 $\mu g.g^{-1}$ dw). These results are from the same order of magnitude as those previously published for seals from the Wadden Sea [2,3]. Profiles of PCBs of the 13 animals, CB 153>CB 138>CB 146> CB 187>CB 180, were in good agreement with previously published data for seals collected between 2006 and 2008 [1]. For PBDEs, BDE 47 was the predominant congener followed by BDE 100. Concentrations of sum PCBs (median: 52,401 pg/ml) were more than 150 times higher than levels of sum PBDEs (median: 326 pg/ml), and more than 6 times higher than concentrations of sum HO-PCBs (median: 5,028 pg/ml).

Analyses of *in vitro* immune responses and correlation patterns are still under process but preliminary results suggest no effect of trace elements and organic contaminant on the hematology profile. Other factors such as location, age (i.e. pups, yearlings, adults) and season have been shown to influence blood values [24]. Proliferative responses will be calculated for all seals and will be compared to pollutant concentrations. Further work must be conducted to determine whether a larger sample size can also support our preliminary findings and if specific pollutants are responsible for the decreased lymphocyte proliferation response.

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References

1. Weijs L, Das K, Siebert U, van Elk N, Jauniaux T, Neels H, Blust R, Covaci A (2009); *Environ. Int.* 35: 842-50.
2. Das K, Siebert U, Gillet A, Dupont A, Di-Poï C, Fonfara S, Mazzucchelli G, De Pauw E, De Pauw-Gillet M-C (2008); *Environ Health.* 7: 52
3. Griesel S, Kakuschke A, Siebert U, Prange A (2008); *Sci. Total Environ.* 392: 313-23
4. Mos L, Morsey B, Jeffries SJ, Yunker MB, Raverty S, De Guise S, Ross PS. (2006); *Environ. Toxicol. Chem.* 25: 3110-17
5. Neale JC, Kenny TP, Tjeerdema RS, Gershwin, ME. (2005); *Clin Dev Immunol.* 12: 91-97
6. Ross PS, Vos JG, Osterhaus ADME in *Toxicology of Marine Mammals,* (eds J.G. Vos, G. Bossart, M. Fournier, & T. O'Shea): 534-57 (Taylor and Francis Publishers, 2003).
7. Kakuschke A, Valentine-Thon E, Griesel S, Fonfara S, Siebert U, Prange A (2005); *Environ. Sci. Technol.* 39: 7568-75
8. Kakuschke A, Valentine-Thon E, Fonfara S, Griesel S, Siebert U, Prange A (2006); *Mar. Mamm. Sci.* 22: 985-96
9. Kakuschke A, Valentine-Thon E, Griesel S, Rosenberger T, Mundry R, Siebert U, Prange A (2008); *Mar. Pollut. Bull.* 56: 764-69
10. Pillet S, Fournier M, Measures LN, Bouquegneau J, Cyr, D (2002); *Toxicol. Appl. Pharmacol.* 185: 207-17
11. de Swart RL, Ross PS, Timmerman HH, Vos HW, Reijnders PJH, Vos JG, Osterhaus ADME (1995); *Clin Experim Immunol.* 101: 480-86
12. De Guise S, Martineau D, Béland P, Fournier M (1998); *J Toxicol Env Health.* 55: 479-93
13. Levin M, Leibrecht H, Mori C, Jessup D, De Guise S (2007); *Vet Immunol Immunopathol.* 119: 269-77
14. Ross PS, de Swart RL, Addison RF, van Loveren H, Vos JG, Osterhaus, ADME (1996); *Toxicology.* 112: 157-69
15. Pillet S, Lesage, V, Hammill, M, Cyr, DG, Bouquegneau, J-M Fournier, M. (2000); *Mar. Pollut. Bull.* 40: 921-27
16. Neale JC, Van De Water, JA, Harvey, JT, Tjeerdema, RS Gershwin, ME. (2002); *Dev Immunol.* 9: 215-21
17. Neale JC. Contaminant-induced immune alterations in the Pacific harbor seal, *Phoca vitulina richardsi*, of the central coast and San Francisco Estuary. (University of California, Davis, 2003).
18. Ross PS, Beckmen, KB Pillet, S. in *Toxicology of Marine Mammals,* (eds J.G. Vos, G. Bossart, M. Fournier, & T. O'Shea): 570-91 (Taylor and Francis Publishers, 2003).
19. Kakuschke A, Valentine-Thon E, Griesel S, Rosenberger T, Mundry R, Siebert U, Prange A (2008); *Mar. Pollut Bull*. 56: 764-69.
20. Lahvis GP, Wells RS, Kuehl DW, Stewart JL, Rhinehart HL, Via CS (1995); *Environ Health Perspect.* 103: 67-72
21. De Swart RL, Kluten RMG, Huizing CJ, Vedder LJ, Reijnders PJH, Visser IKG, UytdeHaag FGCM, Osterhaus ADME (1993); *Vet. Immunol. Immunopathol.* 37: 217-30
22. Weijs L, Das, K, Siebert, U, Neels, H, Blust, R Covaci, A. (2008); *Organohalogen Compounds.* 70, 837-40
23. De Swart RL, Ross PS, Vedder LJ, Boink FBTH, Reijnders PJH, Mulder PGH, Osterhaus ADME (1995); *Can J Zool.* 73: 2035-43
24. Hasselmeier I, Fonfara S, Driver J, Siebert U (2008); *Aquat. Mamm.* 34: 149-56