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Effects of Organochlorines, Individually and in Mixtures, on B-Cell Proliferation in Marine Mammals and Mice

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Organochlorines (OC) are lipophilic and stable, and therefore accumulate in tissues of top predators, such as marine mammals. While the immunomodulatory effects of individual OC have been studied in lab animals, their effects in other species (such as marine mammals) and the possible interactions between chemicals in mixtures are not well understood. This study investigated the immunomodulatory effects of four polychlorinated biphenyls (PCB, IUPAC numbers 138, 153, 169, and 180), as well as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), individually and in mixtures, in marine mammals and mice. Mitogen-induced B lymphocyte proliferation was mostly modulated by non-coplanar PCBs, for which general mechanisms underlying toxicity are poorly understood. Simple additive effects of OC in mixtures were found only in mice, while both synergistic and antagonistic interactions between OC were found in marine mammals. The toxic equivalency (TEQ) approach, which is currently used to assess the dioxin-like toxicity of OC mixtures, failed to predict immunotoxicity in mice and marine mammals, likely due to the complexity of interactions between OC and effects via dioxin-independent pathways. The commonly used mouse model failed to predict the immunotoxicity due to OC in the marine mammals tested. In addition, clustering data suggested that phylogeny might not help predict the toxicity of OC. Lymphoproliferative response was modulated in most species tested suggesting the possibility of increased susceptibility to infectious diseases in these animals. These findings may be helpful in more accurately characterizing the immunotoxic potential of OC in different target species and help in more relevant risk assessment.

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Organochlorines (OC), including polychlorinated biphenyls (PCB), were produced in the United States and other countries over a 50-yr period from the 1930s. As a result of improper disposal practices and unintentional releases, OC are now ubiquitous environmental contaminants. The lipophilicity of OC and with the resistance to metabolism and excretion facilitate bioaccumulation of these chemicals in organisms (Kidd et al., 1998; Ross et al., 2000; Safe et al., 1985). Investigations are needed to better characterize the toxicological effects of OC due to ample evidence of OC exposure in various organisms.

The well-documented immunotoxicity of OC (Safe, 1993) is an important consideration in the assessment of potential adverse health effects since the immune system plays a central role in the defense against pathogens (Krzystyniak et al., 1995). Adverse effects upon exposure to OC were reported for both the adaptive and the innate immune system in experimental animals (Davis & Safe, 1989; Smithwick et al., 2003; Stack et al., 1999; Tryphonas, 1994; Tryphonas et al., 1991; Vos & Luster, 1989). The immunomodulatory effects of OC were also investigated in free-ranging animals. For instance, there was a negative correlation between mitogen-induced lymphocyte proliferation and OC blood levels described in wild adult bottlenose dolphins (Lahvis et al., 1995), and positive correlations between B- and T-lymphocyte proliferation and total PCB concentrations in blubber in harbor seal pups (Levin et al., 2005a). The high incidence of neoplasms, pneumonia, opportunistic infections, and mastitis in beluga whales in the

St. Lawrence Estuary suggested impaired immune responses due to high concentrations of OC (De Guise et al., 1995, 1998; Martineau et al., 1994). In addition, it was suggested by many that PCB might have compromised the immune system in striped dolphins and harbor seals, contributing to the morbillivirus mass mortalities. In a semi-field study, captive harbor seals fed highly contaminated fish were compared to seals fed less contaminated fish. Harbor seals with a highly OC-contaminated diet had several impaired immune system functions including (1) decreased mitogen- and antigen-induced lymphocyte proliferation, (2) reduced mixed lymphocyte reaction, (3) suppressed natural killer cell activity, and (4) diminished delayed-type hypersensitivity and antibody responses to ovalbumin (de Swart et al. 1994; Ross et al., 1995, 1996).

Numerous studies investigated the effects of individual contaminants, but only a handful of studies examined the effects of contaminants in mixture (De Rosa et al., 2004). When mice were exposed to dieldrin and carbofuran individually, immune functions were suppressed (Flipo et al., 1992). However, when these insecticides were tested in mixture, the immune parameters returned to either control or above-control values, indicating antagonistic interactions between these insecticides on the immune responses (Flipo et al., 1992, Vial et al., 1996). Chitosan and copper individually induced a local inflammatory acute phase response by stimulating production of total ceruloplasmin and lysozyme levels as well as phagocytic ability in healthy carp, but the mixture of the two produced a reduced effect compared to the effect that the compounds induced individually (Dautremepuits et al., 2004). Mixtures of OC produced only minor effects on immune function (decreased proliferation of splenic T cells), reproductive hormone levels, or general indices of reproductive function in sexually mature male rats, suggesting that mixtures of OC do not exert an adverse effect on immune function or reproductive physiology in male rats (Wade et al., 2002), although individual OC compounds are known to produce adverse effects on immune function and reproductive physiology. Mixtures of OC compounds exerted complex interactions (antagonistic and/or synergistic) on two innate immune functions, phagocytosis and respiratory burst, when tested in marine mammals (Levin et al., 2005c, 2007). The findings with regard to the biological effects and interactions of compounds in mixtures are neither consistent nor well understood, despite the fact that it represents "real-life" environmental exposure.

The toxic equivalency (TEQ) approach was developed to estimate the toxicity of OC mixtures, as the sum of the relative (dioxin-like) toxicity of its components. This conventional TEQ approach was utilized to facilitate risk assessment and regulatory control of exposure to OC mixtures (Van den Berg et al., 1998). The TEQ approach was successful at predicting the toxicity of real-world as well as synthetic mixtures, suggesting mostly additive interactions between chemicals. However, since this approach assumes that the effects of compounds are additive, it may fail to predict the toxicity of compounds in mixture when nonadditive effects such as synergistic and antagonistic interactions are involved.

To understand the interactions of OCs in mixture as well as to investigate the ability of the conventional TEQ approach to predict immunotoxicity, this study investigated the effects of OCs, individually and in mixtures, on LPS-induced B-cell proliferation. Our study also tested differences between species and the ability of the mouse model to predict effects in several marine mammal species.

MATERIALS AND METHODS

Animals

Blood samples from captive marine mammals were obtained opportunistically from several facilities (Mystic Aquarium, Mystic, CT; Sea World, San Diego, CA, and San Antonio, TX; U.S. Navy Marine Mammal Program, San Diego, CA; California Department of Fish and Game, Santa Cruz, CA). Blood samples were also obtained from wild harbor seals (Fisheries and Oceans Canada; Sidney, BC, Canada). The following species were tested: pilot whale (*Globicephala melaena*), beluga whale (*Delphinapterus leucas*), and bottlenose dolphin (*Tursiops truncatus*) for cetaceans, harbor seal (*Phoca vitulina*) and Northern fur seal (*Callorhinus ursinus*) for pinnipeds, and sea otter (*Enhydra lutris*) for mustelidae. While previous exposure to OCs was not quantified in the animals tested, the study design (comparison of different exposures to the unexposed cells of the same animal that are used as control) accounts for previous exposure and any other potential confounding factor.

Female B6C3F1 mice (*Mus musculus*), the most commonly used model in immunotoxicology (Luster et al., 1992), were obtained from Charles River Laboratories, MA. Mice were maintained at 18–26°C with relative humidity between 40 and 70%, and a light/dark cycle at 12-h intervals. Animals were housed 5 mice per cage containing sawdust (hardwood) bedding and sampled at 2 to 4.5 mo of age. For quality control, B6C3F1 mice were tested simultaneously with each marine mammal species (see later discussion).

Chemicals and Reagents

Cells were cultured in complete culture medium, consisting of Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with 1 m*M* sodium pyruvate, 100 μ*M* nonessential amino acids, 25 m*M* HEPES, 2 m*M* L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (all from Gibco BRL), along with 10% fetal bovine serum (Hyclone, Logan, UT).

PCB IUPAC 138, 153, 169, and 180 (purity >98.4%) and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD, purity >98%) were purchased from Ultra Scientific (North Kingston, RI). Non-coplanar PCB IUPAC 138, 153, and 180 were chosen because they are the most abundant PCB congeners found in

tissues of St. Lawrence beluga whales and other marine mammal populations (Muir et al., 1990; Tanabe, 1988). PCB 169 was chosen because it is a coplanar congener that is known to be highly toxic (Hennig et al., 2002). TCDD was chosen because it is the most potent immunotoxicant of the halogenated aromatic hydrocarbons (Holsapple et al., 1991). PCB, resuspended in dimethyl sulfoxide (DMSO; Sigma, St Louis, MO), and TCDD, purchased in toluene, were used to prepare working solutions in complete culture medium. PCB congeners and TCDD were tested at concentrations of 5 ppm and 0.05 ppb, respectively, when tested individually, or that same concentration for each component when tested in mixtures (for example, PCB 138 + 153 included 5 ppm of PCB 138 and 5 ppm of PCB 153). The concentrations used are environmentally relevant (within the range found in marine mammal tissues) and when tested individually did not significantly affect the proliferation of beluga whale lymphocytes (De Guise et al., 1998). All 26 possible combinations containing 2, 3, 4, and 5 of the OC compounds were tested along with individual chemicals and unexposed control cells. The final DMSO and toluene concentrations did not exceed 0.4 and 0.001%, respectively, and these concentrations of DMSO did not affect B-cell proliferation in mice or beluga whales.

Sample Collection and Lymphocyte Isolation

Blood samples from marine mammals were collected into heparinized tubes (Vacutainer, Becton Dickinson, Franklin Lakes, NJ). The blood was kept cool on ice and processed within 1 d after collection. Whole blood was centrifuged for 20 min at 220 × g, and the buffy coat was collected and resuspended into complete culture medium. The peripheral blood mononuclear cells (PBMC) were isolated by density-gradient centrifugation on Ficoll-Paque plus (Amersham Biosciences, Uppsala Sweden) for 35 min at 990 × g. The mononuclear cells were resuspended in complete culture medium, washed twice, and enumerated with their viability assessed before exposure to OCs using the exclusion dye trypan blue.

B6C3F1 mice were euthanized by CO_2 inhalation followed by cervical dislocation for assurance of death as approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Connecticut. The spleen was harvested aseptically from each animal, and a single-cell suspension was prepared in complete culture medium using two pairs of forceps. Mononuclear cells were isolated by density gradient centrifugation on Ficoll-Paque plus for 15 min at 720 × g. The mononuclear cells were resuspended in complete culture medium, washed twice, and enumerated with their viability assessed before exposure to OC using the exclusion dye trypan blue.

Lipopolysaccharide (LPS) Induced Lymphocyte Proliferation

In vitro lipopolysaccharide (LPS)-induced B-cell proliferation is one of the assays validated by the National Toxicology Program to assess immunotoxicity (Luster et al., 1988). This assay was performed according to standard methods (Brousseau et al., 1999; Dean et al., 1987; Pallardy et al., 2000; Smialowicz, 1995) as used before in marine mammal immunology and immunotoxicology (De Guise et al., 2003). LPS was also chosen since it is a T-cell-independent polyclonal stimulator of B-cell proliferation through its interaction with the CD14 surface protein found on B cells (Raetz, 1993). Briefly, mononuclear cells in complete culture medium were plated in triplicate (2×10^5 cells/well) in 96-well flat-bottom tissue culture plates (Falcon, Becton Dickinson). Mononuclear cells from each animal were stimulated with 0.05 μg/ml purified *Escherichia coli* 0111:B4 LPS (Sigma) with and without exposure to individual or mixtures of OC. Cells were incubated at 37°C with 5% CO_2 for a total of 66 h. LPS-induced lymphocyte proliferation was evaluated as the incorporation of 5-bromo-2′-deoxyuridine (BrdU), a thymidine analogue, added for the last 18 h of incubation, and further detected with a monoclonal antibody and a colorimetric enzymatic reaction (cell proliferation enzyme-linked immunosorbent assay [ELISA] BrdU (colorimetric), Roche Diagnostics, Alameda, CA) as per manufacturer's instructions using an ELISA plate reader (Multiskan EX v.1.0) at 690 nm with a reference wavelength of 450 nm. The suboptimal concentration of LPS used was determined in preliminary experiments as one inducing significant (compared to unstimulated control) but clearly suboptimal (compared to the concentration providing optimal stimulation) proliferation in the species tested. Further toxicity testing was performed upon LPS stimulation, and did not include the use of unstimulated cells, as the study design compares the effects of OC on proliferation to unexposed (but LPS-stimulated) cells from the same individuals (repeated measures study design, see below).

The conditions used for LPS-induced B lymphocyte proliferation assay were not optimized (time, LPS concentration, etc.) for each species, but the same conditions were used for all species tested, since differences in experimental conditions would not have allowed to determine whether differences in proliferation represented differences in susceptibility between species or differences attributable to differences in testing conditions.

Cell Viability Upon Exposure to OC

Viability of splenocytes was measured after a 66-h exposure to OC by adding 0.05 mg/ml of propidium iodide (Molecular Probes, Eugene, OR) to the cell suspension and quantifying fluorescence at 630 nm (FL-3) using a FACScan (Becton Dickinson, Rutherford, NJ) flow cytometer and the Cell Quest software (Becton Dickinson Immunocytometry System, San Jose, CA).

TEQ Value

In order to test whether the TEQ approach could accurately predict the toxicity of mixtures, TEQ values were determined

following Van den Berg et al. (1998), as later revised (Van den Berg et al., 2006) (Table 1, second column). Briefly, TEQ values were determined as the sum of the toxicity of each component of the mixture [TEQ=$\sum$ (TEF × [OC])], where TEF estimates the toxicity of a compound relative to TCDD.

Statistical Analysis

For B-cell proliferation, the optical densities (OD) were read directly from the ELISA plate reader and the triplicates for each OC/OC mixture for each animal were averaged. For quality control, the ODs from all mice were run on SPSS for Macintosh statistical package (v. 8.0, Chicago) to detect outliers amongst each variable from box plots. This was performed to detect and eliminate experiments for which the variability was greater than expected for any technical reason. This quality control program would ensure that technical errors on one given day would not translate in misinterpretation of the data for several individuals from any given marine mammal species run on that day as changes specific to that species (as the

TABLE 1

Immunomodulatory Effects of in Vitro Exposure to Ocs on LPS-induced B Cell Proliferation in Marine Mammals and Mice (% Change from Unexposed Control). PCBs and TCDD were Tested at 5 ppm and 0.05 ppb each, Respectively, Individually or in Mixtures

	TEQ (ng/g)	B6C3F1 Mouse (N = 65)	Beluga whale (N = 18)	Pilot whale (n = 5)	Bottlenose dolphin (N = 10)	Harbor seal (N = 11)	Northern Fur Seal (n = 13)	Sea otter (n = 10)
PCB 138	0	↓ (22%)						
PCB 153	0	↓ (16%)						
PCB 169	150	↓ (17%)						
PCB 180	0	↓ (29%)						
TCDD	0.05	↓ (10%)						
138+153	0	↓ (34%)		↑ (131%)				
138+169	150	↓ (31%)						
138 + 180	0	↓ (41%)						
138 + TCDD	0.05	↓ (11%)						
153 + 169	150	↓ (33%)		↑ (117%)				
153 + 180	0	↓ (45%)		↑ (155%)	↑ (58%)	↑ (44%)	↑ (45%)	
153 + TCDD	0.05	↓ (22%)						
169 + 180	150	↓ (45%)						
169 + TCDD	150.05	↓ (25%)						
180 + TCDD	0.05	↓ (23%)		↑ (132%)	↑ (61%)	↑ (29%)		
138 + 153 + 169	150	↓ (61%)						
138 + 153 + 180	0	↓ (70%)						
138 + 153 + TCDD	0.05	↓ (33%)			↑ (48%)		↑ (45%)	
138 + 169 + 180	150	↓ (61%)					↑ (42%)	
138 + 169 + TCDD	150.05	↓ (40%)						
138 + 180 + TCDD	0.05	↓ (40%)						
153 + 169 + 180	150	↓ (57%)			↑ (51%)		↑ (49%)	
153 + 169 + TCDD	150.05	↓ (28%)			↑ (46%)		↑ (42%)	
153 + 180 + TCDD	0.05	↓ (42%)					↑ (40%)	
169 + 180 + TCDD	150.05	↓ (39%)						
138 + 153 + 169 + 180	150	↓ (77%)						
138 + 153 + 169 + TCDD	150.05	↓ (52%)					↑ (43%)	
138 + 153 + 180 + TCDD	0.05	↓ (68%)						
138 + 169 + 180 + TCDD	150.05	↓ (51%)						
153 + 169 + 180 + TCDD	150.05	↓ (68%)						
138 + 153 + 169 + 180 + TCDD	150.05	↓ (77%)						

↓ significant decrease, $p<0.05$.
↑ significant increase, $p<0.05$.
no significant difference, $p>0.05$.

mouse data would be different from the other "normal" data from mice in other experiments). If only one outlier data point in mice (beyond an inner fence on either side, as determined objectively by the computer software) was found for a particular variable (unexposed control, individual OC or OC mixture), it was eliminated as well as the marine mammal data for the same variable (unexposed control, individual OC or OC mixture) processed on the same day as part of the same experiment. If two or more outliers were detected in a given mouse, the data from that mouse (for all 32 variables) were rejected as part of our quality control program, and eliminated from the data set as well as all the data from marine mammals tested on the same day as part of the same experiment. For each species, using all animals (some animals may have been tested more than once at different points in time), a repeated-measure one-way analysis of variance (RM ANOVA) with Dunnett's test was used to compare the different experimental exposure groups to the unexposed controls, since the same cells were divided into 31 exposure groups and 1 unexposed control, providing an intrinsic control for each individual animal. Therefore, the effects of a treatment were determined compared to the unexposed cells of each individual in a species. Sample size was increased for each species until the statistical power as suggested by the statistical software was sufficient (>0.8) to be confident with our negative results. RM ANOVA was also used to determine the effects of OC on cell viability in mice.

In determining whether the TEQ values could accurately predict toxicity, OC treatments were grouped according to their TEQ value and compared using one-way ANOVA. Pearson correlation analysis was used to compare the measured (observed) reduction in proliferation to that calculated as the sum of the response measured for its individual congeners. Forward stepwise regression was used to determine which congeners significantly contributed to changes in lymphocyte proliferation. The relative changes in lymphocyte proliferation for each species, compared to unexposed control, were used as the dependent variables and the concentrations of each congener (ppm) were used as the independent variables. Results are reported as the equation that explains most of the variability using only the independent variables that contributed significantly. RM ANOVA, one-way ANOVA, correlation, and regression analysis were evaluated using the SigmaStat Windows 1.0 (Jandel Scientific, San Rafael, CA) software, using $p < .05$ for statistical significance.

Clustering Analysis

Dendrograms exhibiting relationships between species and OC treatment groups were generated through hierarchical clustering of data based on Euclidean metric distance and average linkage clustering using Multiexperiment Viewer software (MeV; available from the Institute for Genomic Research, http://www.tm4.org/mev.html).

RESULTS

Effects of OC on B-Cell Proliferation

The immunomodulatory effects of OC on B-cell proliferation in marine mammals and mice are summarized in Table 1. In mice, in vitro exposure to all the mixtures, as well as individual OC, induced significant decreases in lymphocyte proliferation.

In marine mammals, in vitro exposure to OC exerted no effect on beluga whale or sea otter lymphocyte proliferation, while some mixtures stimulated an increase in lymphocyte proliferation in pilot whales, bottlenose dolphins, harbor seals, and Northern fur seals. Individual compounds and a mixture of all five OC failed to modulate B-cell proliferation in marine mammals. Ten OC mixtures produced significant effects on marine mammal B-cell proliferation, and five of the mixtures affected more than one species of marine mammals. In pilot whales, four mixtures significantly increased (117–155%) lymphocyte proliferation. In bottlenose dolphins, five mixtures including PCB 153, TCDD, or both significantly elevated (46–61%) lymphocyte proliferation. In harbor seals, two OC mixtures that included PCB 180 significantly increased lymphocyte proliferation. In Northern fur seals, seven OC mixtures that included PCB 153, PCB 180 or both significantly elevated lymphocyte proliferation.,

Cell Viability Upon Exposure to OC

No significant change in splenocyte viability was detected following exposure to OC (data not shown).

Regression Analysis

Forward stepwise regression analysis was utilized in species for which significant effects were detected to determine which OC significantly contributed to the changes in lymphocyte proliferation. The regression equations and adjusted R^2 (adj. R^2) values are summarized in Table 2. In mice, the 3 non-coplanar PCBs and 1 coplanar PCB 169 explained 23% (adj. R^2) of the changes in lymphocyte proliferation. In marine mammals, OC explained only 2–5% of the changes in B-cell proliferation. In cetaceans, one non-coplanar PCB (PCB 138) explained changes in lymphocyte proliferation in pilot whales, while there was no significant equation for bottlenose dolphins. In the two pinniped species tested, variability in B-cell proliferation was also explained by non-coplanar PCB (PCB 138 and/or 153).

Interactions of OC in Mixture

In mice, a significant positive correlation was detected between the predicted effect of OC mixtures, as calculated by adding the effects of their components, and the effect measured upon experimental exposure for the 26 OC mixtures (Figure 1a). The correlation provided a relatively good prediction of the effects of mixtures from that of their components with a high

TABLE 2
Forward Stepwise Regression Analysis to Predict Changes in B Cell Proliferation Compared to Unexposed Control (ΔP)

Species	Regression equation (difference in proliferation compared to control)	Adjusted R^2
Mouse	ΔP = –0.03 (PCB 138) –0.03 (PCB 153) –0.03 (PCB 169) –0.05 (PCB 180) + 0.3	0.23
Pilot whale	ΔP = –0.03 (PCB 138) + 0.64	0.02
Bottlenose dolphin	no significant equation	–
Harbor seal	ΔP = –0.02 (PCB 138) + 0.02 (PCB153) + 0.06	0.05
Northern Fur Seal	ΔP = 0.02 (PCB 153) + 0.14	0.02

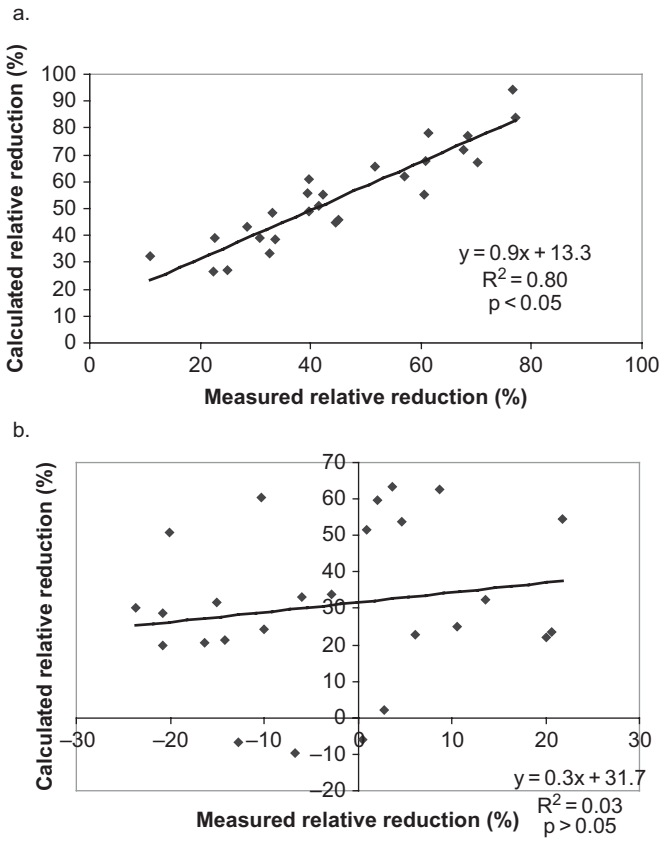

FIG. 1. Correlation analysis between the calculated relative reduction (%) in B-cell proliferation (calculated as the sum of the effects of individual components of a mixture compared to control) and measured relative reduction (%) compared to control for all 26 OC mixtures. A significant positive correlation was detected in mice (a), suggesting additive effects of OC in mixtures. No significant correlation was detected in beluga whales (b), suggesting nonadditive effects of OC in mixtures.

R^2 (.8) and a slope close to 1 (0.9). No significant correlations were detected in beluga whales (Figure 1b) as well as for the other marine mammals tested (data not shown).

Predicting Toxicity Utilizing TEQ Values

The TEQ approach was utilized to predict the toxicity of OC mixtures relative to TCDD. TEQ values, however, could not accurately predict the effects on B-cell proliferation in any of the species tested, since higher TEQ values did not correspond with enhanced toxicity, measured as an increase or a decrease in B-cell proliferation compared to control (Table 1). Additionally, no significant difference was detected between the OC treatments that were grouped according to their TEQ value in beluga whales (Figure 2), mice, or other marine mammals tested (data not shown).

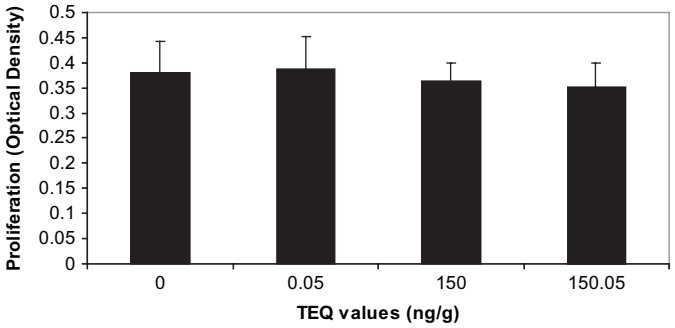

FIG. 2. LPS-induced B-cell proliferation (expressed as mean optical density ± SD) for OC treatments grouped according to their TEQ values. No significant difference between the four groups in beluga whales were detected, suggesting that TEQ values did not accurately predict the immunomodulatory effects on B-cell proliferation upon in vitro OC exposure.

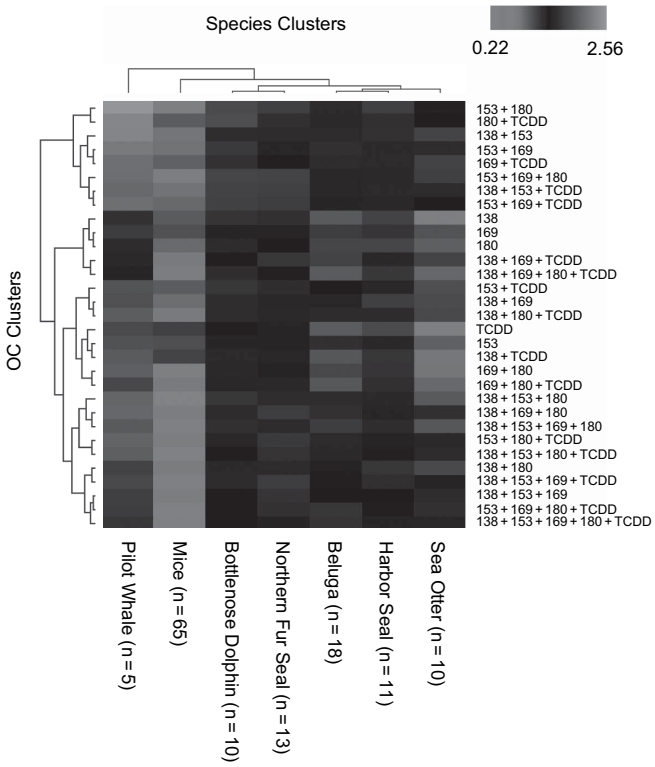

FIG. 3. Dendrogram representing clustering by species and OC based on modulation of B-cell proliferation compared to unexposed control. Brighter shades of green indicate greater reduction of lymphocyte proliferation compared to control, brighter shades of red indicate enhancement of lymphocyte proliferation compared to control, and black indicates no changes compared to control.

Clustering of Species and OC According to Their Immunomodulatory Effects

Figure 3 illustrates how marine mammals and mice clustered based on relative changes in LPS-stimulated B-cell proliferation upon exposure to OC compared to controls. Northern fur seals and bottlenose dolphins clustered together, and beluga whales, harbor seals, and sea otters clustered together. Mice did not cluster closely with marine mammal species, but pilot whales clustered furthest from all the species tested. It is noteworthy that species did not group according to phylogeny. OC, individually or in mixtures, clustered without any obvious pattern when all species were taken into consideration.

DISCUSSION

Our study demonstrated a marked (up to 77%) suppression of mouse B-lymphocyte proliferation upon exposure to all OC and OC mixtures tested. This suppression appeared to mostly result from additive interactions between congeners (see Figure 1a), and to be at least in part predicted by the concentration of PCB in the mixture (see Table 2). The reduction of lymphocyte proliferation seen in mice was not the result of a cytotoxic effect due to OC, since viability was not affected by OC exposure, but rather the OC affected the ability of lymphocytes to proliferate. These results are not surprising in view of the broad immunosuppressive characteristics of organochlorines in general and of PCB in particular upon testing in lab animals (Brouwer et al., 1989; Kunita et al., 1985; Loose et al., 1977; Thomas & Hinsdill, 1978; Vos & Luster, 1989). While the biochemical targets and mechanisms responsible for immune modulation induced by OC are still under investigation, Smithwick et al. (2004) demonstrated that the inhibition of LPS-induced splenocyte proliferation in C57B1/6 mice was driven by *ortho*-substituted (non-coplanar) PCB congeners and that those compounds, unlike non-*ortho*-substituted (coplanar) congeners, interrupt the cell cycle progression from G0/G1 to S phase with a decreased expression of the cell cycle regulatory protein cyclin D2. Our results, which suggest that *ortho*-substituted PCBs (138, 153, and 180) mostly explained the changes in lymphocyte proliferation rather than the non-*ortho*-substituted OC (PCB 169 and TCDD), are compatible with the suggestion that *ortho*-substituted PCB may interrupt the cell cycle progression from G0/G1 to S phase in mice, where decrease in lymphocyte proliferation was detected.

PCB are also known to suppress humoral immunity in guinea pigs, rabbits, rhesus monkeys, and mice (Dean et al., 1989). Since upon activation B cells undergo proliferation before differentiating into antibody-producing plasma cells or memory cells (Goldsby et al., 2001), a PCB-induced reduction in the ability of B cells to proliferate may be one of the mechanisms involved in the reduction in antibody production. It is also not surprising that exposure to PCB results in higher susceptibility to viral diseases in several species of lab animals (Dean et al., 1989; Imanishi et al., 1980; Koller & Thigpen, 1973).

The OC tested did not suppress B lymphocyte proliferation in marine mammals, in which they either had no effect (beluga whale and sea otters), or increased B-cell proliferation (pilot

whales, bottlenose dophins, harbor seals, and Northern fur seals). Such increases were observed only with some of the mixtures tested (Table 1), with effects that appeared nonadditive in nature (see Figure 1b) and poorly predicted by the concentrations of the different chemicals in the mixtures (see Table 2), all of which is in sharp contrast with the effects described in mice. A rise in B-cell proliferation might also result in adverse health effects. B-cell activation requires two signals, and one signal without the other may result in an induction of anergy, an active state of nonresponsiveness (Schwartz 2003). A continuous low-grade stimulation by OC may possibly mimic a first signal, and in the absence of the second signal might lead to anergy and possibly the inability to mount an appropriate response to pathogens. Additionally, if OC produce enough stimulatory signal to tilt the balance toward survival of the self-reactive lymphocytes and allow those self-reactive cells to escape the removal process through apoptosis, it may lead to activation of self-reactive clones of B cells, generating a humoral-mediated response against self-antigens (Goldsby et al., 2001). These reactions might lead to autoimmune responses such as hemolytic anemia, Hashimoto's thyroiditis, and Addison's disease that result from antibody-mediated damage to self-cells or organs (Goldsby et al., 2001). While autoimmunity is not frequently documented in polluted marine mammals, it is possible that B-cell anergy might be involved as one of the mechanisms underlying the often suspected OC-mediated immunomodulation leading to enhanced susceptibility to infectious disease in marine mammals. For example, the OC levels detected in many of the diseased marine mammals during the morbillivirus epizootic that devastated northern European populations of common and gray seals in 1987 and 1988 were extremely high (Aguilar & Borrell, 1994). Although several studies demonstrated associations between immune functions and concentrations of OC (Beckmen et al., 2003; Lahvis et al., 1995; Levin et al., 2005a; Lie et al., 2005), the direct cause-and-effect relationship has not been well demonstrated in marine mammals.

The TEQ approach was developed to predict the toxicity of OC in mixtures. However, this approach is contingent upon two assumptions. The first assumption is that the toxicity of OC is mediated through the aryl hydrocarbon receptor (AhR). TCDD and coplanar PCBs have affinity for the AhR, while non-coplanar PCBs have low to no affinity for AhR. (DeRosa et al., 1998; Safe, 1994). This study, as well as several others (Levin et al., 2005b, 2005c; Mori et al., 2006), demonstrated that OC toxicity in marine mammals was driven mostly by the non-coplanar PCBs, as shown from the regression analysis (Table 2), suggesting that not all immunomodulatory effects of OC are mediated through the AhR. The second assumption in utilizing the TEQ is that it estimates the overall toxicity of OC mixtures by adding the effects of the components of the mixture. Our results demonstrated simple additive effects in mice, but not in marine mammals, suggesting more complex interactions between OC in mixtures in these species. For example, the demonstration of significant effects for some mixtures in the absence of effects for their individual components suggests synergistic interactions, while the alleviation of effects upon addition of additional OC to a mixture suggests antagonistic interactions. Since our results suggest complex interactions between OC in mixture and effects not necessarily mediated through the AhR, it is not surprising that the toxicity on the lymphoproliferative response upon exposure to OC was not proportional to the TEQ values (Figure 2) in this study.

B6C3F1 mice have been the model most commonly used to test the immunotoxicity of chemicals for extrapolation to other species. However, mice failed to predict the effects observed in all the marine mammal species tested in the present and previous studies, due to marked differences between species and between immune assays when assessing susceptibility to OC (Levin et al., 2005c, 2007; Mori et al., 2006). The dendrogram (Figure 3) further illustrates that mice did not cluster closely with the marine mammal species tested, nor did marine mammal species cluster according to phylogeny (pinnipeds vs. cetaceans), further suggesting that phylogeny may not help predict toxicity. Although differences detected between mice and marine mammals might be due to the different origin of cells (spleens for mice and blood for marine mammals), this is unlikely, since spleen is well known to include lymphocyte trafficking from peripheral blood.

In summary, the immunotoxicity of OC in mixtures involves complex interactions and mechanisms underlying toxicity that are poorly understood. It was not possible to predict the toxicity of mixtures of OC in marine mammals from (1) the commonly used mouse model, (2) adding the effects of individual components of a mixture, or (3) the TEQ approach. These findings are disturbing and not accounted for by the current risk assessment paradigms (Faustman & Omenn, 1995). The current study should provide new, target species-specific information relative to hazard identification at the cellular level that might be useful for alternative approaches for more accurate and relevant risk assessment.

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