Arch Environ Contam Toxicol (2016) 70:121–132
DOI 10.1007/s00244-015-0178-z

In Vitro Exposure of Harbor Seal Immune Cells to Aroclor 1260 Alters Phocine Distemper Virus Replication

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Received: 2 February 2015 / Accepted: 6 June 2015 / Published online: 4 July 2015

Abstract In the last 30 years, several large-scale marine mammal mortality events have occurred, often in close association with highly polluted regions, leading to suspicions that contaminant-induced immunosuppression contributed to these epizootics. Some of these recent events also identified morbillivirus as a cause of or contributor to death. The role of contaminant exposures regarding morbillivirus mortality is still unclear. The results of this study aimed to address the potential for a mixture of polychlorinated biphenyls (PCBs), specifically Aroclor 1260, to alter harbor seal T-lymphocyte proliferation and to assess if exposure resulted in increased likelihood of phocine distemper virus (PDV USA 2006) to infect susceptible seals in an in vitro system. Exposure of peripheral blood mononuclear cells to Aroclor 1260 did not significantly alter lymphocyte proliferation (1, 5, 10, and 20 ppm). However, using reverse transcription-quantitative polymerase chain reaction (RT-qPCR), lymphocytes exposed to 20 ppm Aroclor 1260 exhibited a significant decrease in PDV replication at day 7 and a significant increase at day 11 compared with unexposed control cells. Similar and significant differences were apparent on exposure to Aroclor 1260 in monocytes and supernatant. The results here indicate that in harbor seals, Aroclor 1260 exposure results in a decrease in virus early during infection and an increase during late infection. The consequences of this contaminant-induced infection pattern in a highly susceptible host could result in a greater potential for systemic infection with greater viral load, which could explain the correlative findings seen in wild populations exposed to a range of persistent contaminants that suffer from morbillivirus epizootics.

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Along the Northeast coast of the United States, the three species of seal most commonly seen are harbor seal (*Phoca vitulina concolor*), gray seal (*Halichoerus grypus*), and harp seal (*Phoca groenlandica*). Hooded seal (*Cystophora cristata*) and, less commonly, ringed seal (*Phoca hispida*) also frequent the area (Hannah J, International Marine Mammal Association 2000; Harris et al. 2001). Although nearly extirpated in New England due to bounty hunts, protection in the 1960s and 1970s enabled harbor seals and gray seals to regain historical grounds in the Northeast United States (Lelli et al. 2009; Wood et al. 2011; Waring et al. 2013).

With an increasing population of marine mammals there exists an increase in the occurrence of unusual mortality events (UMEs), which, by definition, occur when a greater-than-expected number of mortalities are recorded (NOAA 2006). The most notable of these large-scale mortality events in recent history includes one in which half the

population of harbor seals in European waters died in 1988 and again in 2002. The cause of these devastating mortality events was a morbillivirus, specifically phocine distemper virus (PDV) (Osterhaus and Vedder 1988; Jensen et al. 2002). Many more recent events also identified morbilliviruses as a cause or contributing factor (Geraci 1989; Duignan et al. 1996; Kennedy et al. 2000; Earle et al. 2011; NOAA 2014, Duignan et al. 2014). The close association of these mortality events with highly polluted regions led researchers to suspect that the immunosuppressive effects of various products of industry contributed to these epizootics (Safe 1989, 1992; de Swart et al. 1994; Martineau et al. 1994; Ross et al. 1994).

The immunotoxicity of these persistent organic pollutants (POPs) was documented in harbor seals during a feeding exposure experiment in which seals were fed fish captured in contaminated and less contaminated areas (Ross et al. 1992, 1994; de Swart et al. 1994, 1995). Many subsequent in vitro investigations of in marine mammals have indicated that immunomodulations of the innate and adaptive immune system—including effects on natural killer cell (NK) activity, cytokine response, respiratory burst, phagocytosis, and lymphocyte proliferation (LP) (De Guise et al. 1996, 1998; Neale et al. 2005; Mori et al. 2006; Levin et al. 2007a, b; Dufresne et al. 2010; Frouin et al. 2010) occurred on exposure to a range of persistent contaminants. Correlative studies based on immune assays, histopathological findings, and measured concentrations of POPs in tissues also suggest cellular and humoral immunity, and endocrine function can be influenced by these contaminants (Brouwer et al. 1989; Beckmen et al. 2003; Beineke et al. 2005; Levin et al. 2005a; Mos et al. 2006; Schwacke et al. 2011). Epidemiological studies examining the effects of polychlorinated biphenyls (PCBs) also suggest that animals with greater burdens of contaminants are at increased risk of dying from infectious disease (Jepson et al. 1999; Hall et al. 2006).

Not all studies suggest that POPs induce negative affects on measurable immune system functions. In contrast to the above-mentioned findings, direct in vivo experimental exposure of harbor seals to PCB and subsequent PDV exposure did not result in differences between exposed and nonexposed groups and resulting infection (Harder et al. 1992). Similarly, no association was found in gray seals between general infection status and PCB burdens (Hall et al. 1997). A recent retrospective study on the 2000–2001 Caspian seal mortality event due to CDV suggests that concentrations of organochlorine contaminants had no association with CDV status despite PCB and organochlorine pesticide concentrations being similar to those that result in immune suppression in harbor seals (Kennedy et al. 2000, Kajiwara et al. 2002; Wilson et al. 2014).

Given these discrepancies, interpretation of results from correlative in vivo and in vitro studies should consider the class of contaminants being addressed, ranges of exposure, age, sex, nutritional status, species specific susceptibility, and environmental parameters (Hutchinson and Simmonds 1994; Ross et al. 1996; Hammond et al. 2005; Levin et al. 2005b; Mori et al. 2006; Harkonen et al. 2007). Confounding factors make it difficult to understand the relationship between observed immunosuppression and an increase in susceptibility to infectious disease in wildlife, yet these studies are important in adding to the weight of evidence regarding the effects of legacy and emerging contaminants on marine mammal health (Ross et al. 1996; Schwacke et al. 2011).

In the northeast United States, recovering seal populations are exposed to a wide array of POPs (Shaw et al. 2014; Weijs et al. 2014). Concentrations of PCBs measured in the blubber of harbor seals from the northeast United States region ranged from 5.1 to 151 μg/g (ppm) lipid weight (lw) (Shaw et al. 2005) representing levels greater than the 17 μg PCB/g lipid lw threshold for adverse effects including alterations in immune response (De Swart et al. 1996; Ross et al. 1996; Kannan et al. 2000).

In 2006, a new North American strain of PDV (PDV USA 2006) was isolated and determined to be the cause of mortality of harbor and gray seals during the Northeast United States Pinniped Unusual Mortality Event (Matassa et al. 2008; Earle et al. 2011). As in the 1988 and 2002 mortality of European seals, the role of contaminant exposures, susceptibility to PDV, and ultimate mortality is still unclear. This study aimed to (1) address the potential for a mixture of PCBs, specifically Aroclor 1260, to alter harbor seal T-lymphocyte proliferation, which is an important component of the acquired immune system necessary for combating viral infection, and to directly influence viral replication after contaminant exposure as well as to (2) assess if exposure can result in changes in susceptibility to PDV on in vitro infection.

Materials and Methods

Study Design

Aroclor 1242, 1254, and 1260 were the major Aroclors produced in North America (Sather et al. 2001). Aroclor 1260 was chosen to test a mixture of dioxin-like and nondioxin-like congeners found in seals of the northwest Atlantic. Aroclor 1260 (PCB 1260; lot number NT01023; Ultra Scientific, North Kingstown, Rhode Island, USA) stock solution was initially resuspended in dimethyl sulfoxide (DMSO) at 5000 ppm (50 mg/10 ml). The highest concentration of Aroclor 1260 used for the experiment was

20 ppm with a maximum DMSO concentration of 0.4 %. Therefore, Aroclor 1260 dilutions used in all experiments were diluted to the appropriate concentration using complete culture media (see later text) with equivalent concentrations of DMSO (0.4 %) in all experimental and control dilutions. Concentrations chosen are within those found in blubber of harbor seals in the same geographic region (Shaw et al. 2014).

Animals Investigated and Sampling

Blood samples from eight adult wild captured harbor seals were collected as part of an ongoing population assessment by the National Marine Fisheries Service (NMFS) Northeast Fisheries Science Center (NEFSC) between 2011 and 2012. Blood samples from four stranded harbor seals were collected from seals previously released into the wild from the University of New England Marine Animal Rehabilitation and Conservation Program (UNE MARC) and the Riverhead Foundation for Marine Research and Preservation (Riverhead) between 2010 and 2012 (Table 1). All blood was collected from the extradural vein or plantar venous plexus of the hind flipper and collected into sterile Vacutainer tubes containing sodium heparin (Becton Dickson, Franklin Lakes, New Jersey, USA), shipped overnight on cold packs, and processed within 24 h. RNA from peripheral blood mononuclear cells (PBMCs) was processed as stated later in the text to detect PDV in PBMCs before use in the LP and infection experiment.

PBMCs were collected from heparinized blood by Ficoll density gradient centrifugation. To compare blood leukocytes before and after PBMC isolation, erythrocytes from a subsampled 1-ml aliquot of blood were first lysed using 0.15 M ammonium chloride lysing buffer solution (Brousseau et al. 1999) at room temperature (37 °C). Briefly, 9 ml of lysing buffer solution was added to 1 ml of blood and rocked gently for 2 min. The sample was washed thrice at $500\times g$ for 10 min with Hanks Balanced Salt Solution (HBSS; Life Technologies, Grand Island, New York, USA). Blood warmed to room temperature for PBMC isolation was diluted 1:1 in HBSS, placed 1:1 over Ficoll 1077 (Amersham Biosciences, Uppsala, Sweden), and centrifuged at $990\times g$ for 45 min. The interface layer was isolated using a sterile pipette and washed 10:1 in HBSS at $500\times g$ for 10 min. Erythrocytes were lysed twice using lysing buffer. Leukocytes were resuspended in HBSS and washed three times with HBSS at $500\times g$ for 10 min each. The PBMCs were counted and the viability of cells assessed using the exclusion dye trypan blue. The proportion of leucocyte subpopulations was assessed by flow cytometry and cells cryopreserved in ice cold fetal bovine serum (Hyclone, Logan, Utah, USA) with 10 % DMSO (Sigma-Aldrich, St. Louis, Missouri, USA).

LP Assay

Cryopreserved PBMCs from four wild-capture and six stranded harbor seals ($n = 7$) were washed thrice in Dulbeco's Modified Eagle Medium (DMEM; Gibco BRL, Grand Island, New York, USA) for LP. This DMEM medium was supplemented with (all from Gibco BRL) 1 mM sodium pyruvate, 100 μM nonessential amino acids, 25 mM HEPES, 2 mM L-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin along with 10 % fetal bovine

Table 1 Seal blood samples

Source	ID	Sex	Age class	Stranding/capture location	Assay	SN titers PDV
NEFSC	019 Pv	Male	Adult	Rockland, ME	PDV	NA
NEFSC	47 Pv	Female	Adult	Chatham, MA	PDV	1:32
NEFSC	051Pv	Female	Adult	Cupsogue, Long Island, NY	PDV	1:64
NEFSC	028Pv	Female	Adult	Chatham, MA	PDV	1:32
UNE MARC	MARC 12-278	Male	Yearling	Biddeford, ME	PDV	NA
NEFSC	015 PV	Male	Adult	Chatham, MA	LP	Toxic
NEFSC	019 Pv	Male	Adult	Chatham, MA	LP	NA
NEFSC	007 Pv	Male	Adult	Chatham, MA	LP	Toxic
NEFSC	001 Pv	Male	Adult	Chatham, MA	LP	Negative
Riverhead	NY4180-10	Male	Yearling	Bellport, NY	LP	Negative
UNE MARC	MARC10-074	Male	Weanling	Rye, NH	LP	Negative
UNE MARC	MARC10-076	Female	Weanling	Nubble Light, York, ME	LP	Negative

Samples were collected between 2010 and 2012

NEFSC samples from wild capture harbor seals, *UNE MARC* blood samples from stranded seals collected from seals before release into the wild from UNE MARC and Riverhead, *assay* samples used for PDV infection LP, *NA* no data or samples available

serum (Hyclone, Logan, Utah, USA) and plated (1×10^6 cells/ml final concentration 100 µl/well) in triplicate in 96-well flat-bottom tissue culture plates (Falcon; Becton Dickinson, Franklin Lakes, New Jersey, USA). The purity of mononuclear cells was determined by flow cytometry based on the forward- and side-scatter profile. Cells from the same individual seal were exposed in vitro to Aroclor 1260 at 1, 5, 10, and 20 ppm or DMSO vehicle only (0 ppm). The selection and range of Aroclor 1260 used was based on toxicity and previously published in vitro PBMC exposures using Aroclor 1254 and 1260 (Davis and Safe 1989; Hammond et al. 2005; Brousseau and Fournier 2010; Dufresne et al. 2010). Because the removal of pathogens is largely dependent on T-cell activation, proliferation, and differentiation into effector and memory cells, the T-cell mitogen concanavalin A (Con-A; Sigma, St. Louis, MO, USA) was selected and used at the suboptimal concentration (0.1 µg/ml), which was shown to be more sensitive in detecting immunotoxicity than greater, or optimal, concentrations (Mori et al. 2006). Cells were exposed with and without Con-A. Cells were incubated at 37 °C with 5 % carbon dioxide (CO_2) for a total of 66 h. LP was evaluated by the incorporation of 5-bromo-2′-deoxyuridine (BrdU), which was added for the last 18 h of incubation, and detected with a monoclonal antibody and a colorimetric enzymatic reaction (Cell Proliferation ELISA BrdU (colorimetric), Roche Diagnostics GmbH, Mannheim, Germany) per the manufacturer's instructions using an enzyme-linked immunosorbent assay plate reader (Multiskan EX v 1.0) at 690 nm with a reference wavelength of 450 nm. The stimulation index was calculated as the ratio of the measured optical density between unstimulated *versus* stimulated cells.

Infection of Seal PBMCs With PDV Pre-exposed to Aroclor 1260

Cryopreserved cells from four wild capture harbor seals and one stranded harbor seal (total $n = 5$) were washed thrice in preinfection exposure supplemented DMEM. This medium included DMEM/Nutrient Mixture F-12, HEPES (DMEM/F-12, HEPES; Gibco BRL) supplemented with 200 µg/ml penicillin/streptomycin, 0.25 µg/ml Fungizon, and 10 % cosmic calf (CCS) fetal bovine serum (Hyclone), hereafter referred to as "preinfection DMEM." The purity of mononuclear cells was determined by flow cytometry based on the forward- and side-scatter profile. Cells were plated in triplicate (5×10^4 cells/well) with 20 ppm Aroclor 1260 and 0.1 µg/ml Con-A to stimulate proliferation in a 96-well round-bottom plates (Falcon, Becton Dickinson, Lincoln Park, New Jersey, USA). Infected and noninfected cells were incubated in two separate incubators as previously described (Bogomolni et al. 2015). Cells were then washed: Plates were centrifuged for 5 min at $500\times g$, the medium was replaced, and the cell solution was gently resuspended, and further incubated for 24 h in medium with 10 % CCS and human recombinant interleukin (IL)-2 (IL-2 h) at 100 IU/ml (Sigma) to activate IL-2 receptors as previously described (Siebelink et al. 1992). Plates were then washed (5 min at $500\times g$) and the medium removed for subsequent infection. Cells were infected with a multiplicity of infection of 1.0 with PDV USA 2006 for 1 h at 37 °C with 5 % CO_2. Control cells underwent the same procedures with the exception that an equivalent volume of medium without virus (100 µl) and the cells were placed in a second separate incubator. Virus or control medium was then removed from the cells: Wells were gently mixed with 200 µl medium, centrifuged (5 min at $500\times g$) to remove remaining virus or control medium, and gently mixed with 200 µL of new medium. Cells were maintained in 200 µl preinfection DMEM supplemented with 100 IU/ml IL-2 h. The medium used for virus infection contained 2 % serum, double the concentration of penicillin/streptomycin (400 µg/ml), and an additional 0.5 µg/ml gentamicin. Cells were maintained by adding 20 µl of this medium supplemented with IL-2 at days 5, 7, and 9.

RT-qPCR

Control and infected cells were sampled for RNA in separate bio-safety cabinets. For RNA collection, three samples were collected from each triplicate well using trypsin: tissue culture supernatant (S), lymphocytes (L) derived from the supernatant pellet after centrifugation for 30 s at $5223\times g$, and monocytes (M), which adhered to the bottom of the well. Plates were placed in the corresponding incubator at 37 °C with 5 % CO_2 and 200 µl of trypsin (Hyclone) for 7–10 min. Contents of each well were then collected into one microcentrifuge tube. Trypsin was subsequently removed by centrifugation for 30 s at $5223\times g$. Samples for RNA extraction were resuspended with RLT buffer (Qiagen, Valencia, California, USA). RNA extraction from seal PBMCs and for the fractions of the infection experiment was performed using a Qiashredder column (Qiagen) and RNeasy plus mini kit (Qiagen). This kit includes a DNA eliminator column. RNA quantity (ng/µl) was determined using a Nanodrop 1000 Spectrophotometer (Thermo Scientific). Samples were cryopreserved at −80°C until analysis.

PDV and glyceraldehyde phosphate dehydrogenase (GAPDH) primers and probes were used in a duplex reaction using Qiagen Quantifast Duplex RT-PCR +R in MicroAmp Optical 9-well reaction plates (Applied Biosystems) in the 7500 Real Time Cycler (Applied Biosystems) as previously described (Bogomolni et al. 2015). Copy numbers of PDV and GAPDH were determined using 50 ng/µl of sample RNA in duplicate. For

each run, at least eight log dilutions of each plasmid standard were used as the positive control. Samples, no template control, and standards were also run in duplicate. Each 20-µl sample reaction contained the following: 10 µl Quantifast Master mix, 0.4 µl ROX (an added reference dye designed to normalize the fluorescent reporter signal), 0.6 µl PDV forward primer (5′-ACC TCG ATG GGC AAT GTG TT-′3) and 0.6 µl PDV reverse primer (5'-GTC TTA CCG TAG ATC CCT TCT GAG AT-′3), 0.4 µl PDV Taqman probe (5′-[FAM]CAT GTC CCT CAT ATC AAA ACC TTC GGA GG [TAMRA]-'3), 0.4 µl GAPDH forward primer (5′-GTC TTC ACT ACC ATG GAG AAG G-′3) and 0.4 µl GAPDH reverse primer (5′-TCA TGG ATG ACC TTG GCC AG-′3), 1.92 µl GAPDH Taqman probe with LNA bases (5′-[HEX]G[+ C]CA{+ A]GAGGG[+ T]C[+ A]T[+ C]A[-TAMRA]-′3), 0.2 µl RT-mix, and ≤5 µl RNA with sterile RNAse-free water. Standards were run under the same conditions with an additional 3.08 µl water and 1 µl of each of the PDV and GAPDH standard ds-DNA plasmid in tenfold serial dilutions from 1^8 to 10^8 copies. The reaction was initiated with an RT step at 50 °C for 20 min, followed by a denaturation step at 95 °C for 5 min, and amplification for 40 cycles each of denaturation at 95 °C for 15 s and annealing and extension at 60 °C for 32 s. Each fluorescent reporter signal was measured against ROX. The lowest level of detection was the lowest amplified dilution of the standard. The baseline cycle threshold value was manually set at the point of initiation of the exponential phase of the reaction for each of the two standards detected.

The $\log_{10}$ $TCID_{50}$ of PDV USA 2006 was calculated using tenfold dilutions of virus grown on SLAM-transfected Vero cells and the results analyzed using the Spearmann–Karber titre calculator. The known $TCID_{50}$ stock virus was then made into tenfold dilutions, and RNA was extracted from these dilutions using the Qiagen Mini Viral RNA Isolation Kit. PDV RNA was then run in a duplex RT-qPCR reaction as described previously from these dilutions and used to calculate $TCID_{50}$ equivalents from measured ds-DNA standards, and therefore, infectious PDV equivalents in duplex reaction samples.

Statistics

Wild-capture and stranded animals were pooled as one population for use in these experiments. For LP, one-way repeated-measures analysis of variance (ANOVA) was performed to compare the exposed with the unexposed treatments using $p < 0.05$ for statistical significance. Dunnet's method was used to determine differences between multiple pairwise comparisons *versus* control (0 ppm). For the infection study, outliers were determined using Minitab (Minitab Inc., State College, Pennsylvania, USA) for each run on the ABI 7500. A generalized linear mixed model two-way repeated-measures ANOVA was performed for each cell fraction (S, L, M) to compare within means of treatment (exposed or unexposed to Aroclor 1260) and between day of infection (days 5, 7, 9, and 11). Statistics were analyzed using SigmaPlot 12.5 (Systat Software, San Jose, California, USA). Datasets that lacked normality and equal variance were transformed using a Johnson transformation using Minitab (Minitab). The Johnson transformation determines an optimal transformation from three different distribution families (Kotz and Johnson 1993). All error bars represented as mean ± SD.

Results

Animals Investigated and Sampling

RT-qPCR of PBMC samples indicated that no animals used in the study had detectable PDV RNA, i.e., they were considered to not show evidence of current infection. GAPDH copies in PBMCs were within the range of detection of the standards indicating suitable quality RNA for subsequent amplification tests.

LP Assay

Flow cytometry was used to determine the proportions of cell subpopulations on purification of mononuclear cells using Ficoll gradient centrifugation. Subpopulations can be distinguished by relative size (forward scatter) and complexity (side scatter). Granulocytes were large and complex (granular), lymphocytes were smaller and less complex, and monocytes were slightly larger than lymphocytes and less complex than granulocytes. Ficoll gradient centrifugation allowed selective loss of granulocytes and enrichment in mononuclear cells (lymphocytes and monocytes) in harbor seal samples. This purification resulted in 71–98 % mononuclear cells after Ficoll gradient centrifugation (Fig. 1).

Exposure of PBMCs to Aroclor 1260 did not significantly alter LP at any of the tested concentrations when compared with the unexposed controls (Fig. 2). Subsequently, a level of 20 ppm was used for infection experiments based on previously published literature and sum PCB exposure concentration in tissues from harbor seals of the northeast United States. (Addison and Brodie 1977; Shaw et al. 2005).

RT-qPCR

Aroclor 1260–exposed lymphocytes exhibited a 3.9-fold decrease in PDV (average difference of 1185 copies of PDV plasmid) at day 7 ($p < 0.001$) and a 2.4-fold increase

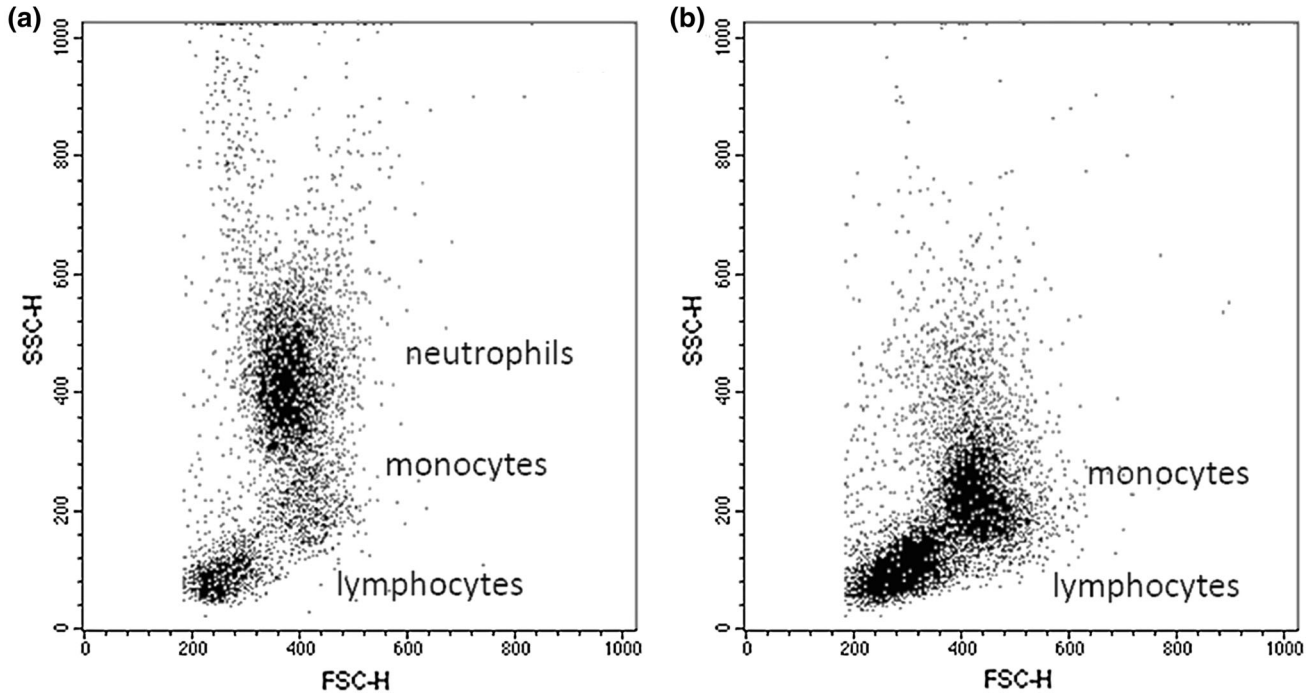

Fig. 1 PBMC isolation. Representative scatter plots of the flow cytometric profile of harbor seal peripheral blood leukocytes **a** before and **b** after Ficoll gradient centrifugation shows the selective loss of granulocytes and enrichment in mononuclear cells (lymphocytes and monocytes) in harbor seal cell isolation

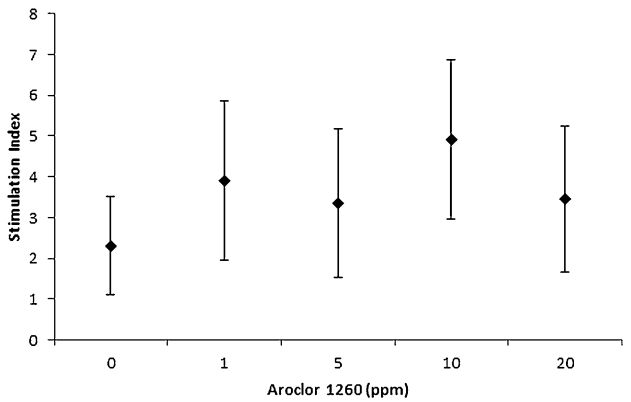

Fig. 2 Lymphocyte proliferation. Exposure of harbor seal PBMCs ($n = 10$) to Aroclor 1260 with 0.1 μg/ml Con-A did not modulate LP significantly at any tested concentration between unstimulated and stimulated PBMCs (mean ± SD)

at day 11 (average difference of 1177 copies of PDV plasmid) ($p = 0.003$) compared with unexposed control cells (Fig. 3a). PDV quantity in cells pre-exposed to Aroclor 1260 increased through the experiment with more virus at day 9 ($p = 0.019$) and 11 ($p = 0.048$) compared with day 7 in the exposed lymphocytes. PDV quantity in unexposed lymphocytes did not change over time.

Differences were apparent between the Aroclor 1260 pre-exposed and control monocytes with pre-exposed cells having 1.7 times less virus (average difference of 524 copies of PDV plasmid) at day 7 ($p = 0.002$) and a 4.1-fold increase (average difference of 12,474 copies of PDV plasmid) at day 11 ($p < 0.001$) (Fig. 3b). In monocytes pre-exposed to Aroclor 1260 preexposed, virus increased between days 5 and 11 ($p = 0.005$), days 7 and 9 ($p = 0.015$), as well as days 7 and 11 ($p = 0.001$). PDV quantity in monocytes that were not exposed to Aroclor 1260 did not change over time.

Viral quantity was also lower at day 7 with a 1.7-fold decrease with an average of 56 copies PDV plasmid ($p = 0.005$) and a 1.7-fold increase at day 11 ($p = 0.005$) with an average of 87 copies of PDV plasmid for the pre-exposed *versus* control supernatant fractions (Fig. 3c). The supernatant from pre-exposed cells had increases in virus throughout the time course of infection between days 5 and 9 ($p = 0.048$), 5 and 11 ($p = 0.008$), 7 and 9 ($p = 0.011$), and 7 and 11 ($p = 0.001$), but no such increase was observed in supernatant from control cells.

The difference in viral quantity between unexposed cells and monocytes pre-exposed to Aroclor 1260 t day 7 was 1.7-fold with an average difference of 524 copies of PDV plasmid. For lymphocytes at day 7, this was 3.9-fold with an average difference of 1185 copies.

GAPDH quantity in control and experimental cells was measurable at the end of the infection (day 11). Copies of GAPDH plasmid in the Aroclor pre-exposed and nonexposed cells were not significantly different comparable

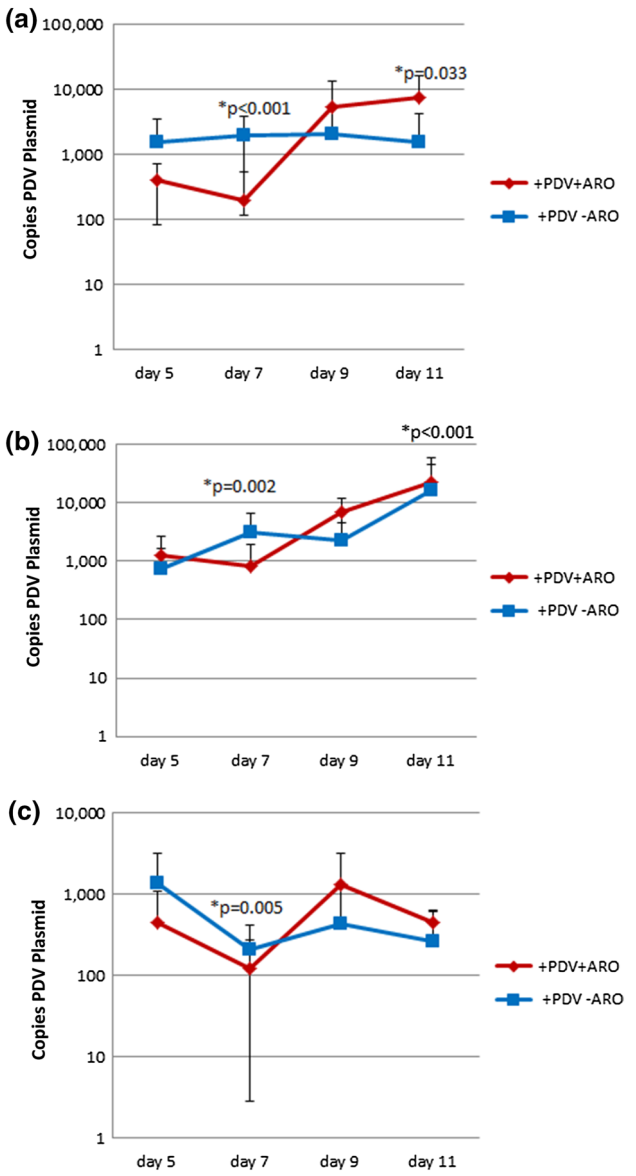

Fig. 3 Quantity of PDV during in vitro infection. PDV quantity measured by RT-qPCR (copies of PDV plasmid) in **a** lymphocyte, **b** monocytes, and **c** supernatant fractions from harbor seal cells exposed to 20 ppm Aroclor 1260 (PDV+, Aroclor 1260+) and unexposed control cells (PDV+, Aroclor 1260–). Samples were taken at days 5, 7, 9, and 11 after infection. Results are presented as mean ± SD ($n = 5$) *($p < 0.05$)

ranging from 4413 ± 3859 copies in supernatant to 213 ± 522 in lymphocytes and 283 ± 440 in monocytes.

Assay Sensitivity Based on $TCID_{50}$ Equivalents of Standard PDV ds-DNA Plasmid Standard

The PDV virus isolate was calculated to have a $TCID_{50}$ of 5.72 ($log10TCID_{50}$/ml), which is equivalent to 331,131 infectious particles/ml. The limit of detection and sensitivity of the assay was 0.5 (equivalent to 12 copies of PDV standard) to 524,807 infectious particles with an equivalent of 46,445,300 copies of PDV standard (Fig. 4).

Discussion

Since the first investigation in 1988 into what had become a growing number of epizootics due to morbillivirus, there has been extensive research focused on understanding the relationship between anthropogenic contaminants and disease due to underlying immune suppression. Simmonds (1992) suggested that the evidence pertaining to the scale, frequency, location, correlation to contaminant burden, clinical signs, and our understanding of toxicology of these contaminants warranted considering these events as something other than natural occurrences (Simmonds 1992). Therefore, the goal of this study was to test the hypothesis that exposure to an anthropogenic contaminant can influence the functioning of T lymphocytes, an important component of the acquired immune system necessary for combating viral infection, and also directly influences viral replication after contaminant exposure. T-lymphocyte proliferation in harbor seals was assessed after exposure to Aroclor 1260, a mixture of highly chlorinated PCBs, and the effects on PDV viral replication were also measured.

At the concentrations tested, Aroclor 1260 did not significantly modulate harbor seal T-lymphocyte proliferation using the mitogen Con-A. In comparison, in vitro proliferation of gray seal T lymphocytes was significantly decreased on exposure to 15 ppm Aroclor 1254 (Dufresne et al. 2010). Studies in a single gray seal indicated that Aroclor mixtures, specifically Aroclor 1260, caused a decrease in T-lymphocyte proliferation of blood and lymph derived T cells at concentrations between 50 and 100 ppm and an increase of 30 % for proliferation in lymph node–derived T cells at 6.25–12.5 ppm (Brousseau and Fournier 2010). Levin et al. (2005a) reported a that a positive correlation between mitogen-induced T-lymphocyte proliferation and blubber total PCBs in weaned harbor seals and noncoplanar PCBs significantly contributed to the changes in LP. A general suppressive effect was noted with exposure of harbor seal PBMCs to PCB 156 and PCB 80, although this was not statistically significant (Neale et al. 2002).

The importance of species differences in interpreting immune response to simple chemical mixtures, including potential synergistic and antagonistic effects, was shown by Mori et al. (2006). Species differences in immunotoxic response on exposure to PCB mixtures were evident for eight species of marine mammal species. In addition, noncoplanar PCBs were shown to modulate immune function, suggesting a nonaryl hydrocarbon receptor (AhR)-mediated mechanism of action for these PCBs on immune cells. Therefore, toxic equivalents, which are

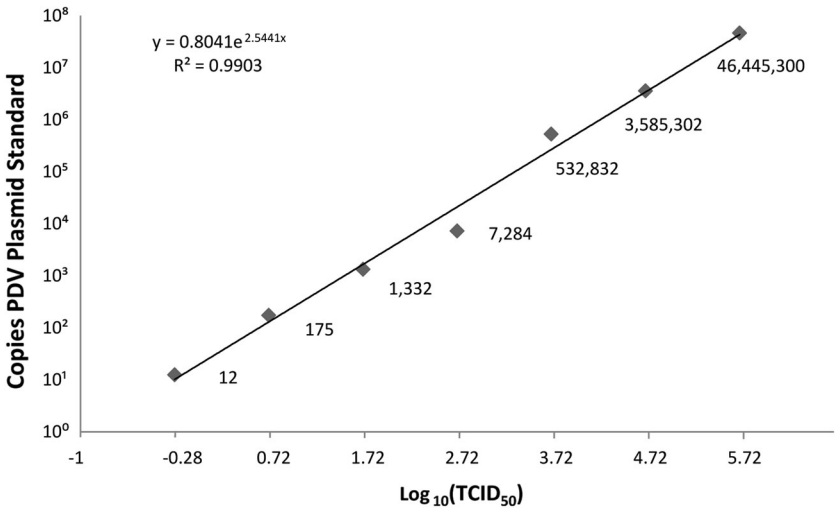

Fig. 4 Assay sensitivity based on $TCID_{50}$ equivalents of standard PDV ds-DNA plasmid standard. The PDV USA 2006 virus isolate used in the experiment was calculated to have a $TCID_{50}$ of 5.72 ($log10TCID_{50}$/ml), which is equivalent to 331,131 infectious particles/ml. The limit of detection and sensitivity of the assay was 0.5 (which is equivalent to 12 copies of PDV standard) to 524,807 infectious particles with an equivalent of 4.64×10^7 copies PDV standard

commonly used to assess the toxicity of PCBs, did not capture nondioxin-like immunotoxic effects in marine mammals. Aroclor 1260, an Aroclor mixture of lower toxicity in mice, was shown to have the greatest toxic potential in gray seals based on inhibition of 50 % of proliferative response (Brousseau and Fournier 2010). A tissue matrix–dependent effect of PCBs, as well as species differences in immunotoxic response and the importance of mixtures of chemicals, should be taken into account when attempting to understand the effects of chemicals on immune response.

During this infection experiment, there were significant lower numbers of PDV copies in cells exposed to Aroclor 1260 at day 7 in monocytes and lymphocytes, as well as in the supernatant, compared with unexposed controls. The reasons for this decrease are not likely due to direct chemical cytotoxic effects. The potential cytotoxicity caused by the concentration of Aroclor 1260 used during these in vitro exposures was considered when designing the experiment. Concentrations were chosen to be environmentally relevant and thus less likely to cause cytotoxic effects. In guinea pigs, Aroclor 1260 at 50 ppm was shown to be immunosuppressive by decreasing humoral and cell-mediated immunity including decreased leukocyte counts in peripheral blood. This was thought to be caused by direct PCB effects on immune cells (Vos and Driel-Grootenhuis 1972). Concentrations assessed here were lower than this value, and the results of our LP assay did not support direct chemical cytotoxicity. In addition, GAPDH measured in cells was within detectable levels throughout the experiment. Previous studies indicate that in controlled exposure of harbor seal PBMCs (without stressor, without virus), cellular GAPDH is decreased at day 11, which was attributed to the attrition of cells from the primary culture (Bogomolni et al. unpublished data). Similarly, PBMCs in this study exhibited decreased GAPDH at day 11 in control and in PDV-infected cells. All other time points exhibited high copy numbers of GAPDH. It is therefore unlikely that the decrease in PDV at day 7 is due to the lack of viable host cells, especially because PDV increased at a later time point.

The decrease in virus is also not believed to be due to immunotoxic effects. This in vitro system likely fails to provide the cellular interactions and microenvironment (such as a lymph node) to optimize the generation of a primary immune response, and the relatively small number of cells used (5×10^4 cells per well) is unlikely to include PDV-specific cells given the diversity of T cells. Furthermore, no effects on T-cell proliferation were observed on in vitro exposure to similar doses in our study.

The delay in infection could be due to several factors including differences in host cell activation and expression, activation of cell surface receptors, or differences in host cell chemical signaling. After in vitro stimulation with antigen or mitogen, activated T cells express SLAM, or CD150, which enables an increase in morbillivirus infection (Farina et al. 2004; Yanagi et al. 2009). PDV USA 2006 preferentially binds to these SLAM-expressing immune cells (Ohishi et al. 2012; Melia et al. 2014). The importance of this activated receptor was illustrated when a laboratory-modified strain of CDV, which was unable to recognize the SLAM receptor, was not able to spread infection (Von Messling et al. 2006). Activation of other cell receptors, such as IL-2R and ProHB-ECF, can also influence morbillivirus infection (Yanagi et al. 2009; Melia et al. 2014). It is therefore possible that differences in virus replication in Aroclor-exposed cells could be explained by transient suppression of, delay of, or other alteration in the timing of cell activation and cell surface expression of receptors relevant to infection with PDV.

Documentation exists for the ability of PCBs and polycyclic aromatic hydrocarbons (PAHs) to interfere with the production of IL-2 and T-cell receptor signaling in laboratory animals as well as harbor seals (Exon and Koller 1985; Neale et al. 2005). Exposure of Con A–stimulated harbor seal PBMCs to both PAHs and PCB significantly decreased the expression of protein tyrosine kinases and cytokines produced through T-cell receptor signaling, specifically IL-2, needed for the activation and clonal expansion of T cells (Neale et al. 2005). In relation to subsequent exposure to PDV, activation of T cells from a naïve state is important for the activation of other host receptors, specifically CD150, the host receptor necessary for the cell to be receptive to PDV infection (Tatsuo and Yanagi 2002).

AhR is another important host cell receptor with growing interest for its role in contaminant exposures, viral replication, and immune suppression (Lawrence and Kerkvliet 2006; Head and Lawrence 2009; Stockinger et al. 2014). AhR regulates the expression of genes that encode cytochrome P450 enzymes (CYPs); the induction of these enzymes is involved in the metabolism of various endogenous and exogenous substances, including PCBs and PAHs, that are known to be immunosuppressive. Activation of AhR with TCDD also has been shown to suppress the clonal expansion and differentiation of influenza virus-specific CD8+ T lymphocytes (cytotoxic T cells) important for the production of interferon-γ (BP et al. 2005) as well as CD4+ T lymphocytes (T-helper cells) (Lawrence et al. 2000). The mechanism that induces these changes is not known, but it is believed to be due to differences in gene expression (Head and Lawrence 2009).

Aroclor 1260 may be suppressing clonal expansion and activation of T cells through AhR-mediated or -independent mechanisms, which in turn decreases infection, at least transiently, by preventing the activation of cell surface receptors necessary for PDV to infect the cell. At day 11, the increase in viral load could have occurred if the effects of Aroclor were transient in nature or affected by the timing of activation. It is also possible that another mechanism, such as cytokine expression, is involved at later time points. In vivo morbillivirus exposures indicate that cytokine profiles can change dramatically during infection. Transcripts of proinflammatory cytokines were upregulated in CDV infection in dog with no change in measured anti-inflammatory cytokines (Markus et al. 2002). In CDV-exposed ferrets, those that mounted a greater anti-inflammatory response survived (Svitek and von Messling 2007). In ferrets that died from CDV infection, a suppressed cytokine response was noted within the first days of infection compared with those that survived. This was similarly found in mink infected with CDV (Nielsen et al. 2009).

In vivo exposures of seals to PDV, with or without PCBs, were undertaken soon after the 1988 European epizootic (Harder et al. 1990, 1992). The duration of cell-associated viremia, PDV-antigen distribution in tissues of fatally infected seals and the humoral immune response to PDV shown no differences between PCB exposed and unexposed control seals (Harder et al. 1992). Although these studies provide evidence for the lack of immunosuppressive effects of some PCBs on susceptibility to disease in vivo, our recent understanding of the complex nature of mixtures and immunomodulation underscores the utility and ethical approach of in vitro exposures over future in vivo studies to understand this relationship.

Conclusions

Studies that have focused on mixtures of contaminants, rather than single chemicals, give us an insight into what may actually occur in real-world exposures (Levin et al. 2005b, 2007a; De Guise et al. 2006; Mori et al. 2006). Few studies exist that show the influence of these contaminants, let alone in the context of complex real-world exposures, on the susceptibility to infection in marine mammals. This study builds on a growing list of in vitro studies and addresses both the exposure to mixtures of POPs (Aroclor 1260) and subsequent susceptibility to infection by PDV. The results here indicate that exposure to Aroclor 1260 affects the replication of PDV in harbor seals cells with exposure resulting in a decrease in virus early during infection, and an increase during late infection. The consequences of this contaminant-induced infection pattern in harbor seals, a highly susceptible host, could result in a greater potential for systemic infection with greater viral load and therefore possibly affect an animal's resistance to the disease caused by PDV. These finding could help explain the correlative findings seen in wild populations exposed to a range of persistent contaminants that suffer from morbillivirus epizootics.

Acknowledgments This project was possible thanks to the John H. Prescott Marine Mammal Rescue Assistance Grant Program (Grant No. NA10NMF4390260) and with support from the Sounds Conservancy Quebec Labrador Foundation Grant, the Switzer Foundation Environmental Leadership Fellowship, and he NOAA/UCONN Oceans and Human Health I-RICH Fellowship. Samples were obtained under NOAA NMFS Marine Mammal Parts Authorization, NOAA NEFSC Permit No. 17670, and PDV under United States Department of Agriculture Permit No. 123319. Many thanks to Judy St. Leger of SeaWorld Parks and Entertainment for serology data obtained on wild captured seals, to Erika Cote, Rebecca Gast, Guillermo Risatti, Shannon Prendiville, Kristen Patchett, the Marine Animal Rehabilitation and Conservation Center at UNE, the Riverhead Foundation for Marine Research and Preservation, and the United States Northeast/Greater Atlantic Region Marine Mammal Stranding Network staff and volunteers.

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