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IMMUNOMODULATORY EFFECTS OF IN VITRO EXPOSURE TO ORGANOCHLORINES ON T-CELL PROLIFERATION IN MARINE MAMMALS AND MICE

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*Marine mammals bioaccumulate various environmental contaminants such as organochlorines (OCs), which biomagnify via the food web. While the immunomodulatory effects of individual OCs have been studied, the effects of mixtures are not well understood. The immunomodulatory effects of polychlorinated biphenyl (PCB) 138, 153, 169, and 180 as well as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and all possible mixtures were examined in marine mammals and mice. Lymphocyte proliferation was significantly modulated by OCs in all species tested, mostly by non-coplanar PCBs, as shown using regression analyses. Correlation analyses showed significant correlations (interpreted as additive effects) between OCs in mice, killer whales, and Steller sea lions. Nonadditive synergistic and antagonistic interactions between OCs were detected in most of the species tested. Toxic equivalency (TEQ) values used for OC toxicity assessment failed to predict the immunomodulatory effects measured in mice and marine mammals. The commonly used mouse model failed to predict immunomodulatory effects in other species. Clustering data suggested that phylogeny does not predict toxicity of OCs. Overall, our data suggest the presence of species-specific sensitivities to different mixtures, in which OCs interactions may be complex and that may exert their effects through dioxinlike or dioxin-independent pathways. Lastly, lymphocyte proliferation, an important part of adaptive immunity, was significantly modulated in mice and marine mammals,*

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suggesting the possibility of increased susceptibility to diseases. These findings will be useful to better characterize the risk associated with OC exposure and possibly lead to new conservation and management strategies.

Organochlorines (OCs), such as polychlorinated biphenyls (PCBs), are found in the environment and wildlife, long after their production and use have been banned (DeRosa et al., 1998; Safe, 1994). This is a concern because marine mammals, being on top of a complex food chain, often bioaccumulate OCs through diet due to their stability and lipophilic properties (Troisi et al., 2001). As a consequence, OCs have been found in marine mammal tissues worldwide (Addison & Stobo, 2001; Gaskin et al., 1974; Holden & Marsden, 1967; Simmonds et al., 2002; Sladen et al., 1966; Taruski et al., 1975).

Over the past three decades, ample evidence has accumulated to suggest that OCs affect the immune system of mammalian species. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), the most immunotoxic of aromatic hydrocarbons, induces thymic atrophy in most experimental species (Andersson et al., 1991; Davis & Safe, 1988; Kerkvliet et al., 1990). PCBs also cause lymphoid depletion in chicks (Andersson et al., 1991; Fairbrother et al., 2004; Fox & Grasman, 1999), decrease in the number of T-cell-mediated cytotoxic activity in mice (Kerkvliet et al., 1990), and decrease in the number of T cells and the T-helper/T-suppresser cell ratio in nonhuman primates (Tryphonas et al., 1989). PCB-induced immunosuppression results in a higher sensitivity of experimental animals to a wide variety of infectious agents, including gram-negative bacteria (endotoxin), protozoa, and viruses (Imanishi et al., 1980; Koller & Thigpen, 1973; Loose et al., 1978; Thomas & Hinsdill, 1978). This is a concern in marine mammals since OC-mediated immunosuppression could be a contributing factor to marine mammal stranding events and die-offs attributed to infectious agents (Domingo et al., 1995; Kennedy et al., 2000).

In “real life,” OCs exist in complex mixtures of various congeners rather than individual compounds, complicating the risk assessment process. Thus, the toxic equivalency factors (TEFs), which estimate the toxicity of a compound relative to TCDD, have been developed and introduced to determine the toxic equivalency (TEQ) of a mixture as the sum of the toxicity of its components (concentration of OC $\times$ TEF) and facilitate risk assessment and regulatory control of exposure to these mixtures (Van den Berg et al., 1998). This approach assumes that the combined effects of the different congeners are additive. However, some compounds may not act in an additive way, but rather exert antagonistic or synergistic effects (Biegel et al., 1989; Davis & Safe, 1989). If antagonistic and synergistic effects occur when compounds are mixed, the conventional TEQ approach would fail to accurately predict the toxicity of compounds in mixture. In this study, the immunomodulatory effects on concanavalin A (Con A)-induced T lymphocyte proliferation upon

in vitro exposure to different OC mixtures were compared to the effects of individual compounds in marine mammals and mice to better understand the interactions of OCs in mixture and compare the susceptibility of different species.

MATERIALS AND METHODS

Animals

Blood samples from captive marine mammals were obtained opportunistically from several aquariums (Mystic Aquarium, Mystic, CT, Shedd Aquarium, Chicago, Sea World, San Diego, CA, and San Antonio, TX, and U.S. Navy Marine Mammal Program, San Diego, CA), as well as blood samples from wild animals (Marine Environmental Research Institute, Blue Hill, ME, and California Department of Fish and Game, Santa Cruz, CA). The following species were tested: killer whale (*Orcinus orca*), beluga whale (*Delphinapterus leucas*), bottlenose dolphin (*Tursiops truncatus*), and Commerson's dolphin (*Cephalorhynchus commersonii*) for cetaceans, harbor seal (*Phoca vitulina*), Northern fur seal (*Callorhinus ursinus*), and Steller sea lion (*Eumetopias jubatus*) for pinnipeds, and sea otter (*Enhydra lutris*) for mustelidae. Female B6C3F1 mice (*Mus musculus*), 2 to 4.5 mo of age, the most commonly used model in immunotoxicology (Luster et al., 1992), were purchased from Charles River Laboratories, MA. For quality control, B6C3F1 mice were tested simultaneously with each marine mammal species, except for harbor seals and five Northern fur seals.

Organochlorines

Five OCs were tested in vitro on marine mammal and mouse leukocytes. PCBs IUPAC 138, 153, 169, 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 PCBs 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. Finally, TCDD was chosen because it is the most potent immunotoxicant of the halogenated aromatic hydrocarbons (Holsapple et al., 1991). PCBs were resuspended in dimethyl sulfoxide (DMSO; Sigma, St. Louis, MO) to prepare stock solutions. OCs were then added to Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, Grand Island, NY) supplemented with (all from Gibco BRL, Grand Island, NY) 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, along with 10% fetal bovine serum (Hyclone, Logan, UT), thereafter referred to as complete DMEM. All possible combinations (26) of 2, 3, 4, or 5 of these compounds were tested along with individual chemicals and unexposed control cells. PCB congeners and TCDD were tested at concentrations of 5 ppm and

0.05 ppb, respectively, which are relatively low, environmentally relevant concentrations that individually did not significantly affect the proliferation of beluga whale lymphocytes (De Guise et al., 1998). The final DMSO concentration did not exceed 0.4%.

Sample Collection and Isolation of Lymphocytes

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 DMEM. 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 PBMC were resuspended in complete DMEM, washed twice, and enumerated with their viability assessed 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 Committees (IACUC) at the University of Connecticut. The spleen was harvested aseptically from each animal, and a single-cell suspension was prepared using two pairs of forceps in complete DMEM. Mononuclear cells were isolated by density gradient centrifugation on Ficoll-Paque plus gradient for 15 min at 720 × g. The mononuclear cells were resuspended in complete DMEM, washed twice, and enumerated with their viability assessed before exposure to OCs using the exclusion dye trypan blue.

Lymphocyte Proliferation

Con A-induced T-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). Briefly, mononuclear cells in complete DMEM were plated (2×10^5 cells/well) in 96-well flat-bottom tissue-culture plates (Falcon, Becton Dickinson, Franklin Lakes, NJ) in triplicate for each treatment group (including unexposed control) for every individual animal. Cells were incubated at 37 °C with 5% CO_2 for a total of 66 h with the T-cell mitogen concanavalin A (Con A; Sigma, St. Louis, MO). Con A was used at a suboptimal concentration (0.1 μg/ml), as well as at an optimal concentration (1 μg/ml) in preliminary experiments, to stimulate T-lymphocyte proliferation. 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 ELISA BrdU [colorimetric], Roche Diagnostics, Alameda, CA) as per manufacturer's instructions using an enzyme-linked immunosorbent assay (ELISA) plate reader (Multiskan EX v.1.0) at 690 nm with a reference wavelength of 450 nm.

Cell Viability After Exposure to OCs

To confirm that the OCs were not cytotoxic to the proliferating lymphocytes in culture, 0.05 mg/ml of propidium iodide (Molecular Probes, Eugene, OR) was added to the mononuclear cell suspension, and mice splenocyte viability was assessed with a FACScan (Becton Dickinson, Rutherford, NJ) flow cytometer using CellQuest software (Becton Dickinson Immunocytometry System, San Jose, CA).

TEQ Values

In order to test whether the TEQ approach was accurate in predicting the toxicity of mixtures, TEQ values were determined following Van den Berg et al. (1998) (Table 1, second column). Briefly, TEQ values were determined as the sum of the toxicity of each component of the mixture [$TEQ = \Sigma (TEF \times [OC])$], where TEF estimates the toxicity of a compound relative to TCDD.

Statistical Analysis

The optical densities (OD) were read directly from the ELISA plate reader and the triplicates of each animal were averaged. For quality control, ODs of all mice were run on SPSS for Macintosh statistical package (v. 8.0, Chicago) to detect outliers amongst each treatment group 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 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 in a particular treatment group (individual OC or OC mixture), it was eliminated as well as the data for the same treatment group (individual OC or OC mixture) in the marine mammal samples 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 treatments) 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 group 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. RM ANOVA was also used to determine the significance of OCs on cell viability in mice. In determining whether the TEQ

TABLE 1. Immunomodulatory Effects of In Vitro Exposure to OCs on Con A-Induced T-Cell Proliferation in All Species Tested (% Change From Unexposed Control)

	TEQ (ng/g)	B6C3F1 Mouse (n = 62)	Killer whale (n = 3)	Beluga whale (n = 24)	Bottlenose dolphin (n = 11)	Commerson's dolphin (n = 3)	Harbor seal (n = 21)	Northern Fur Seal (n = 7)	Steller sea lion (n = 5)	Sea otter (n = 5)
PCB 138	0	↓ (31%)								
PCB 153	0							↑ (58%)		
PCB 169	50									
PCB 180	0	↓ (29%)								
TCDD	0.05						↑ (87%)	↑ (53%)		
138 + 153	0	↓ (28%)		↑ (43%)			↑ (102%)	↑ (75%)	↑ (36%)	↑ (48%)
138 + 169	50	↓ (26%)		↑ (30%)			↑ (89%)	↑ (67%)	↑ (28%)	
138 + 180	0	↓ (49%)								
138 + TCDD	0.05	↓ (19%)					↑ (109%)	↑ (60%)		
153 + 169	50			↑ (49%)		↑ (6 1%)	↑ (143%)	↑ (80%)	↑ (31%)	↑ (63%)
153 + 180	0	↓ (25%)		↑ (55%)		↑ (68%)	↑ (150%)	↑ (80%)	↑ (45%)	↑ (72%)
153 + TCDD	0.05			↑ (27%)			↑ (79%)		↑ (29%)	
169 + 180	50	↓ (25%)					↑ (74%)	↑ (58%)	↑ (31%)	
169 + TCDD	50.05	↑ (17%)		↑ (51%)	↑ (31%)		↑ (216%)	↑ (84%)	↑ (33%)	↑ (60%)
180 + TCDD	0.05			↑ (58%)		↑ (66%)	↑ (207%)	↑ (94%)	↑ (57%)	↑ (67%)
138 + 153 + 169	50	↓ (52%)					↑ (45%)			
138 + 153 + 180	0	↓ (50%)	↓ (63%)				↑ (86%)			
138 + 153 + TCDD	0.05	↓ (27%)		↑ (46%)		↑ (62%)	↑ (213%)	↑ (79%)	↑ (25%)	↑ (89%)
138 + 169 + 180	50	↓ (51%)		↑ (39%)			↑ (147%)	↑ (61%)	↑ (29%)	
138 + 169 + TCDD	50.05	↓ (34%)					↑ (87%)	↑ (54%)	↑ (34%)	
138 + 180 + TCDD	0.05	↓ (34%)					↑ (109%)	↑ (56%)	↑ (28%)	
153 + 169 + 180	50	↓ (31%)		↑ (40%)			↑ (153%)	↑ (53%)	↑ (29%)	
153 + 169 + TCDD	50.05			↑ (51%)			↑ (168%)	↑ (88%)	↑ (36%)	
153 + 180 + TCDD	0.05	↓ (31%)					↑ (78%)		↑ (30%)	
169 + 180 + TCDD	50.05	↓ (20%)					↑ (80%)		↑ (32%)	
138 + 153 + 169 + 180	50	↓ (60%)	↓ (49%)		↓ (29%)		↑ (87%)			
138 + 153 + 169 + TCDD	50.05	↓ (38%)		↑ (31%)			↑ (116%)	↑ (64%)	↑ (29%)	
138 + 153 + 180 + TCDD	0.05	↓ (59%)	↓ (51%)				↑ (47%)			
138 + 169 + 180 + TCDD	50.05	↓ (50%)								
153 + 169 + 180 + TCDD	50.05	↓ (36%)					↑ (55%)			
138 + 153 + 169 + 180 + TCDD	50.05	↓ (63%)	↓ (59%)		↓ (32%)		↑ (35%)			
P value		6×10^{-127}	2×10^{-9}	8×10^{-48}	8×10^{-18}	4×10^{-8}	2×10^{-130}	8×10^{-10}	3×10^{-16}	3×10^{-8}

↓ significant decrease, $p < .05$.
↑ significant increase, $p < .05$.
no significant difference, $p > .05$.

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

To obtain dendrograms expressing relationships between the various mixtures and species, data were run on advanced clustering software (Genesite, BioDiscovery, CA) using hierarchical clustering based on centroid cluster linkage and Euclidean distance metric.

RESULTS

Concanavalin A Concentration

In determining the concentration of Con A for inducing T-cell proliferation, both optimal (1 μg/ml) and suboptimal (0.1 μg/ml) concentrations were tested. The suboptimal concentration had similar specificity and higher sensitivity compared to the optimal concentration of Con A in beluga whales, as it allowed the detection of adverse effects for more mixtures, as well as the detection of increased magnitude of the effects (data not shown). Significant effects detected at optimal concentrations were always found with the suboptimal concentrations ($p < .05$), demonstrating conservation of specificity in the effects detected. Thus, a suboptimal concentration, rather than an optimal concentration, was chosen to test all species in this study.

Effects of OCs on T-Cell Proliferation in Mice and Marine Mammals

A representative bar graph (mean ± standard error of mean [SEM]) of the analysis of the data is presented for bottlenose dolphins (Figure 1). Statistical analysis was performed using RM ANOVA to assess differences between exposed groups and unexposed control. The magnitude of the change compared to control was presented in Table 1 for statistically significant differences.

The immunomodulatory effects of OCs on lymphocyte proliferation in marine mammals and mice are summarized in Table 1. In mice, significant

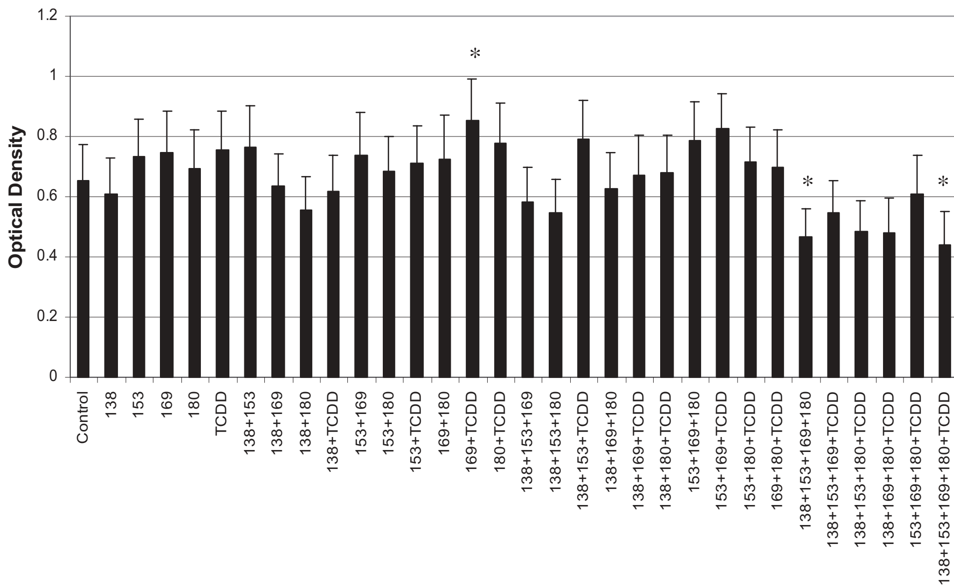

FIGURE 1. Con A-induced lymphocyte proliferative responses in bottlenose dolphins ($n = 11$). Bars represent optical densities (OD) expressed as mean ± SEM. Significant increase or decreases in lymphocyte proliferation compared to unexposed control were detected for 3 OC mixtures; asterisk indicates significant at $p < .05$.

decreases in lymphocyte proliferation after OC exposure were seen with 21 mixtures, all of which included PCB 138 or PCB 180, with the exception of mixture PCB180 + TCDD. All mixtures that contained both PCB 138 and PCB 180, with the exception of the mixture PCB138 + 180 + TCDD, reduced mouse T-cell proliferation by 49% or more. Only 1 mixture, PCB138 + 153 + 169, that did not include both PCB 138 and PCB 180 reduced proliferation by 50% or more.

With regard to marine mammals, lymphocyte proliferation in killer whales was significantly reduced (49–63%) by the four OC mixtures that included all three non-coplanar PCBs. In bottlenose dolphins, the two mixtures that included all four (coplanar and noncoplanar) PCBs significantly decreased lymphocyte proliferation compared to control. In sea otters, all six mixtures affecting proliferation included PCB 153, TCDD, or both. Significant increases in lymphocyte proliferation after exposure to most OC mixtures tested were detected in three of the pinniped species in this study. Several mixtures of OCs modulated lymphocyte proliferation in beluga whales and Commerson's dolphin; however, no obvious congener pattern was detected in those species either.

The mixture containing only the two coplanar OCs (PCB169 + TCDD) induced a significant increase in lymphocyte proliferation in seven of the nine species tested, with the most marked effects (216% increase compared to control) in harbor seals.

Changes in lymphocyte proliferation were not associated with changes in cell viability in mice, as the percent of cell viability for each OC treatment groups was not significantly different compared to the cell viability of unexposed control cells. Although cell viabililty in other species was not measured, it is unlikely that a decrease in viability would be associated with an increase in proliferation as noted in most species.

Regression Analysis

Forward stepwise regression analysis was utilized to determine which OC congeners significantly contributed to explaining the changes in lymphocyte proliferation in each species tested. The regression equations and adjusted R^2 values (adj. R^2) are summarized in Table 2. For mice, 3 non-coplanar PCBs explained 7% (adj. R^2) of the changes in lymphocyte proliferation. For the cetacean species tested, 2 non-coplanar PCBs (138 and 180) explained 13–38% (adj. R^2) of the changes in proliferation except for beluga whales, for which the best regression included only PCB 153 and explained only a small proportion of the change in lymphocyte proliferation (adj. R^2 = 1%). The three pinniped species also all included at least one non-coplanar PCB, but the regressions explained only a small proportion of the changes in lymphocyte proliferation (adj. R^2 = 2–4%). Harbor seals were the only species in which one coplanar OC (TCDD) significantly contributed to explaining the variability in lymphocyte proliferation. For sea otters, none of the OCs contributed significantly to a regression to explain the overall changes in lymphocyte proliferation.

Interactions of OC Chemicals in Mixture

Significant positive correlations between the predicted effect of a mixture, as calculated by adding the effects of its components, and the effect measured on experimental exposure for the 26 treatment groups were detected in mice and killer whales (Figure 2, a and b), suggesting that the OC chemicals in mixtures had additive effects. A significant negative correlation was detected in

TABLE 2. Forward Stepwise Regression Analysis Performed for Each Species Tested to Predict Changes in T-Cell Proliferation Compared to Unexposed Control (ΔP)

Species	Regression equation (difference in proliferation compared to control)	Adjusted R^2
Mouse	$\Delta P = -0.06(\text{PCB } 138) - 0.05(\text{PCB } 180) - 0.02(\text{PCB } 153) + 0.33$	.07
Killer whale	$\Delta P = -0.08(\text{PCB } 138) - 0.05(\text{PCB } 180) + 0.26$	.38
Beluga	$\Delta P = 0.05(\text{PCB } 153) + 0.66$	.01
Bottlenose dolphin	$\Delta P = -0.06(\text{PCB } 138) - 0.03(\text{PCB } 180) + 0.27$	.13
Commerson's dolphin	$\Delta P = 0.05(\text{PCB } 138) - 0.05(\text{PCB } 180) + 0.49$	.14
Harbor seal	$\Delta P = 7489.42(\text{TCDD}) - 0.05(\text{PCB } 138) - 0.04(\text{PCB180}) + 1.29$	.04
Northern Fur Seal	$\Delta P = 0.04(\text{PCB } 180) + 0.74$	.02
Steller sea lion	$\Delta P = -0.02(\text{PCB } 138) + 0.3$	.04
Sea otter	No significant equation	—

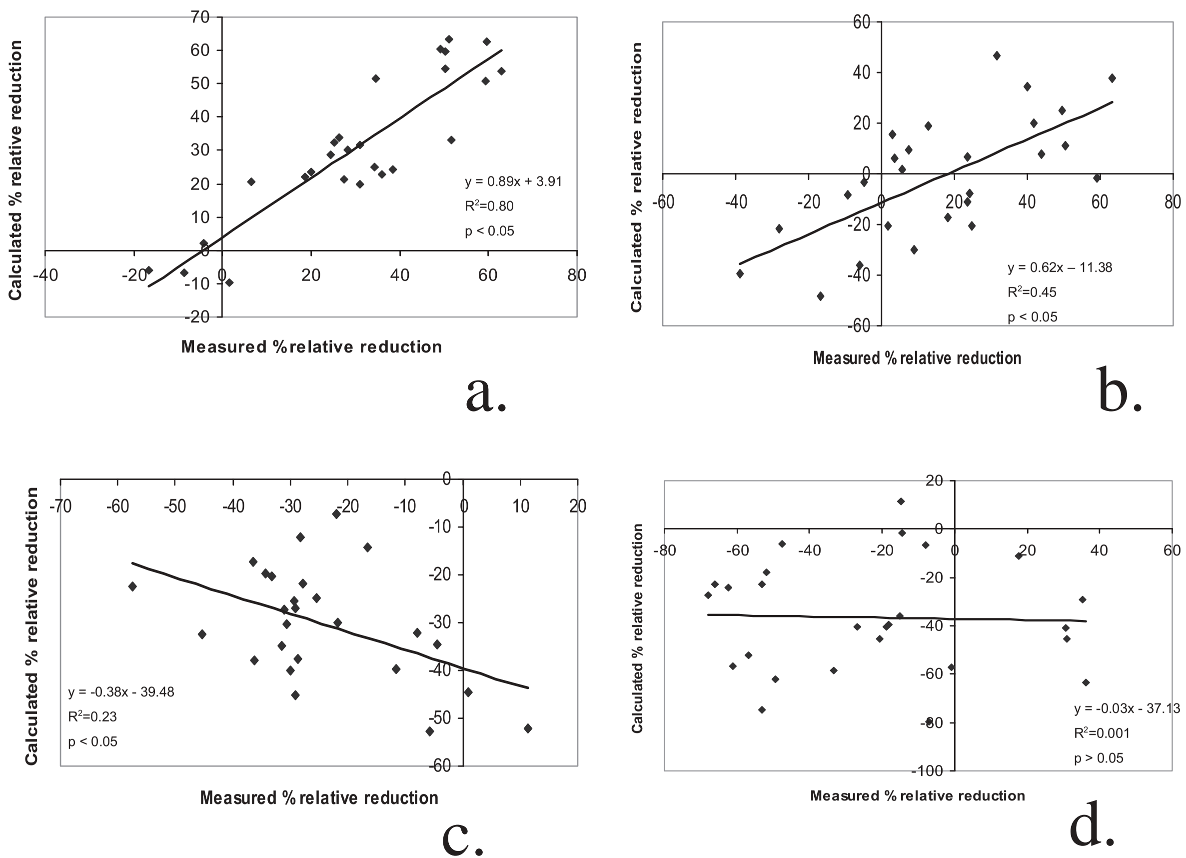

FIGURE 2. Correlation analysis between the calculated (%) relative reduction (the sum of the effects of individual congeners compared to control) and measured (%) relative reduction in all 26 mixtures. Significant positive correlations were detected in (a) mice and (b) killer whales, suggesting additive effects. The significant negative correlation in (c) Steller sea lions has no predictive value. No significant correlation was detected in (d) Commerson's dolphins, suggesting nonadditive interactions of OCs in mixtures.

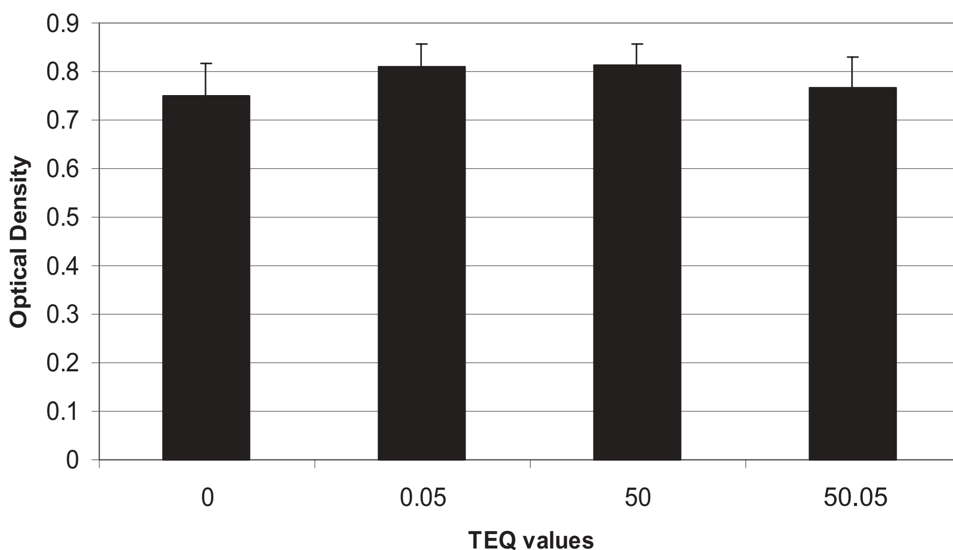

FIGURE 3. Lymphocyte proliferation (expressed as mean ± SEM) for OC treatments grouped according to their TEQ values showed no significant difference between the four treatment groups in beluga whales, suggesting that the TEQ values did not accurately predict the immunomodulatory effects on T-cell proliferation upon in vitro OC exposure.

Steller sea lions (Figure 2c), while no significant correlations were detected in Commerson's dolphins (Figure 2d) and the other marine mammals tested (data not shown). These findings suggest that the OC congeners in mixture had nonadditive interactions in these species.

Predicting Toxicity Utilizing TEQ Values

TEQ values, used for predicting the toxicity of OC mixtures relative to TCDD, could not accurately predict the effects on T-cell proliferation in all species tested (Table 1). Higher TEQ values did not correspond with increased toxicity, measured as increase or a decrease in T-cell proliferation, compared to control (Table 1). Furthermore, no significant difference was detected amongst the OC treatments that were grouped according to their TEQ value in beluga whales (Figure 3), mice, or other marine mammals tested (data not shown).

Species and OC Clusters

Figure 4 illustrates how different species of marine mammals and mice in this study clustered based on relative changes in lymphocyte proliferation. Mice clustered together with killer whales and bottlenose dolphins, and the rest of the species clustered together except for harbor seals, which clustered

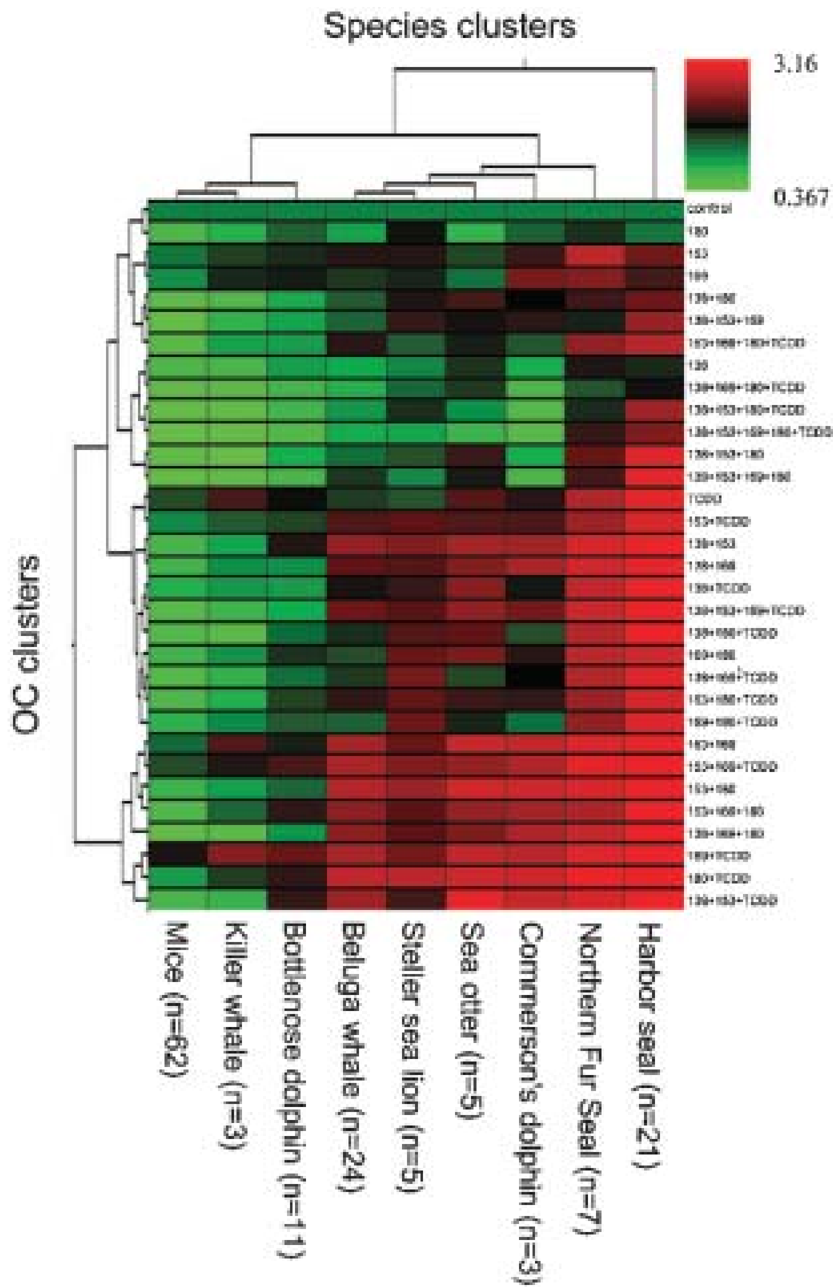

FIGURE 4. Dendrogram representing clustering by species and OCs based on modulation of lymphocyte proliferation compared to unexposed control. Brighter shades of green indicate greater reduction of lymphocyte proliferation compared to control, brighter shades of red indicate greater enhancement of lymphocyte proliferation compared to control, and black indicates no difference compared to control.

furthest from all the other species. No clear grouping of species by phylogeny (rodent, cetacean, pinniped, or mustelidae) was evident. OCs individually or in mixtures clustered without any obvious pattern when all species were taken into consideration.

DISCUSSION

The studies testing the effects of contaminants on the immune system of marine mammals have recently been reviewed (De Guise et al., 2003). Most field studies reported associations between immune functions and concentrations of chemicals, without cause-and-effect relationships. Although the direct determination of the effects of environmental contaminants on the immune system of wild marine mammals is difficult, some studies utilized different approaches, including in vitro exposure and animal models in addition to semifield studies. Overall, the present data suggest that environmental contaminants with known immunotoxic effects in laboratory animals, and that are present in high concentrations in tissues of marine mammals, may induce immunotoxic effects in these free-ranging marine mammals (De Guise et al., 2003).

Several studies have evaluated the effects of OCs on mitogen-induced lymphocyte proliferation in variety of species, with results varying from no modulation, to suppression or enhancement. Exposure of human lymphocytes to PCBs in vitro resulted in no significant change in mitogen-induced lymphocyte proliferation (Fernlof et al., 1997). On the other hand, accidental in vivo exposure of humans to PCBs in the Yu-Cheng incident resulted in significant enhancement of T-lymphocyte proliferation (Lu & Wu, 1985). Significant increase in T-cell proliferation, as detected in our study on exposure of mouse cells to mixture of two coplanar PCBs, was also found in mouse and rat splenocytes exposed to Aroclor 1254, in vitro and in vivo, respectively (Lubet et al., 1986; Smith et al., 2003). Our results showed significant decrease in Con A-induced mice splenocyte proliferation with exposure to most noncoplanar OCs, as for lipopolysaccharide (LPS)-induced mouse splenocyte proliferation upon in vitro exposure to PCB mixtures (Stack et al., 1999). Rhesus monkeys (*Macaca mulatta*) exposed to Aroclor 1254 orally for 55 mo also showed declines in lymphoproliferation with Con A (Tryphonas et al., 1991). With regard to marine mammals, harbor seals (*Phoca vitulina*) chronically exposed to polyhalogenated aromatic hydrocarbons from fish in a semifield condition had reduced T-lymphocyte proliferation (Ross et al., 1996), while exposure to most OCs increased proliferation in our study. Reduced proliferation was also reported in beluga whale cells exposed in vitro to mixtures of OCs at concentrations in the range of those observed in tissues of St. Lawrence belugas (De Guise et al., 1998) while most OC exposures increased proliferation in our study. Those differences between studies could be related to qualitative or quantitative differences in (1) exposure, (2) interactions of chemicals in mixtures, (3) differences in the animals used as source of cells, or (4) possible influence of metabolic activity in in vivo studies.

The TEQ approach has been developed to predict the toxicity of complex mixtures from that of its individual compounds. The TEFs have been validated experimentally for relatively simple mixtures containing from one to four different polychlorinated dibenzo-*p*-dioxins (PCDDs) and/or polychlorinated dibenzofurans (PCDFs) and/or PCBs (Birnbaum et al., 1987). For a complex mixture of dioxinlike PCBs, when appropriately derived, TEF/TEQ can be used to predict the toxicity of a complex mixture of OCs (Birnbaum & DeVito, 1995). There was no evidence in the data for nonadditive interactions, which is in agreement with most studies showing additive effects. However, these effects were only seen when relatively high levels of TCDD are used and the interactive effects may be dose and tissue specific. Thus, the use of TEF/TEQ for dioxinlike chemicals fills a risk assessment vacuum and allows for the estimation of health risks for complex mixtures (Birnbaum & DeVito, 1995). The TEQ approach, however, may not be adequate in predicting toxic effects of compounds in mixture if the interactions of OC compounds in mixture are more complex than simply additive—for example, if there were antagonistic (Davis & Safe, 1989) or synergistic interactions (De Guise et al., 1998), or if some effects were mediated by dioxin-independent mechanisms (Levin et al., 2004). Our results support the idea that the TEQ approach did not predict the modulation of T-cell proliferation in marine mammals or mice.

The immunotoxic effects of OCs with a structure similar to that of TCDD, such as coplanar PCBs, are mediated through the cytosolic aryl hydrocarbon receptor (AhR) (DeRosa et al., 1998; Safe, 1994). However, AhR-mediated toxicity may not hold true for all OCs. For instance, non-coplanar or non-TCDD like PCBs, such as PCB 138 and PCB 180, each induced a significant decrease in mouse lymphocyte proliferation in this study. On the other hand, PCB 153, another non-coplanar PCB, did not have any effect on mouse lymphocyte proliferation. This suggests the possibility of differences in the immunotoxic effects amongst the non-coplanar PCBs. When two or more non-coplanar PCBs were present in a mixture, that mixture always induced a significant decrease in mouse lymphocyte proliferation compared to the control, suggesting the possibility of a common receptor. The mixture of the two coplanar congeners (TCDD and PCB 169), on the other hand, increased lymphocyte proliferation in seven of the nine species tested. These results suggest that not all immunomodulatory effects of OCs are mediated through the AhR, and that different OCs may mediate different effects.

Evidence that OCs may mediate AhR-independent toxicity was also shown in marine mammals. In killer whales, when the three non-coplanar PCBs were present in a mixture, with or without coplanar OCs, the mixture always decreased lymphocyte proliferation compared to control. In all species, except for bottlenose dolphins, modulation of T-cell proliferation was observed with some OC mixtures that did not contain coplanar OCs, suggesting non-coplanar PCB congeners exerted immunotoxic effects through an AhR-independent mechanism, suggesting the possibility of a receptor that mediates the toxicity for non-coplanar PCBs.

To better understand which OC congener contributed to the changes in lymphocyte proliferation, regression analysis was utilized. Regression equations for the marine mammals further supported that the changes in lymphocyte proliferation were mostly explained by the non-coplanar PCBs (in particular PCBs 138 and 180) rather than the coplanar OCs, with the exception of harbor seals, the only species for which coplanar OCs significantly contributed to explaining immunomodulatory effects. However, no one model was very predictive to explain all the changes in lymphocyte proliferation for a particular species, as the adjusted R^2 values were low (1 to 38%). This highlights the difficulty in designing models to predict the complex interactions of OCs in a mixture.

The nature of the interactions of chemicals in mixture, such as additive or nonadditive, was investigated. Mice, killer whales, and Steller sea lions showed statistically significant additive effects when OCs were in mixtures, but the correlations in killer whales and Steller sea lions were not as strong as in mice (killer whale $R^2 = .45$; Steller sea lions $R^2 = .22$; mouse $R^2 = .80$). This indicates that the calculated toxicity in killer whales and Steller sea lions may not predict the measured toxicity as precisely as in mice, and that the statistical relationship may not be completely linear. Although statistically significant, the correlation between the predicted and measured relative reduction in proliferation in Steller sea lions was negative, offering little predictive value. All other species tested failed to show statistically significant relationships suggesting non-additive interactions (Figure 2d). Therefore, the interactions of OCs in mixture may be complex, and using a simple additive model may not be the most appropriate way to predict toxicity of mixtures in all species.

Nonadditive interactions could consist of either synergistic or antagonistic effects, and examples for both of these types of interactions were found. Examples of synergistic effects were detected in all species tested. In belugas, none of the 5 individual OCs induced significant effects compared to the control; however, 12 mixtures exerted a significant effect compared to the control, indicating a greater effect than the sum of the components of a mixture, suggesting synergistic interactions. Similar synergistic interactions were seen in killer whales, bottlenose dolphins, Commerson's dolphins, and sea otters. In mice, PCB 169 and TCDD produced no effects individually but significantly increased proliferation when mixed together. In both harbor seals and Northern fur seals, PCB 138 and 169 produced no effects individually but significantly increased proliferation when mixed.

Antagonistic interactions were also detected in all species tested with the exception of killer whales. For instance, the mixture of PCB 169 and TCDD significantly increased proliferation compared to control. However, when PCB 180 (in belugas, bottlenose dolphins, Northern fur seals, and sea otters), PCB 153 (in mice), or PCB 138 and 180 (in harbor seals) were added to the previous mixture, it alleviated the effects suggesting antagonistic interactions. Taken together, these findings suggest that interactions between OCs in mixtures are complex. This is an important finding because in reality OCs are found in complex and diverse mixtures rather than as individual congeners.

Differences in the immunomodulatory effects of OC mixtures as well as single compounds between mice and the marine mammal species were observed. Mice and killer whales showed significant decrease in lymphocyte proliferation upon exposure to OCs, whereas most marine mammals tested showed significant increase in lymphocyte proliferation with exposure to OCs. Individual OCs or mixtures did not always induce similar immunomodulatory effects in all species, suggesting interspecies differences in their susceptibility and sensitivities to different OCs (Table 1).

B6C3F1 mice, thus far, have been a commonly used model to predict the toxicity of chemicals for risk assessment. However, in this study, mice failed to consistently predict the effects observed in the marine mammals tested. Although the differences detected between mice and marine mammals could be due to the different origin of cells (spleens for mice and blood for marine mammals), it is unlikely since spleen is well known to include lymphocytes trafficking from the peripheral blood. Using the mouse model to predict immunomodulatory effects in other species could result in two types of error. Predicting that a chemical or a mixture is toxic, based on results obtained in mice, when in fact the chemical is nontoxic or safe in other species, would overestimate the risk and result in a conservative approach. On the other hand, predicting that a chemical or a mixture is nontoxic or safe based on results obtained in mice, when in fact the chemical is toxic or non-safe in other target species, could cause a serious problem and result in underestimation of the risk and observation of unexpected toxicity in those target species. Moreover, the dendrogram (Figure 4) illustrates the diversity in susceptibility to OCs between species, as evident by the rather random grouping of species and the broad diversity of effects, suggesting that future attempts to use one (such as B6C3F1 mice) or a few species to predict toxicity and risk for broad categories of more or less closely related species, as is the case for the current strategies for human health and ecological risk assessment, should be avoided.

In our study, both significant decrease and increase in lymphocyte proliferation compared to unexposed control were found in the species tested (Table 1). Lymphocytes play an essential role in the acquired immune response against pathogens, and significant differences in the ability of lymphocyte to proliferate upon stimulation may be of concern. Interaction of mature, immunocompetent lymphocytes with the appropriately presented antigen stimulate the cell to proliferate and differentiate into effector and memory cells (Goldsby et al., 2001). A reduction of the ability of lymphocytes to proliferate upon encounter with a pathogen may lead to a decreased acquired immune response, and consequently increased susceptibility to diseases (Imanishi et al., 1980). Thus, our data showing decreased lymphocyte proliferation in mice, killer whales, and bottlenose dolphins may be indicative of species more susceptible to diseases upon exposure to OCs. Increase in lymphocyte proliferation, detected in seven of the species tested, could also be indicative of adverse health issues. T-cell activation occurs by contact with antigen displayed by an antigen-presenting cell (APC). However, this interaction, by itself,

is not sufficient to fully activate naive T cells. Naive T cells require two distinct signals for activation, proliferation, and subsequent differentiation into effector and memory cells. "Signal one" is generated by interaction of antigenic peptide with the T-cell receptor–CD3 complex, and "signal two" is delivered by a antigen nonspecific costimulatory molecule provided primarily by interactions between CD28 on the T cell and members of the B7 family on the APC (Greenfield et al., 1998). The presence of signal one without signal two leads to a tolerance mechanism in which the lymphocyte enters an active state of unresponsiveness or anergy following an antigen encounter, a situation in which the cells remain alive for an extended period of time but remain unable to mount a functional immune response (Schwartz, 2003). A continuous low-grade stimulation by OCs could mimic signal one, in the absence of signal two, and lead to anergy, which would prevent an appropriate response to pathogens. In addition to the possibility of T cells becoming anergic, T cells bearing a high-affinity receptor for self-MHC molecule alone or self-antigen plus self-MHC molecules, which are usually eliminated in the thymus in the course of the negative selection process, could receive enough stimulatory signal from OCs to tilt the balance toward survival and escape removal through apoptosis, possibly resulting in autoimmunity. Several autoimmune diseases are mediated directly by T cells, such as rheumatoid arthritis, insulin-dependent diabetes mellitus, and multiple sclerosis (Goldsby et al., 2001). Thus, significant increase and decrease in lymphocyte proliferation could both result in increased susceptibility to diseases.

Another study has tested the immunomodulatory effects of the same individual and mixtures of OC on phagocytosis, as part of the innate immune system (Levin et al., 2005). In that study, none of the OCs significantly modulated mouse phagocytosis, while 2 individual non-coplanar PCBs and 22 of the 26 mixtures modulated lymphocyte proliferation in mice in the current study. The majority of the OCs enhanced lymphocyte proliferation in marine mammals in the current report, while the majority of the OCs reduced phagocytosis. Regression analysis revealed that in both studies, non-coplanar PCBs, rather than coplanar OCs, explained most of the changes in immune functions, suggesting immunomodulatory effects for non-coplanar OCs in both the innate and acquired arms of the immune system. However, not one model was well suited for predicting toxicity of any other species, emphasizing that OCs in mixtures are complex and difficult to model. Both the TEQ approach and the commonly used mouse model failed to predict the toxicity of OCs in all species tested in both studies. Clustering data in both studies suggest that OC toxicity cannot be predicted by phylogeny. However, differences in clustering between the two studies show that the effects of OCs can depend on the immune function studied.

In conclusion, OC compounds in mixtures induce complicated interactions that are not yet well understood. Furthermore, each marine mammal species tested in this study had different sensitivities to the immunomodulatory effects of OC mixtures. It was not possible to predict the immunomodulatory effects that the OC mixtures induced in most of the marine mammals either

from (1) the commonly used mouse model, (2) the effects of individual chemicals in each species, or (3) the TEQ approach. In fact, predicting the toxicity of OC mixtures in marine mammals from those in mice would have resulted in either overestimation or underestimations of immunotoxic risk to marine mammals. Further assessment is needed to understand the mechanism of OC-mediated toxicity and to understand the differences in immunomodulatory potential between species. Further investigation on the mechanism of OC toxicity should aid in more precise and relevant risk assessment as well as global conservation and management strategies for the overall anthropogenic effects on marine mammal populations.

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