PAH- and PCB-induced alterations of protein tyrosine kinase and cytokine gene transcription in harbor seal (*Phoca vitulina*) PBMC

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Abstract
Mechanisms underlying *in vitro* immunomodulatory effects of polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls (PCBs) were investigated in harbor seal peripheral leukocytes, via real-time PCR. We examined the relative genetic expression of the protein tyrosine kinases (*Fyn* and *Itk*), which play a critical role in T cell activation, and IL-2, a cytokine of central importance in initiating adaptive immune responses. IL-1, the macrophage-derived pro-inflammatory cytokine of innate immunity, was also included as a measure of macrophage function. Harbor seal PBMC were exposed to the prototypic immunotoxic PAH benzo[a]pyrene (BaP), 3,3',4,4',5,5'-hexachlorobiphenyl (CB-169), a model immunotoxic PCB, or DMSO (vehicle control). Exposure of Con A-stimulated harbor seal PBMC to both BaP and CB-169 produced significantly altered expression in all four targets relative to vehicle controls. The PTKs *Fyn* and *Itk* were both up-regulated following exposure to BaP and CB-169. In contrast, transcripts for IL-2 and IL-1 were decreased relative to controls by both treatments. Our findings are consistent with those of previous researchers working with human and rodent systems and support a hypothesis of contaminant-altered lymphocyte function mediated (at least in part) by disruption of T cell receptor (TCR) signaling and cytokine production.

Keywords: Polycyclic aromatic hydrocarbon, polychlorinated biphenyl, *Phoca vitulina*, protein tyrosine kinase, cytokine, interleukin

Introduction
The immunotoxic effects of polycyclic aromatic hydrocarbons (PAHs) and halogenated aromatic hydrocarbons (HAHs), such as polychlorinated biphenyls (PCBs), have been investigated in rodent and human systems where they have been found to induce a broad range of adverse effects (reviewed in White 1986, Vos and Luster 1989, Kerkvliet and Burleson 1994, White et al. 1994). These ubiquitous marine pollutants tend to bioaccumulate through the food web and concentrate in tissues of marine mammals, whose long life span and high content of adipose tissue make them an ultimate sink for lipophilic contaminants. The highest levels are found in species, such as the harbor seal (*Phoca vitulina*), occupying top trophic levels and inhabiting heavily polluted coastal waters. PAHs and HAHs have been demonstrated to decrease host resistance to bacterial, viral, and parasitic disease in rodent models, and contaminant-induced immunosuppression is speculated to have contributed to the high mortality observed in several marine mammal populations during recent morbillivirus epizootics (Osterhaus and Vedder 1989, Hall et al. 1992, Aguilar and Borrell 1994, Tanabe et al. 1994, Van Loveren et al. 2000).

Altered immune parameters associated with environmental exposure to PAHs and PCBs have been reported for several species of pinnipeds and cetaceans and include reduced antigen-specific antibody responses, depressed T-lymphocyte proliferative
responses to mitogen, depressed plasma retinol, increased white blood cell count and depressed red cell parameters, and frequent infections with mildly pathogenic bacteria and high rates of cancer (Martineau et al. 1994, Lahvis et al. 1995, Beckmen et al. 2003, Jenssen et al. 2003, Neale et al. 2005). Experiments in which captive harbor seals were fed heavily-contaminated (especially with PCBs) fish also supported a hypothesis of contaminant-induced immunological impairment. In addition to an increase in neutrophil counts in peripheral blood, the following parameters were depressed in seals fed contaminated fish: plasma retinol and thyroid hormone levels, natural killer cell activity, mitogen- and antigen-induced T cell proliferative responses, mixed lymphocyte reaction, and delayed-type hypersensitivity and specific serum antibody responses (Reijnders 1986, Brouwer et al. 1989, De Swart et al. 1996, Ross et al. 1996, Van Loveren et al. 2000).

Specific mechanisms of PAH- and PCB-induced immunotoxicity in these organisms, however, remain largely unexplored. Signal transduction and the T lymphocyte/IL-2 pathway have been proposed as sensitive targets of both PAHs and HAHs (Exon et al. 1985, House et al. 1987, Kerkvliet and Brauner 1987, House et al. 1989, Clark et al. 1991, Davila et al. 1995). The removal of pathogens is largely dependent on the process of T cell activation, proliferation, and differentiation into armed effector and memory cells. Activation of T lymphocytes via T cell receptor (TCR) signaling begins with protein tyrosine kinase (PTK) activation, which initiates a cascade of intracellular signaling that transfers the signal to other molecules and eventually carries it to the nucleus, where gene transcription for the production of cytokines and other mediators of lymphocyte function occurs (a very similar process occurs in B cells). Previously, we demonstrated that certain PCB and PAH compounds suppressed in vitro mitogen-stimulated T cell proliferation in the harbor seal (Neale et al. 2002). To explore potential mechanisms of this effect, here we exposed harbor seal leukocytes to the prototypic immunotoxic PAH, benzo[a]pyrene (BaP), and 3,3',4,4',5,5'-hexachlorobiphenyl (CB-169), a model immunotoxic PCB, in vitro, and examined, via real-time PCR, the relative expression of certain genes important in T cell activation and function.

We selected targets relevant to early TCR signaling and downstream cytokine production (these topics are reviewed in Weiss and Litmann 1994, Kung and Thomas 1997, Qian and Weiss 1997, Janeway et al. 1999, Abbas and Lichtman 2003). These included the PTKs Fyn and Lck, the T cell growth factor, IL-2. The earliest biochemical events within the T cell that follow clustering of the TCR complex and coreceptors upon antigen stimulation are (1) the activation of PTKs including the Src-family kinases Fyn and Lck, associated with the cytoplasmic domains of the clustered CD3 and coreceptor proteins, and (2) phosphorylation by these PTKs of tyrosines in the ITAMs of the CD3 and ζ chains. ZAP-70 binds to phosphotyrosines of the ζ chain and phosphorylates adapter proteins. The Tec family kinase Itk, once activated, binds phosphorylated adapter proteins and can then phosphorylate and activate phospholipase C gamma 1 (PLCγ1). Active PLCγ1, in turn, hydrolyzes membrane PIP2 to generate IP3 and DAG, which then activate two distinct downstream signaling pathways in T cells. A third signaling pathway following phosphorylation of the adapter protein LAT by ZAP-70 is the Ras-MAP kinase pathway. All three eventually converge to generate active transcription factors that stimulate expression of various genes responsible for cellular responses.

Among the earliest detectable cellular responses to antigen recognition and TCR signaling is the secretion of cytokines. IL-2, the growth factor for antigen-stimulated T cells, is responsible for T cell clonal expansion after antigen recognition, primarily through autocrine activity. IL-2 also increases synthesis of other cytokines in T cells, promotes the proliferation and differentiation of NK cells, and acts as a growth factor and stimulus for antibody synthesis in B cells. In addition, we investigated genetic expression of IL-1, the macrophage-derived pro-inflammatory cytokine of innate immunity, as a measure of macrophage function. We included this target cytokine not only because the macrophage has been suggested as a primary target of PAH immunotoxicity in previous studies (Myers et al. 1987, 1988), but also because of the numerous roles served by macrophages in the effector phases of adaptive immune responses.

Materials and methods

Free-ranging harbor seals (2 female pups, 1 male pup, 1 female yearling) were captured using tangle nets near a haul-out site in San Francisco Bay, California, on 5/31/04 and 6/1/04 (NMFS Scientific Research Permit No. 555-1565). Seals were physically restrained. Blood was drawn from the extradural venous sinus into sterile evacuated blood collection tubes containing acid citrate dextrose. Peripheral blood mononuclear cells (PBMC) were isolated from whole blood within 8 h of collection following methods detailed previously (Neale et al. 2002, 2004). White cell pellets were suspended in complete medium (RPMI 1640 medium with 2 mM L-glutamine, 0.08% gentamicin, and 10% fetal bovine serum). Cell viability was assessed via trypan blue exclusion and viable cells counted microscopically using a hemacytometer. PBMC (4 × 10⁶ cells/ml) were incubated (37°C, 5% CO₂, humidified atmosphere) in 48-well microtiter plates containing 250 μl cell suspension per well.
Following overnight incubation, wells were supplemented with 250 μl medium solutions containing concanavalin A (Con A; Sigma-Aldrich, St. Louis, MO, USA), a polyclonal T cell activator in the harbor seal, and either BaP (Sigma-Aldrich), CB-169 (AccuStandard, New Haven, CT, USA), or the vehicle control (anhydrous dimethylsulfoxide, DMSO; Sigma-Aldrich), in triplicate for each individual and treatment. Final concentrations were 5 μg/ml Con A and 20 μM BaP, CB-169, or DMSO. Cells were incubated for 4 h. Plates were then centrifuged at 200g for 2 min, supernatants were removed, and 250 μl TRIZol reagent (GibcoBrl/Life Technologies, Gaithersburg, MD, USA) added to each well with repetitive pipetting to ensure complete cell lysis. After 5 min incubation at room temperature, contents of wells were transferred to 1.5-ml microcentrifuge tubes. RNA was isolated (phenol/chloroform separation) and precipitated from the aqueous phase with isopropanol, according to the manufacturer’s instructions. The RNA pellet (approximately 20 μg) was then redissolved in 20 μl diethyl-pyrocarbonate (DEPC)-treated RNase-free water (hereafter, “water”), pooled from triplicate samples, and incubated at 60°C for 10 min. For reverse transcription, approximately 5 μg RNA (in 5 μl water) was added to a mixture of 1 μl (0.5 μg) oligo(dT)₁₂–₁₈ primers, 1 μl dNTP Mix (10 mM), and 5 μl water. This mixture was incubated at 70°C for 10 min then cooled on ice. Next, 4 μl of 5 × First Strand Buffer, 2 μl of dTT (0.1 M) and 1 μl (10 units) RNase inhibitor were added to the first mixture and incubated at 42°C for 2 min. Finally, 1 μl (200 units) Superscript II reverse transcriptase was added for a total volume of 20 μl. This was incubated for 42°C for 50 min. The reverse transcriptase enzyme was heat-killed at 70°C for 15 min. All RT reagents supplied from Invitrogen (Carlsbad, CA, USA).

We designed the following real-time PCR primers (5’–3’) for amplification of harbor seal cDNA (forward/reverse): IL-1β, TGAGCT GTACCGAGAGTCTCAA/AAACCTTCTATCTCCCCTTCAA; IL-2, GAAACTAAAGGGATCTGAAAC/GTGTAGAGTCCA/AAACCCTTCTATTCCCCTTCCA; IL-2, GGGATGACAAGGTTCGTCCTTG; and the internal control/housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, for target gene adjustment; these ratios were then compared between treatments and controls using 2-tailed, paired t-tests. To strengthen our analysis, we performed identical real-time PCR using another common housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase, for target gene adjustment; these results were qualitatively and quantitatively similar to those obtained using B2M correction and are therefore not presented here.

**Results**

Expression of PTKs was less variable among individuals than expression of interleukins (Table I). Fyn was least variable and IL-2 most variable overall; greatest inter-individual differences were observed in BaP and DMSO exposures for IL-2, related largely to lower Ct ratios (corresponding to greater cDNA copy number) for Harbor Seal 2. PBMCs from this individual also produced greater IL-1 transcripts in all samples than those of the other three seals. Exposure of Con A-stimulated harbor seal PBMC to both the model PAH and PCB produced significantly altered expression in all four targets relative to vehicle controls (Figure 1). Fyn and Itk were
both up-regulated; expression of \textit{Itk} was significantly increased for PBMC exposed to BaP \((p < 0.01)\) and CB-169 \((p = 0.01)\). Likewise, \textit{Fyn} expression was significantly increased in CB-169 samples \((p = 0.03)\); the increase was marginally nonsignificant for BaP exposures \((p = 0.08)\). mRNAs for IL-2 were decreased relative to controls by BaP \((p = 0.01)\) and CB-169 \((p = 0.02)\). Likewise, IL-1 transcription was depressed by both treatments as well \((BaP \ p < 0.01, \ CB-169 \ p = 0.03)\).

### Discussion

Experimental studies using laboratory rodents and human cell lines have elucidated PAH- and HAH-sensitive endpoints of immune dysfunction of both humoral and cell-mediated immunity, and have provided evidence for various mechanisms of action in these mammalian systems (reviewed in Kerkvliet and Burleson 1994, White et al. 1994, Davila et al. 1995). Most studies have focused on the prototypic
immunotoxic PAHs BaP and 7,12-dimethylbenz[a]anthracene (DMBA) and their metabolites, and the prototypic immunotoxic HAH, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD). The following proposed mechanisms whereby PAHs mediate their immunosuppressive actions are not mutually exclusive, and include interaction with the intracellular aromatic hydrocarbon (Ah) receptor; disruption of lymphocyte signaling; altered interleukin production; disruption of intracellular calcium mobilization; and metabolic activation to reactive metabolites. Many of the effects of TCDD and structurally related HAHs appear to be mediated via binding to the Ah receptor; toxicity may be caused by the products of as-yet-unidentified genes induced, along with the CYP1A1 enzyme, following Ah receptor activation. However, in vivo, ex vivo, and in vitro studies of TCDD immunotoxicity have produced conflicting data with respect to target cell types and specific immune responses to exposure; these discrepancies may relate to in vitro culture conditions, genetic differences among experimental species or strains at the Ah locus, and direct vs. indirect (e.g. via effects on the endocrine system) effects on lymphoid tissues.

This study is, to our knowledge, the first to investigate effects of in vitro PAH or PCB exposure on PTK or cytokine targets in a marine mammal. Previous research on rodent and human cells, however, does provide a basis for comparison. Similarly to our findings, exposure to DMBA in a human T cell line resulted in the activation of Lck kinases, as well as increased tyrosine phosphorylation of PLCγ1, increased formation of IP3, and a sustained rise in intracellular calcium; these authors speculated that xenobiotic-induced phosphorylation mimicked antigen-receptor activation in T cells and could lead to alterations in antigen responsiveness in vivo (Archuleta et al. 1993). In another study from the same research group, the PTKs Lyn and Syk were activated in a human B cell line exposed to BaP metabolites, and this effect also was associated with increased intracellular calcium (Mounho and Burchiel 1998). Stimulation of tyrosine phosphorylation also was reported for murine B lymphocytes exposed to TCDD (Kramer et al. 1987, Clark et al. 1991).

Decreased production of IL-2 following PAH and PCB exposures also has been reported previously. IL-2 activity of rat splenocytes was significantly suppressed following in vitro exposure to a PCB mixture (Exon et al. 1985). Steppan et al. (1993) observed a profound suppression of IL-2 and INF-γ production in CB-169-treated mice. Similarly for PAHs, splenocytes isolated from mice exposed in vivo to DMBA or BaP showed suppressed IL-2 production (House et al. 1987, Lyte et al. 1987). In vitro exposure of mouse splenocytes to DMBA or BaP also resulted in suppression of IL-2 production (House et al. 1987, Thurmond et al. 1988, Pallardy et al. 1989).

PTKs and IL-2 apparently have not been simultaneously investigated in previous studies of PAH or HAH immunotoxicity. So far as PAH- and PCB-induced increases in Fyn and Itk transcription corresponded to increased synthesis of these proteins—thus presumably stimulating TCR signaling—depression of IL-2 gene transcription indicated incomplete TCR signal transduction. The mechanistic link between the observed phenomena is not clear and the many intervening biochemical events suggest various scenarios; potential intermediate targets (direct or indirect) include regulatory kinases and/or phosphatases, elements of the ras/MAP kinase cascade and the Ca\(^{2+}\)-dependent DAG/PKC and IP\(_3\)/calcineurin pathways, and transcription factors such as NF-AT (nuclear factor of activation in T cells), which binds to the promoter region of the IL-2 gene and is necessary to activate its transcription (Archuleta et al. 1993, Qian and Weiss 1997, Chakravarti et al. 1998, Yu et al. 2002).

The strong suppression of IL-1 expression suggested a direct inhibitory effect of the model compounds on the macrophage. Alternatively, effects of BaP and CB-169 on macrophages could have been indirect, i.e. via effects on T\(_{H1}\) cells resulting in suppressed expression of INF-γ (a factor of macrophage activation), which in turn could reduce macrophage production of IL-1 (Steppan et al. 1993). The apparent effect on macrophage function suggests that exposure to these compounds could compromise antigen presentation/processing and macrophage support of T cell function in humoral immunity, as has been shown in BaP exposures using a mouse model (Myers et al. 1988). The ability of the model PAH and PCB to decrease production of inflammatory chemoattractive mediators such as IL-1 would imply that exposure could result in decreased host resistance to infection.

Here we have begun to explore mechanisms underlying in vitro immunomodulatory effects of PAHs and PCBs in harbor seal PBMC, focusing on alterations in gene transcription of PTK, which play a critical role in T cell activation, and IL-2, a cytokine of central importance in initiating adaptive immune responses. Our findings are consistent with those of previous researchers working with human and rodent systems and support a hypothesis of contaminant-altered lymphocyte function mediated (at least in part) by disruption of TCR signaling and cytokine production. The potential for contaminant-induced immune alterations has particularly important implications for marine mammals experiencing pathogen exposure concomitant with chronic, multiple exposures to relatively high levels of persistent organic pollutants (POPs) such as certain of the PAHs and PCBs. Reported levels of POPs in free-ranging harbor seals inhabiting many polluted areas of Europe and North America are sufficiently high to suggest that many populations may be at risk of immunotoxicity,
which could manifest as diminished host resistance and increased incidence and severity of infectious disease (Reijnders 1994, Ross et al. 1996, Neale 2004, Ross et al. 2004, Neale et al. 2005). Continued investigation of the cellular and molecular pathways of PCB- and PAH-induced immune dysfunction in marine mammals will enhance our understanding of individual and population health and promote marine mammal conservation.

Acknowledgements

We are greatly indebted to JT Harvey and crew (Moss Landing Marine Laboratories) for seal captures. This project was supported by the California Department of Fish and Game’s Oil Spill Response Trust Fund through the Oiled Wildlife Care Network at the Wildlife Health Center, School of Veterinary Medicine, University of California, Davis.

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