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T Lymphocyte-Proliferative Responses of a Grey Seal (*Halichoerus grypus*) Exposed to Heavy Metals and PCBs *in Vitro*

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Abstract

This study investigated *in vitro* the effects of methylmercury chloride (CH_3HgCl), zinc chloride ($ZnCl_2$), cadmium chloride ($CdCl_2$), lead acetate ($Pb(C_2H_3O_2)_2$), and PCBs (Aroclor mixtures) on the proliferation of T lymphocytes from the thymus, lymph node, and blood from one female grey seal (*Halichoerus grypus*) juvenile. After exposure to heavy metals, a dose-response curve was observed with a decrease in proliferation of both T lymphocytes from blood and the lymph node. Exposure to Aroclor mixtures led to a mostly reduced proliferation of thymocytes and T lymphocytes from the lymph node and blood. Lymph node cells seem less sensitive to heavy metals than peripheral blood lymphocytes. Lymph node lymphocytes are more sensitive to PCBs than peripheral blood lymphocytes but less than thymocytes. These results suggest that the sensitivity of T lymphocytes from one grey seal to contaminants may be due to inherent tissue/matrix differences in the sensitivity of these cells to contaminants; however, an individual response cannot be excluded in the present case. That is, samples from a single individual are not extrapolated to the species as a whole in this paper but discussed relative to exposure response by tissues.

Key Words: heavy metals, PCBs, grey seal, *Halichoerus grypus*, T lymphocytes, peripheral blood, immunotoxicity, thymus, lymph node

Introduction

The immune system of mammals is comprised of different organs and tissues that are functionally interdependent throughout the entire lifespan by means of the lymphatic system and blood. The thymus is the main central lymphoid organ and is responsible for the production of immunocompetent T cells, while lymph nodes and the spleen are secondary lymphoid organs where lymphocyte responses initiate and develop. There is a current debate about the potential adverse effect of environmental contaminants on the immune system and therefore on the health status of marine mammals (Ross, 2002). Many marine mammal species are at particular risk of immunotoxicity because of their high trophic level and long lifespan, resulting in the bioaccumulation of elevated levels of contaminants. In Eastern Canada, contaminants found in various marine mammal species include organochlorines (Bernt et al., 1999; Hobbs et al., 2002) and heavy metals (Wagemann et al., 1990).

In marine mammals, effects on immune function have been inferred from associations between organochlorine contaminant levels in the blubber or blood and changes in immune blood parameters and immune responses (Beckmen et al., 2003) or from associations with disease (Ross et al., 1995). Possible immunosuppression by other groups of environmental contaminants, such as heavy metals, may also occur. There are few studies in the literature on the immunotoxic effects of heavy metals (such as cadmium, zinc, mercury, and lead) in marine mammals (De Guise et al., 1996a; Pillet et al., 2000; Lalancette et al., 2003; Camara Pellisso et al., 2008).

Despite the growing body of literature on immunotoxic effects in harbour seals (*Phoca vitulina*), far less work has been carried out on grey seals (*Halichoerus grypus*). Few studies have investigated the effects *in vitro* of organic contaminants (Hammond et al., 2005) and heavy metals (Pillet et al., 2000; Lalancette et al., 2003) on grey seals. In order to be able to measure possible deleterious effects of environmental contaminants on the immune system of pinnipeds, a logical first step is to evaluate the proliferation of lymphocytes from different immune tissues

exposed to contaminants. The aim of the present study is to evaluate whether the mitogen-induced proliferative responses of T lymphocytes in peripheral blood and central and peripheral lymphoid tissues (thymus and lymph node) from an individual grey seal are altered after chemical exposure *in vitro*.

Materials and Methods

The female grey seal juvenile sampled in the present study was used in a previous experiment, which will be described briefly. In an experimental study on the biology of the lungworm (*Otostrongylus circumlitus*) (L. Measures, unpub. data), female grey seal pups born and weaned naturally after a 2-wk lactation period were captured in the Gulf of St. Lawrence in February 2000. The 3-wk-old animals were brought to the Maurice-Lamontagne Institute (Mont-Joli, Quebec) and held in captivity using similar husbandry as described in Gajadhar et al. (2004). Grey seals were cared for according to the Canadian Council on Animal Care with an experimental protocol approved by the Animal Protection Committee at the Maurice-Lamontagne Institute. Seals were exposed to *O. circumlitus* infective larvae and killed at intervals to follow lungworm development. One seal at approximately 4 mo of age and weighing 58.5 kg was euthanized by injection of Euthanyl® (MTC Pharmaceutical, Cambridge, Ontario, Canada) 8 d post-exposure to lungworm larvae. Before euthanasia, a blood sample, around 100 ml, was collected using heparinized Vacutainer tubes (Becton-Dickinson, NJ, USA) and kept at 4° C until further processing and analysis within 6 h post-sampling. At necropsy, because no worms were recovered, this seal was considered to be uninfected, and we decided to use tissues for an *in vitro* experiment. In this context, a thoracic lymph node and the thymus were collected and kept at 4° C in culture medium (RPMI 1640; Gibco BRL, Mississauga, Ontario, Canada) supplemented with 10% fetal calf serum (Gibco BRL), 100 U penicillin (Gibco BRL), and 10 mM Hepes (Gibco BRL), until further processing and analysis within 6 h post-sampling. It was assumed that no stress associated with captivity nor the failure to infect this seal with *O. circumlitus* had affected the seal's susceptibility to heavy metals and PCBs.

Cadmium chloride ($CdCl_2$) and zinc chloride ($ZnCl_2$) were purchased from Sigma Chemicals (Oakville, Ontario, Canada). Lead acetate ($Pb(C_2H_3O_2)_2$) was purchased from Fisher Scientific (Montreal, Quebec, Canada). Stock solutions were prepared in distilled water at a concentration of 10^{-2} M. Methylmercuric chloride (CH_3HgCl) was obtained from Ultra Scientific (North Kingston, Ontario, Canada) and was first dissolved in alcohol to achieve a stock solution of 10^{-2} M. Subsequent dilutions were performed with RPMI 1640 medium. The final concentration of alcohol did not exceed 0.5%. Equivalent volumes of alcohol were added to cells (vehicle controls). Aroclors 1016, 1221, 1248, 1254, 1260, 1262, and 1268 (Ultra Scientific) were dissolved at a concentration of 2,000 ppm in RPMI 1640 medium supplemented with dimethyl sulfoxide (10% DMSO) (Sigma Chemicals). Subsequent dilutions were performed with RPMI 1640 medium. The final concentration of DMSO did not exceed 0.025%. Equivalent volumes of DMSO were added to cells (vehicle controls).

Techniques used to isolate immune cells from immune organs and from peripheral blood were previously described in De Guise et al. (1996a) and in Pillet et al. (2002), respectively. The cells were counted and their viability assessed using acridine orange-propidium iodide (Sigma Chemicals) with a fluorescence microscope. Cell viability was always greater than 90%.

PBMCs, thymocytes, and lymph node lymphocytes adjusted to a concentration of 2.63×10^6 cells/ml (95 µl) were plated with 95 µl of concanavalin A (Con A), to which was added either 10 µl of vehicle control (distilled water, alcohol, or DMSO) or 10 µl of the contaminant to be tested (final concentration ranging from 10^{-9} to 10^{-3} M for heavy metals and from 1.57 to 100 ppm for Aroclor mixtures). Cells were incubated 48 h at 37° C and 5% CO_2 in 96-well tissue culture plates. The optimal mitogen concentration used to stimulate T lymphocytes was previously established at 5 µg/ml Con A. Each assay was plated in triplicate. After a 48-h incubation, 0.5 µCi (20 µl) of [^{3}H]-thymidine (6.7 Ci/mmol; ICN, Mississauga, Ontario, Canada) was added to each well, and plates were incubated for a further 18 h. DNA was collected on fiberglass filters with a Titerteck cell harvester, and the amount of radioactivity incorporated was evaluated with a Beckman β-scintillation counter. Raw data were expressed in disintegrations per minute (DPM). Results were presented as the percentage of the vehicle control. Only T lymphocytes were analyzed because B lymphocytes are absent in the thymus. For each analysis, the mean and standard deviation were determined on replicates for different chemicals.

Dose values for inhibition of 50% of the proliferation response (IC_{50}) (Table 1) indicate that no values for lymphocytes from blood exposed to Pb acetate or Aroclors 1016, 1221, 1248, 1254, 1260, 1262, and 1268 could be calculated because the proliferative response did not decrease below 50% for the range of tested concentrations. The same observation applied to thymocytes exposed

to Aroclor 1221 and lymph node lymphocytes exposed to Aroclor 1221, 1254, 1260, 1262, and 1268.

Results

The proliferation of T lymphocytes stimulated by Con A was significantly higher ($p < 0.05$) in blood than in both the thymus and lymph node. Moreover, the stimulation index for Con A was higher for peripheral blood mononuclear cells (PBMC) (109.3) than for T lymphocytes from the lymph node (38.4) and thymocytes (22.6).

The effects of exposure to heavy metals and PCBs on the proliferation of grey seal T lymphocytes are shown in Figures 1 and 2, respectively. The proliferation of grey seal lymphocytes—from blood and the lymph node—exposed to CH_3HgCl was reduced by ~90% at concentrations of 10^{-5} to 10^{-3} M. Zinc chloride inhibited the proliferative response of lymphocytes from blood and the lymph node by 24% and 56%, respectively, following *in vitro* exposures to concentrations of 10^{-4} and to 10^{-3} M. An inhibition by 36% of T cell proliferation with $CdCl_2$ occurred when blood cells were exposed to 10^{-6} up to 10^{-3} M and by 49% when lymph node cells were exposed to 10^{-5} up to 10^{-3} M. In both blood and the lymph node, the proliferative response of T lymphocytes exposed to Pb acetate was inhibited by ~30% at concentrations of 10^{-4} and 10^{-3} M.

For Aroclors 1016 and 1221, a dose-response inhibition of T cell proliferation was observed for the highest concentrations (50 and 100 ppm) in both blood and the lymph node and only at the highest concentration (100 ppm) for Aroclor 1221 in thymus. For Aroclor 1248, a dose-response inhibition of T cell proliferation by 10%, 40%, and 20% was observed following exposure to 12.5 ppm in blood, the lymph node, and thymus, respectively. In blood, the lymph node, and thymus, the proliferative response of T lymphocytes exposed to Aroclors 1254 was inhibited by 23%, 26%, and 34%, respectively, at highest concentrations (50 and 100 ppm). However, a concentration of 3.13 ppm induced a 27% increase of the proliferative response of lymph node cells. For Aroclor 1260, in thymus, a dose-response inhibition of T cell proliferation by 95% was observed for 50 ppm; and in blood and the lymph node, a dose-response inhibition of T cell

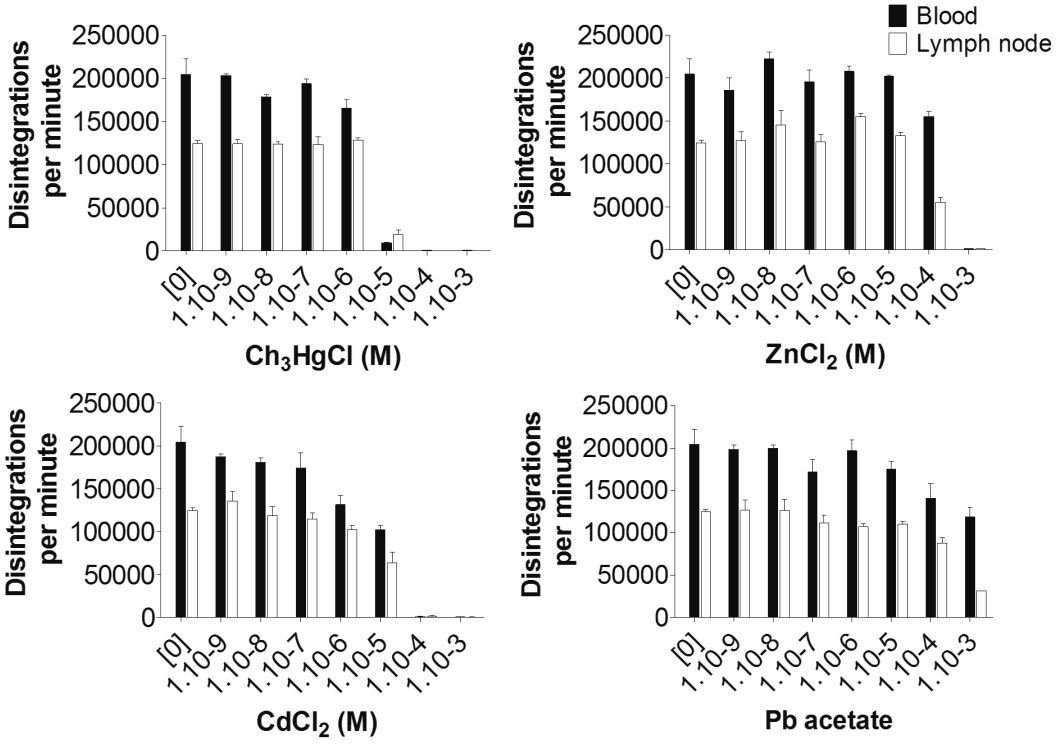

Figure 1. Proliferation assay of grey seal T lymphocytes—from the lymph node and blood—following a 48-h exposure to four metals (triplicate assay); lymphoblastic transformation stimulated by Con A is measured as radioactivity emitted by [^{3}H]-thymidine incorporated in cells in DPM. Results are presented as disintegrations per minute and given as mean ± standard error.

Table 1. Dose values for inhibition of 50% of the proliferative response (IC_{50}) to heavy metals and Aroclor mixtures in a grey seal

Metal	Organs, tissue	IC_{50}	PCB mixture	Organs, tissue	IC_{50}
MeHg	Blood	3.16×10^{-6} M	Aroclor 1016	Thymus	87.5 ppm
	Lymph node	2.18×10^{-5} M		Lymph node	77.0 ppm
			Aroclor 1248	Thymus	78.1 ppm
$ZnCl_2$	Blood	5.97×10^{-5} M		Lymph node	56.7 ppm
	Lymph node	6.00×10^{-4} M	Aroclor 1254	Thymus	64.6 ppm
			Aroclor 1260	Thymus	60.1 ppm
$CdCl_2$	Blood	1.78×10^{-5} M	Aroclor 1262	Thymus	70.3 ppm
	Lymph node	2.98×10^{-5} M	Aroclor 1268	Thymus	79.7 ppm
Pb acetate	Lymph node	5.67×10^{-4} M			

proliferation by 47% and by 35%, respectively, was observed for 100 ppm. However, concentrations of 6.25 and 12.5 ppm induced a ~30% increase of the proliferative response of lymph node cells. For Aroclor 1262, in thymus, a dose-response inhibition of T cell proliferation by 18% was observed for 25 ppm; and in blood and the lymph node, a dose-response inhibition of T cell proliferation by 38% and 23%, respectively, was observed for 100 ppm. A concentration of 1.57 ppm induced a ~25% increase of the proliferative response of lymph node cells. Aroclor 1268 induced an inhibition of T lymphocyte proliferation at 50 ppm in both thymus and blood by 26% and 33%, respectively, and at 100 ppm in the lymph node by 17%. An increase of T lymphocyte proliferation was observed in the lymph node by 33% at 12.5 ppm and in thymus by 30% at 1.57 ppm.

The calculated IC_{50} values (Table 1) for thymocytes exposed to PCB mixtures *in vitro* in terms of toxicity can be ranked as follows: Aroclor 1260 > 1254 > 1262 > 1248 > 1268 > 1016 > 1221.

Discussion

T-lymphocyte proliferations varied between the different immune organs or tissues used according to their source in this study, suggesting that T-lymphocyte proliferation is tissue/matrix dependent as previously demonstrated in beluga whales (*Delphinapterus leucas*) (De Guise et al., 1996b).

Mercury (Hg) is a unique element that, unlike many metals, has no essential biological function. In our experiment, proliferation of grey seal lymphocytes—from blood and the lymph node—was reduced by 10^{-5} M CH_3HgCl and higher. Lalancette et al. (2003) also found that lymphoblastic transformation of lymphocytes from blood in developing grey seals was reduced at a concentration of 10^{-5} M of CH_3HgCl.

Zinc (Zn) is essential in the development and function of the immune system in both humans and rodents, and is likely so in phocids, but little is known about the significance of different levels of Zn in marine mammal tissues (Das et al., 2003). Proliferation of lymphocytes from blood and the lymph node was inhibited by exposure to 10^{-4} M $ZnCl_2$ and higher. Similarly, in human and murine lymphocytes, DNA synthesis decreased at 5×10^{-4} M of Zn ions (Berger & Skinner, 1974) and 10^{-4} M of $ZnCl_2$ (Lawrence, 1981), respectively. The decrease in proliferation of T lymphocytes occurs after an exposure to Zn at concentrations similar to those found in the blood of grey seals (3,150 µg/L = 4.82×10^{-5} M) (Kakuschke et al., 2006).

Cadmium (Cd) is regarded as one of the most toxic heavy metals; however, few studies on its toxic effects have been conducted in marine mammals, despite high levels encountered in several species (Das et al., 2003). In the present study, an inhibition of the proliferative response was noted at 10^{-6} M (and higher) and 10^{-5} M (and higher) $CdCl_2$ in blood and lymph node lymphocytes, respectively. Proliferation of thymocytes and splenocytes from beluga whales was similarly reduced after exposure to 10^{-5} M $CdCl_2$ (De Guise et al., 1996a).

Although the main target for lead (Pb) toxicity appears to be the nervous system, immunotoxic effects have also been reported (Fischbein et al., 1993). In our study, exposure to the highest concentrations (10^{-4} and 10^{-3} M) of $Pb(C_2H_3O_2)_2$ decreased lymphoblastic proliferation of lymphocytes from blood and the lymph node. Although, De Guise et al. (1996a) reported that proliferation of beluga splenocytes and thymocytes was not affected by 10^{-4} M of lead chloride ($PbCl_2$), alterations in mitogenic responses occurred in rat splenocytes at high concentrations (from 10^{-5} to 10^{-3} M) of lead nitrate ($Pb(NO_3)_2$) (Lang et al., 1993).

Polychlorinated biphenyls (PCBs) are highly stable, complex chemicals, most often found in mixtures called Aroclors that due to improper disposal practices have resulted in their wide distribution throughout the environment. Seven

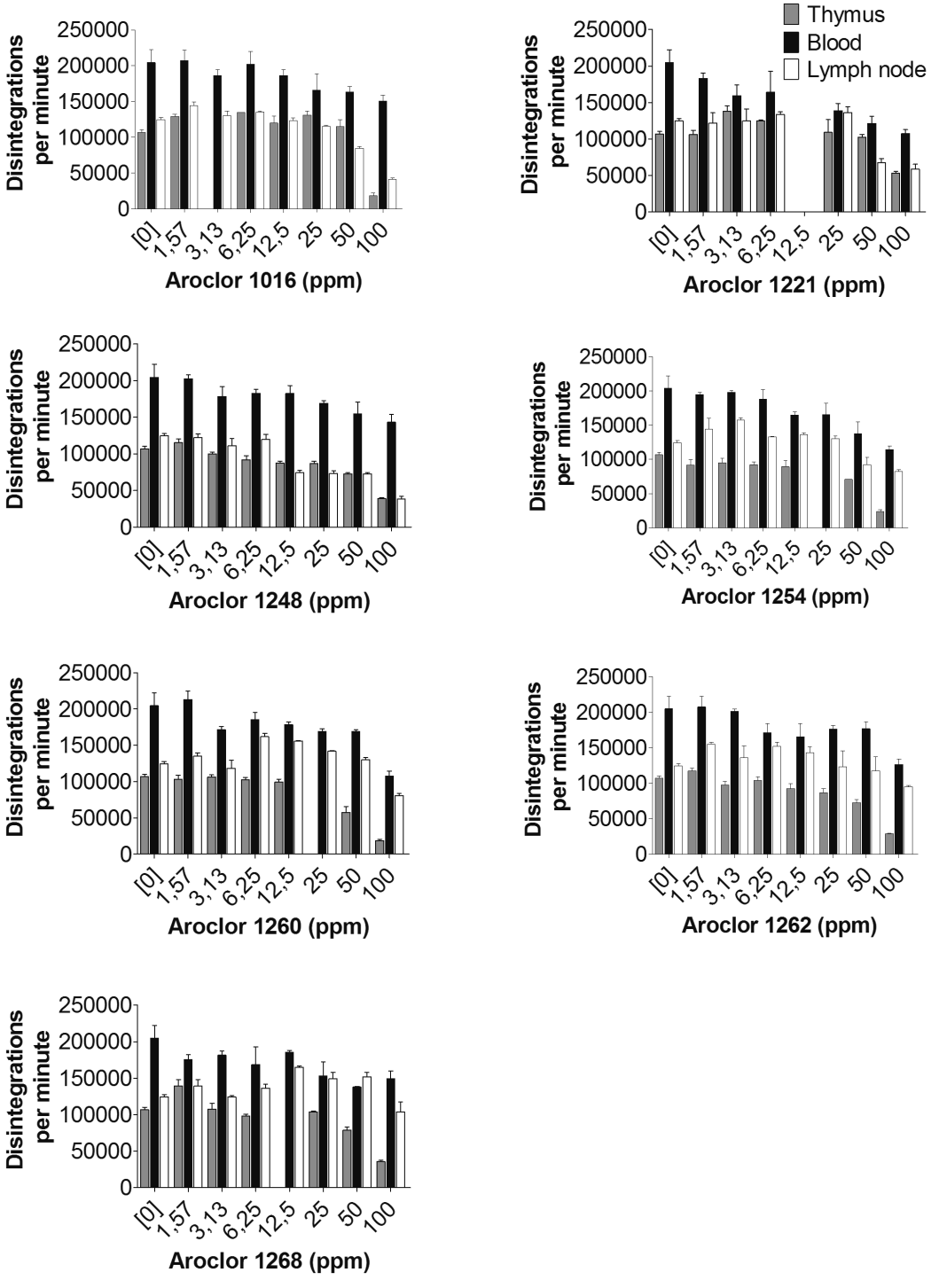

Figure 2. Proliferation assay of grey seal T lymphocytes—from the thymus, lymph node, and blood—following a 48-h exposure to seven Aroclor mixtures (triplicate assay); lymphoblastic transformation stimulated by Con A is measured as radioactivity emitted by [^{3}H]-thymidine incorporated in cells in DPM. Results are presented as disintegrations per minute and given as mean ± standard error.

commercial PCB mixtures, Aroclors 1016, 1221, 1248, 1254, 1260, 1262, and 1268, decreased T-lymphocyte proliferation. However, a proliferative response of T lymphocytes from the lymph node occurred after exposure to Aroclors 1254, 1260, 1262, and 1268 and from the thymus after exposure to Aroclor 1268. Levin et al. (2005) reported that PCBs might also increase the proliferative response of lymphocytes in harbour seals. Therefore, immunotoxic compounds might both suppress and stimulate lymphocytes, but stimulation could reflect a response of only the most immunocompetent cells or the effects of hormesis at low contaminant concentrations. The IC_{50} value for thymocytes was lower than that for lymphocytes from blood and the lymph node (Table 1), suggesting a higher sensitivity to PCB exposure in thymocytes, which may indicate a higher susceptibility of immature thymocytes. The thymus normally processes immature precursor T lymphocytes into the mature immunocompetent T cells of the medulla. A loss of thymocyte production following *in vivo* exposure to contaminants has been demonstrated to Pb in organ pathologies (Handy et al., 2002). The observations and results from this study tend to confirm the results of Beineke et al. (2005), which demonstrated that in the harbour porpoise (*Phocoena phocoena*), lymphoid depletion in the thymus was associated with elevated PCB levels.

Lahvis et al. (1995) indicated that a reduced Con A-induced lymphocyte proliferation in bottlenose dolphins (*Tursiops truncatus*) was correlated with increasing whole blood concentrations of tetrachlorinated to octachlorinated biphenyls. Moreover, in C57BL/6 mice, the more highly chlorinated Aroclors (1260, 1254, and 1248) were more toxic than the lower-chlorinated Aroclors (1242 and 1016) (Davis & Safe, 1989). In this study, tri- and tetra-chlorinated biphenyls predominate in Aroclors 1016 and 1221, tetra- and penta-chlorinated biphenyls predominate in Aroclor 1248, penta- and hexa-chlorinated biphenyls predominate in Aroclor 1254, hexa- and hepta-chlorinated biphenyls predominate in Aroclor 1260 and 1262, and octa-chlorinated homologues were most abundant in Aroclor 1268. The calculated IC_{50} values for thymocytes exposed to PCB mixtures *in vitro* in terms of toxicity can be ranked as follows: Aroclor 1260 > 1254 > 1262 > 1248 > 1268 > 1016 > 1221. These study results seem to suggest that toxicity is not linked to chlorination.

In conclusion, these results suggest that in this individual grey seal, T-lymphocyte proliferation is tissue/matrix dependent. Lymph node cells seem less sensitive to heavy metals than peripheral blood lymphocytes. Lymph node lymphocytes are more sensitive to PCBs than peripheral blood lymphocytes but are less sensitive than thymocytes. Although an immunotoxic effect of the substances has been demonstrated *in vitro* and *in vivo* in other species, especially in laboratory rodents, an individual response possibly not representative of the species as a whole cannot be excluded in the present case. In future studies, access to greater numbers of animals for sampling should be a goal; sample collection from commercially harvested seals could be a good alternative to sacrificing small numbers of seals in order to advance our understanding of the seal immune system and the toxic nature of some important contaminants.

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