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IN VITRO IMMUNOTOXICITY OF BLUBBER-DERIVED CONTAMINANT COCKTAILS IN FRESHLY COLLECTED LYMPHOCYTES FROM SEALS, DOLPHINS AND POLAR BEARS

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Introduction

Humans and wildlife are exposed to a plethora of chemicals, including known and yet uncharacterized contaminants. Most toxicological studies, however, consider effects upon exposure to single compounds. Mixture toxicology is thus becoming an important field of research due to the potential synergistic and antagonistic interactions of chemicals, and effect studies are increasingly interested in realistic chemical mixture exposures (Kortenkamp et al., 2009). In vivo feeding studies using naturally complex mixtures are costly, logistically challenging, and often impossible for certain species due their natural behavior, as well as their protected and endangered status. In vitro toxicology has many advantages in terms of high-throughput capacity, cost-effectiveness, simple system design, mechanistic focus, the ability to cover broad comparable exposure ranges of contaminants, as well as biomarker applicability as early warning signals for larger scale toxic effects (Tryphonas et al., 2004).

Exposure to organohalogenated compounds (OHCs) has been linked with harmful biological effects in both laboratory animals and wildlife, with effects occurring among a wide range of physiological systems and modes of actions (e.g. Safe, 1984). Effects on the immune system are of particular importance given the role of immunity in protecting the host against the adverse effects of invading pathogens. Indeed, the field of immunotoxicology has been around since the 1970s and OHCs have been shown to modulate (suppress and/or stimulate) almost all aspects of the immune system. Recently, Desforges et al. (2016) reviewed what is currently known regarding immunotoxicity in marine mammals and reported that both arms of the immune system (innate and adaptive), including cellular and humoral responses, were modulated by environmental contaminants dose-dependently. Although in vivo studies inherently include contaminant cocktail exposures, the majority of in vitro studies examined individual compounds or simple mixtures of polychlorinated biphenyl (PCB) congeners (Desforges et al., 2016).

The purpose of the present study was to evaluate the immunotoxic effects of realistic OHC cocktails upon in vitro exposure to immune cells from a variety of marine mammal species. As we concurrently report in a poster presentation at this symposium, using a novel method to extract complex OHC mixtures from marine mammal blubber, we produced sufficient cocktail volumes to test concentration-dependent effects on important immune functions, including lymphocyte proliferation, natural killer (NK) cell activity and phagocytosis. This approach offers the most realistic exposure possible for in vitro exposure and therefore represents the most reliable concentration-response estimates for marine mammal (and other animal groups) immunotoxicity.

Materials and Methods

Marine mammal immune cells were collected as part of several field research campaigns and from managed-care facilities under appropriate local laws and permits. Freshly collected lymphocytes were isolated from lymph nodes of harp seals (Pagophilus groenlandicus) and hooded seals (Cystophora cristata) from the East Greenland pack-ice. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood of polar bears (Ursus maritimus) from the Aalborg and Copenhagen Zoos (Denmark). Lymphocytes and granulocytes were collected from free-range bottlenose dolphins (Tursiops truncatus) in Sarasota, Florida, USA. Samples were shipped to our laboratory at the University of Connecticut within 24 h of fresh collection or immediately cryopreserved and kept in liquid nitrogen until analysis.

The contaminant cocktails used in the concentration-response experiments were extracted from polar bear and killer whale blubber (Orcinus orca) sampled under the legal subsistence hunting in East Greenland from the settlements Tasiilaq and Ittoqqortoormiit (2013-2014) using a newly developed freeze-filtration method. Detailed description of the extraction and clean-up method is described in an accompanying abstract. In brief, we extracted the OHC mixture from over 50 grams of blubber, consisting of samples from multiple individuals within a population. This generated a highly concentrated stock solution of chemical cocktail, which included a large number of measured OHCs. Since PCBs are often the most important OHC in terms of toxic effects in marine mammals (Dietz et al., 2015), the exposure concentrations reported herein are benchmarked based on $\sum_{34}$PCB concentrations in wet weight (ww).
In vitro immune assays included mitogen stimulated lymphocyte proliferation, natural killer cell (NK) activity, and phagocytosis. Detailed methodology of these have been previously described for marine mammal cells (Levin et al., 2005; Mori et al., 2006). These have been chosen as they represent important aspects of innate and adaptive immunity against bacterial and viral pathogens. Concentration ranges for the exposure experiments were first assessed for cytotoxicity and included a wide range of concentrations below the cytotoxic threshold. Due to overall OHC pattern similarities, the killer whale chemical cocktail (KW) was used for seal and cetacean exposures and the polar bear chemical cocktail (PB) was used only for polar bears. Polar bears were also exposed to KW to compare cocktail sensitivity.

Results and Discussion

Cytotoxicity
The chemical cocktail stock concentrations of ∑PCBs (34 congeners), ∑organochlorine pesticides (20 compounds) and ∑brominated flame retardants (46 compounds) were very high at 2563, 3331 and 8.6 µg g-1 ww, and 676, 147 and 36.5 µg g-1ww, for KW and PB respectively. Cytotoxicity of each contaminant cocktail was assessed in order to select sub-lethal concentrations for immune assays. Using killer whale PBMCs, we found that the KW cocktail caused significant cytotoxicity above 24 µg g-1 (~95% lymphocyte death). Using polar bear PBMCs, we found that the PB cocktail caused significantly cytotoxic at 2 µg g-1 (~50% lymphocyte death). Based on the cytotoxicity assays, the following KW and PB concentrations were chosen for immunotoxicity testing: KW = vehicle control (0), 0.04, 0.08, 0.4, 0.8, 4, 8 and 16 µg g-1 ∑PCBs; PB = vehicle control (0), 0.002, 0.005, 0.02, 0.05, 0.2, 0.5 and 1 µg g-1 ∑PCBs.

Immunotoxicity
We observed a hormetic effect on mitogen stimulated (concanavalin A) lymphocyte proliferation, whereby lower concentrations stimulated and highest doses suppressed proliferation (Fig. 1). The KW cocktail caused significant reduction in lymphocyte proliferation above 4 µg g-1 for all species tested, including polar bear. The PB cocktail caused significant reduction in proliferation for two of three bears at the highest test concentration of 1 µg g-1. For bottlenose dolphin phagocytosis, there was no difference in the ability of neutrophils or monocytes to phagocytose fluorescent latex beads up to 2 µg g-1 (KW cocktail). Above 2 µg g-1 cell morphology (based on forward and side scatter dot plot using flow cytometry) shifted drastically, such that we could not accurately isolate phagocytes from other cells and debris by flow cytometry. Therefore, we cannot conclude that higher concentrations did not affect phagocytosis. The ability of NK cells to destroy target cells (YAC or K-562) was unaffected by the PB cocktail on polar bear cells up to the highest tested concentration (1 µg g-1) but increased dose-dependently for harp and hooded seals exposed to the KW cocktail.

Conclusion
To our knowledge, the present study is the first to demonstrate that exposure to realistic and species-relevant contaminant cocktails caused modulation of important in vitro immune functions. Moreover, effects occurred at concentrations much lower than reported in previous in vitro assays using single compounds or simple mixtures (see review by Desforges et al., 2016). These results suggest that marine mammals exposed to complex OHC mixtures may be at higher risk of immunomodulatory effects than previously documented.

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