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The Effects of Tributyltin (TBT) and 3,3′,4,4′,5-Pentachlorobiphenyl (PCB-126) Mixtures on Antibody Responses and Phagocyte Oxidative Burst Activity in Channel Catfish, *Ictalurus punctatus*

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Abstract. The organotin tributyltin (TBT) is an antifouling biocide used in marine paints and is a common pollutant in harbor estuaries. We previously demonstrated that the immune system of channel catfish, *Ictalurus punctatus*, is a sensitive target organ of TBT. Exposure strongly suppresses humoral immune responses. Harbor estuaries often contain polychlorinated biphenyls (PCBs) due to their ubuquitous distribution. The coplanar congener 3,3′,4,4′5′-polychlorinated biphenyl (PCB-126) is also immunotoxic to channel catfish, but it suppresses only the innate immune responses and only at high doses. In this study we exposed channel catfish to TBT, PCB-126, or both in mixtures, with canola oil (CO) serving as the carrier control. Antibody responses to *Vibrio anguillarum* and phagocyte oxidative burst activity were measured after (1) a single dose of 0.01 or 1 mg/kg of each or both in combination, and (2) six injections of 1.7 or 170 μg/kg of each (or in combination) given every 3 days over a 16-day period to yield a cumulative dose of 0.01 or 1 mg/kg, respectively. We measured antibody responses to *V. anguillarum* 21 days after immunization and oxidative burst activities 14 and 21 days after the final treatment. The highest dose of TBT suppressed antibody responses after a single exposure. The high dose of PCB-126 also suppressed antibody responses. The addition of PCB-126 to TBT doses did not alter the antibody responses beyond the effects of TBT alone. In the repeated exposure group, only the high dose of TBT suppressed antibody responses. In animals exposed to mixtures, high levels of PCB-126 enhanced suppression associated with low levels of TBT, whereas PCB-126 protected against suppression associated with high levels of TBT. Single exposures to TBT or PCB-126 suppressed phagocyte oxidative burst activity. In animals exposed to mixtures, as a single exposure, the addition of a low dose PCB-126 protected against low dose TBT-related oxidative burst activity suppression. In the repeated exposure groups TBT suppressed oxidative burst activity, but only at the highest dose on day 21, while high doses of PCB-126 suppressed activity on day 14. Furthermore, low levels of PCB-126 reversed the suppressed oxidative burst activity associated with high levels of TBT on day 21. Overall, this study demonstrates moderate additivity in terms of the immunotoxicity of TBT and PCB-126 mixtures using these two endpoints of immune function in the channel catfish model.

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Halogenated aromatic hydrocarbons (HAHs), including halogenated dioxins, furans, biphenyls, and naphthalene, are common environmental contaminants, with the polychlorinated biphenyls (PCBs) being the most ubiquitous. Of the 209 congeners of PCBs, the coplanar structures receive the most attention due to their extreme toxicity. This toxicity, for the most part, relates to a structural similarity to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), the most toxic and prototype planar HAH. Mechanistically, planar HAHs mediate their toxicity primarily through the cytosolic aryl hydrocarbon receptor (Ahr), a well-described transcription factor for a variety of gene products, including CYP1A (Hahn 1994; Hahn and Stegeman 1994).

Because of their widespread distribution, PCBs exist in mixtures with other compounds, particularly in harbor estuaries. For example, the organotin antifoulant biocide tributyltin (TBT) is also a common pollutant in these systems (Hugget *et al*. 1992; Chau *et al*. 1997). Like PCBs, TBT is lipophilic and easily bioaccumulates in animal tissues (Lee 1985; Fent 1996). Although TBT exposure leads to endocrine disruption, developmental abnormalities, and immunotoxicity, its proximal mechanism(s) of action involves general membrane perturbation leading to apoptosis (Fent 1996). Like TBT, coplanar PCBs affect reproduction, development, and immune function (Holsapple *et al*. 1991; Walker and Peterson 1991). Unlike coplanar PCBs, TBT has no known specific ligand (receptor).

The most potent planar PCB is 3,3′4,4′,5-pentachlorobiphenyl (PCB-126), a congener with a TCDD equivalent factor of 0.1 (Kafafi *et al*. 1993). Because TCDD has an assigned TEF of 1.0, PCB-126 should exhibit TCDD-like toxicities at low levels. Regarding the immunotoxicity of planar HAHs, Spitzbergen *et al*. (1986a, 1986b) noted only moderate sup-

pression of the antibody response after exposure to TCDD in rainbow trout. These fish demonstrated enhanced susceptibility to to infectious hematopoietic necrosis (IHN) virus, suggesting that planar HAHs target only the nonspecific aspects of fish immune systems. In support of this interpretation, Rice and Schlenk (1995) demonstrated that PCB-126 does not affect the antibody response of channel catfish, *Ictalurus punctatus*, to *Edwardsiella ictaluri*, yet nonspecific cellular cytotoxicity and phagocyte activity are suppressed. In contrast, PCB-126 and other planar HAHs are potent suppressors of the antibody response in mammals (Holsapple *et al*. 1991).

Tributyltin is also a classical immunotoxic agent in rodents (Vos *et al*. 1984; Smialowicz *et al*. 1989; Kergosien and Rice 1998). In the toadfish, *Opsanus tau*, TBT treatment *in vitro* and *in vivo* impairs phagocyte activation and oxidative burst activity (Rice and Weeks 1989, 1990, 1991). *In vivo*, TBT dramatically suppresses antibody responses to *E. ictaluri* in channel catfish and temporarily inhibits phagocyte oxidative burst and NCC activities (Rice *et al*. 1995). O'Halloran *et al*. (1996) demonstrated that B-lymphocytes were selectively targeted over other leukocytes by organotins. The study described herein extends the work of Rice *et al*. (1995) and Rice and Schlenk (1995) to examine the immunotoxicity of TBT and PCB-126 in mixtures. Because previous studies show that TBT is a more potent immunotoxicant than PCB-126, this study emphasized the influence of PCB-126 on TBT-induced immunotoxicity.

Materials and Methods

Animals and Dosing Regime

Channel catfish, *I. punctatus*, were obtained from the Clemson University Aquaculture facility and housed in 200-L flow-through tanks (2 L/min) maintained at 25–27°C. Each tank was stocked with 20 juvenile fish (50–100 g) of mixed gender and fed daily a standard floating catfish growth chow (Ziegler NH) at 4% body weight. Fish were acclimated to experimental conditions for a minimum of 10 days prior to experiments. Stock solutions of PCB-126 (99% purity, Ultra Scientific Inc., Providence, RI) and TBT (97% purity, Aldrich Chemical Co. Milwaukee, WI) were prepared in DMSO and diluted to working solutions in canola oil. Unless otherwise specified, all other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). The highest nominal concentration of DMSO in any working solution, including the canola oil carrier, was 0.1%. For acute exposures fish were injected IP with a single dose of corn oil (controls), 0.01 or 1 mg/kg TBT or PCB-126, or both in combination. For repeated exposure studies, fish were dosed six times over a 16-day period with 1.7 or 170 μg/kg per dosing of the individual or compounds in combination, or the corn oil carrier, leading to a cumulative dose equal to acute exposures. Specifically, fish were injected with 1 ml of compound per kg at 72-h intervals. Nine treatments (two of PCB-126, two of TBT, four of both in all possible combinations, and the carrier) comprised both studies and each treatment was replicated twice. At the time of receiving the last IP injection of compounds each fish also received 100 μl of antigen solution IP containing 10^7 formalin-killed *Vibrio anguillarum* in phosphate-buffered saline (PBS; pH 7.6).

Five fish per replicate (10 per treatment) were removed 14 and 21 days after the final injection(s) for evaluating phagocyte function. Antigen-specific antibody responses were measured 21 days after the last treatment. Fish were quickly removed from their respective tanks and anesthetized with 100 mg/L 3-aminobenzoic acid ethyl ester methane sulfonate salt (MS-222). Each fish was weighed and its fork length measured for determination of whole body condition factors, then bled from the caudal sinus into a 3-ml vacutainer containg sodium heparin. Anterior kidney tissues were removed from each fish, transfered to a centrifuge tube containing HBSS, then placed on ice until further manipulation for oxidative burst activity evaluations. Plasma samples were collected from each vacutainer after centrifugation and frozen at −80°C until later analysis. Whole body condition factors (K) for each fish were determined by the formula: $K = 100$(body wt in g)/(length in cm)^3 and used as an allometric index of overall health.

Antigen-Specific Antibody Responses

Plasma antibody levels were determined by ELISA using *V. anguillarum*–coated Immulon ELISA plates that were prepared as previously described (Rice *et al*. 1995). Coated plates were washed three times in PBS containing 0.05% Tween-20, incubated at room temperature for 1 h with blocking solution (PBS containing 3% bovine serum albumin, 0.05% Tween-20, and 0.2% sodium azide) to block nonspecific binding sites, and then washed again. To determine relative levels of antibodies specific for *V. anguillarum*, plasma samples were diluted 1:50 in PBS and added to antigen-coated ELISA plates in duplicate in a 100-μl volume per well. To control for possible plate-to-plate variation in the assays, a diluted sample of hyperimmune channel catfish plasma was added to each plate and treated the same as other samples. Following three washings, plates were then incubated for 60 min at room temperature with 9E1 hybridoma supernatants (a gift from Dr. Norman Miller, University Medical Center, University of Mississippi, Jackson, MS). They were washed again three times, and then incubated for another 60 min at room temperature with the final antibody (goat anti-mouse Ig (h + 1)-AP; Fisher, Norcross GA) diluted 1:1,500 in PBS. Following a final washing step, antigen-specfic immunoglobulin was visualized at 405 nm after the addition of *p*-nitrophenyl phosphate (1 mg/ml) dissolved in diethanolamine buffer using a microplate reader.

Phagocyte Oxidative Burst Activity

To isolate anterior kidney phagocytes, tissues in HBSS were first dissociated by repeated aspiration through the barrel of a 1-ml tuberculin syringe using the accompanying plunger. Homogenates were allowed to settle for 2 min to separate the debris from cellular suspensions. Cells from two fish per treatment were then pooled, washed by centrifugation in HBSS, and then layered over a 1.060/1.080 g/ml Percoll density gradient and centrifuged for 25 at 350 *g* to separate phagocytes (mostly neutrophils) from other cells. Phorbol ester-stimulated superoxide anion production by the pooled samples was then determined by employing the nitroblue tetrazolium (NBT) assay as described by Rice and Schlenk (1995). Data were recorded as stimulation indices (SI) calculated as the ratio of stimulated to unstimulated reduction of NBT.

Statistics

Statistics were analyzed using the Instat Stastical software program. Variations within and among treatments were compared by analysis of variance (ANOVA) with an alpha value of 0.05. Optical density data from ELISA assays were arcsine transformed prior to analysis. An ANOVA was followed by the Bonferroni's multiple comparisons test for parametric data sets and by a Dunn's multiple comparisons test for nonparametric data sets.

Results

There were no overt changes in condition factors (K) as a result of treatments (data not shown).

Antibody Responses

Catfish respond to formalin-killed bacteria by exhibiting a maximum plasma antibody response by day 21 postimmunization (Waterstrat *et al*. 1989, 1991). Therefore, antibody responses were measured on day 21 postimmunization in these studies. In fish given a single injection of the compounds, only the highest level of both TBT and PCB-126 resulted in lower antibody responses (Figure 1). Those mixtures containing the highest level of TBT also suppressed antibody responses, but there were no effects beyond what was observed in the highest level of TBT when given alone. In fish given six fractionated doses of TBT or PCB-126 over a 16-day period, specific antibody responses were affected by the highest dose of TBT, but not by either dose of PCB-126 (Figure 2). Mixtures of high PCB-126 and low doses of TBT enhanced the suppression of antibody responses beyond that of low TBT alone. On the other hand, mixtures of high TBT and the high PCB-126 dose increased antibody responses compared to high TBT given alone.

Oxidative Burst Activity

TBT and PCB-126 were potent modulators of phorbol ester-stimulated phagocyte oxidative burst activity. This activity was measured 14 and 21 days after injections. In fish given a single injection of the compounds, both low and high levels of TBT and PCB-126 suppressed this response (Figure 3). By day 21 the SIs were still lower in high TBT and low PCB-126 treated animals. The addition of low PCB-126 to low TBT doses increased the oxidative burst activity compared to both low and high TBT when given alone. No other interactive effects were observed in fish given single dose of mixtures. This study was then followed by another study in which fish were given six fractionated doses of TBT or PCB-126 over a 16-day period. On day 14 postinjections, the oxidative burst activities were lower the high PCB-126 group but not others (Figure 4). In contrast, activities were lower in the high TBT and low PCB-126 groups on day 21. In these fractionated studies, mixtures of TBT and PCB-126 were tested for their effects in comparison to the agents given alone. Neither of the mixtures containing low levels of TBT affected phagocyte oxidative burst activities beyond that of low TBT alone. In contrast, by day 21 the addition of low PCB-126 to the high TBT doses increased activities in comparison to high TBT levels given alone.

Discussion

Tributyltin and co-planar PCBs have been studied extensively for their toxicities and the mechanism(s) associated with these toxicities. Both are immunomodulatory, affect reproductive physiology, and alter normal developmental patterns in all animal models studies to date. For the most part, the coplanar PCBs are active through the Ahr (Safe 1990). As for TBT, it acts as a calcium ionophore (Chow *et al*. 1992; Girard *et al*. 1997), and, as such, can lead to loss of membrane potentials as well as apoptosis. Raffray *et al*. (1993) clearly demonstrated that this effect is the primary mechanism associated with thymic atrophy following exposure to TBT. Although at least two independent laboratories have demonstrated that TBT potentiates PCB-126-induced CYP1A in fish and rodent models (De-

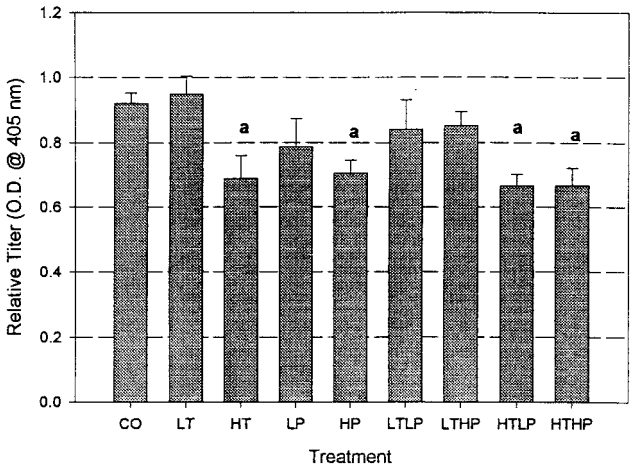

Fig. 1. Relative antibody responses to *Vibrio anguillarum* following treatment with low (L) and high (H) PCB-126 (P) or TBT (T), or combinations as compared to canola oil (CO) control. Animals received a single injection of the compounds and antigen, then sacrificed on day 21 post-treatment. The data represent the mean ± SEM of relative titers as determined by ELISA. Symbols respresent statistical differences compared to the control (a) ($p \leq 0.05$)

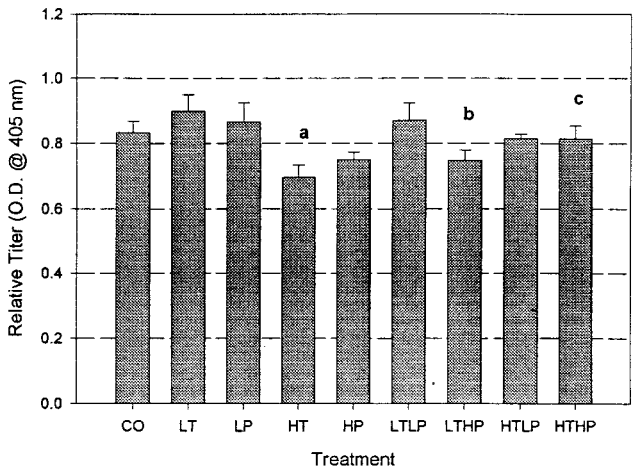

Fig. 2. Relative antibody responses to *Vibrio anguillarum* following repeated treatments with low (L) and high (H) PCB-126 (P), TBT (T), or combinations as compared to canola oil (CO) control. Fish received six injections of the compounds fractionated into 1.7 or 170 μg/kg/injection over a 16-day period and were immunized at the time of the last injection. Fish were sacrificed 21 days post-treatment. Symbols respresent statistical differences compared to the control (a), differences compared to LT (b), or to HT (c) ($p \leq 0.05$)

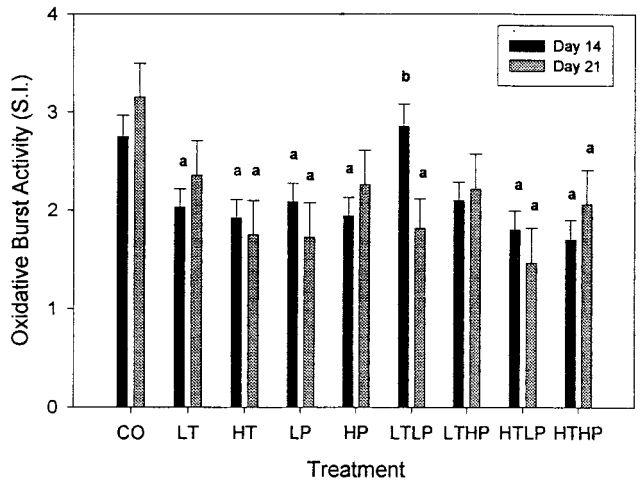

Fig. 3. Phagocyte oxidative burst activity following treatment with low (L) and high (H) PCB-126 (P), TBT (T), or combinations as compared to canola oil (CO) control. Animals received a single injection of the compounds, then sacrificed on days 14 and 21 post-treatment. Symbols respresent statistical differences compared to the control (a) or compared to LT alone (b) ($p \leq 0.05$)

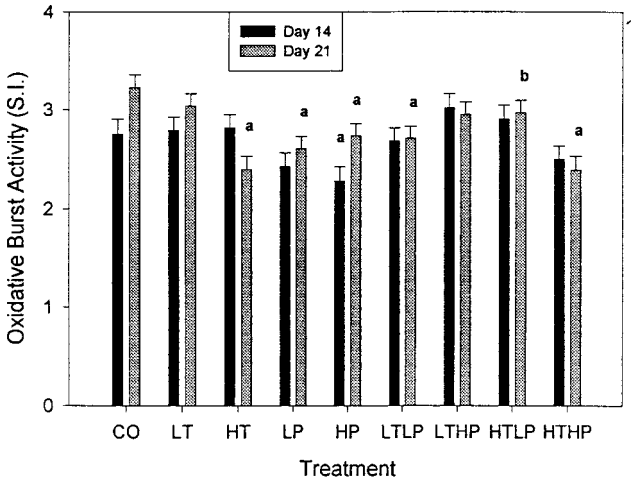

Fig. 4. Phagocyte oxidative burst activity following repeated treatments with low (L) and high (H) PCB-126 (P), TBT (T), or combinations as compared to canola oil (CO) control. Fish received six injections of the compounds fractionated into 1.7 or 170 μg/kg/injection over a 16-day period and were sacrificed 14 and 21 days post-treatment. Symbols respresent statistical differences compared to the control (a) or compared to HT alone (b) ($p \leq 0.05$)

Long and Rice, 1997; Kannan *et al*. 1998), the interactive effects of the two compounds on immune function have not been investigated.

In previous studies (Rice *et al*. 1995), a single dose of 1 mg/kg TBT suppressed the antibody response to *E. ictaluri*, as determined by the ELISpot assay that detects specific antibody secreting cells as an index of humoral immunity. In this study, the same dose of TBT suppressed the antibody response to *V. anguillarum*, using circulating immunoglobulin levels as the endpoint. Such a correlation between the two assays lends confidence to our alternative method for detecting the effects of contaminants on antibody responses in channel catfish, at least under experimental conditions. Moreover, this correlation is also observed in rodent immunotoxicology in that the serum antibody titers to sheep red blood cell antigens correlates with the numbers of specific antibody secreting cells (Temple *et al*. 1993). Unlike our previous studies, however, a single dose of 1 mg/kg PCB-126 suppressed the antibody response. The differences in results between these two studies may be due to the use of a different antigen or to the fact that different strains of channel catfish were used. It is well known that strain differences in rodent Ahr expression exist, resulting in differential immunotoxic responses between the strains. To address this, we carried out preliminary experiments designed to determine if PCB-126 induces hepatic CYP1A in the Clemson strain of channel catfish to the same magnitude as seen in the Mississippi State University strain (Rice and Schlenk 1995). The results clearly demonstrated equal induction. Therefore, it is unlikely that significant differences in Ahr expression exist between the two strains. Other preliminary experiments designed to optimize response kinetics revealed that *V. anguillarum*–specfic antibodies in the Clemson strain reach a peak at day 21, which is in consistent with antibody responses to *E. ictaluri* (Rice and Schlenk 1995; Waterstrat *et al*. 1989, 1991). When, in this study, fish were given a single dose of the compounds as mixtures no effects on the antibody responses were noted as compared to the compounds given alone. Apparently, there are no immunotoxic interactions between PCB-126 and TBT under the given conditions of a single exposure.

In the repeated exposure study, only the highest levels of TBT affected the antibody response. More than likely, fish in the PCB-126-dosed groups compensated for toxicity when the agent is given in such small doses, especially considering that 3 days of nonexposure separated each dosing. By including high levels of PCB-126 in the low TBT dose, the antibody responses were lower than that observed in the low TBT dose when given alone. On the other hand, the suppression of antibody responses by high levels of TBT is reversed by the addition of high doses of PCB-126. From a statistical standpoint, the differences are clear. However, it is unclear at this point how biologically significant this suppression is. As can be seen from the data, the total range of responses for all groups lies within a few optical density points derived from the ELISAs. Moreover, one can only speculate about the mechanisms behind these interactions. The most likely explanation is related to the different pathways for toxicity of each compound. As stated elsewhere, TBT acts like a calcium ionophore and can induce apoptosis, and coplanar PCBs act through the Ahr. Data from Crawford *et al*. (1997) indicate that Ahr-ligands—TCDD in particular—can induce leukocyte cyclooxygenase-2 (COX-2), nitric oxide synthase type II (iNOS), CYP1A, and other proinflammatory proteins. Collectively, the expression of these proteins may be associated with protection against cellular toxicity. Moreover, preliminary data from our lab indicates that Ahr ligands induce the expression of leukocyte major vault protein (MVP) (Xiang and Rice 2000). MVP is the major component of vaults, ribonuclear protein cytoplasmic-nuclear plugs that are involved in the transport of materials in and out of the nucleus. MVP is overexpressed in certain lymphoid neoplasias that acquire chemoresistance. Taken together, it is possible that PCB-126 induces these proteins in

channel catfish leukocytes to the point that it becomes protective against the cytotoxicity of high doses of TBT. The differential responses between animals exposed to LTHP and HTHP may be due to differential effects of the two TBT levels on B-cell and phagocyte physiology.

The phagocyte oxidative burst activity of fish exposed to PCB-126 and TBT was dramatically altered. As previously demonstrated by Rice *et al*. (1995), TBT suppresses this response. However, this study extended the previous observation time of 7 days to 14 and 21 days. Following a single exposure, phagocyte physiology was affected throughout the 21-day period in both TBT- and PCB-126-exposed fish. As with the antibody responses of these fish, there were no toxic interactions between TBT and PCB-126 in mixtures. In fish exposed repeatedly to fractionated doses, the phagocyte oxidative response was altered by the highest dose of PCB-126 on day 14 postinjection, and the highest level of TBT on day 21. As with the antibody responses of these fish, exposure to very low levels of the compounds in mixtures resulted in only a moderate effect. The addition of low levels of TBT to the high level of PCB-126 reversed the suppression associated with TBT alone on day 21 after the last injection. However, as argued in the case of antibody responses, the question of magnitude still remains.

Phagocyte oxidative burst activity seems to be a sensitive target response in channel catfish to TBT (Rice *et al*. 1995). Rice and Weeks (1989, 1990, 1991) demonstrated that the oxidative burst activity of toadfish peritoneal macrophages is extremely sensitive to TBT. At low levels, this compound activates macrophages in the absence of a pharmacological stimulator. At higher doses, TBT is cytotoxic to phagocytes. Subsequent experiments showed that untoward calcium fluxes were induced by TBT, and by chelating extracelluar calcium the effects of TBT on macrophage oxidative burst activity could be reversed. However, this may be the basic nature of metal and organometal-induced toxicity in general. Burnett (1997) demonstrated that metals induce tyrosine kinase activation in red drum lymphocytes through calcium-mediated events. How PCB-126 in mixtures with TBT reverses the organotin's effect on oxidative burst activity in the repeated exposure study is unclear, but the same argument for an induction of proinflammatory and cytoprotective proteins by Ahr ligands can be made.

PCBs and organotins rarely exist in aquatic environments as single compounds, therefore the need to investigate their effects in mixtures is logical. Earlier studies (Fent and Stegeman 1991; Fent and Bucheli 1994; Bruschweiler *et al*. 1996) indicated that TBT disrupts and inhibits the cytochrome P4501A system in fish. DeLong and Rice (1997) demonstrated that TBT potentiated PCB-126-induced CYP1A in mice. Later, Rice and Roszell (1998) demonstrated that low levels TBT potentiate PCB-126-induced hepatic CYP1A protein and EROD activity in channel catfish. High levels of TBT inhibit PCB-126 induced EROD activity without affecting CYP1A protein levels in the model. Kannan *et al*. (1998) validated this observation by showing that TBT potentiates PCB-126-induced CYP1A activity *in vitro* using the rat hepatoma cell line H4IIE. The potentiation of effects of an Ahr-ligand by TBT is unexpected and suggests that mixtures of TBT and PCB-126 may have interactive effects on other physiological systems, including immune function. Elevated physiological levels of calcium leads to the up-regulation of many cellular signal transduction pathways and may be a common mechanism for many classes of immuntoxic xenobiotics (Karrass *et al*. 1996). One of these may enhance the transcription of the Ahr, aryl receptor nuclear translocators (ARNTs), or both. It may even facilitate interactions of the Ahr and its ligand or heat shock chaperones. This potential mechanism needs to be explored, possibly through the use of *in vitro* models, such as the channel catfish leukocyte cell line 42TA, a monocyte line, and the channel catfish B- and T-lymphocyte lines 1G8, 28S.1, respectively (Miller *et al*. 1994).

It is important to realize that any effects of TBT and PCB-126 mixtures on immune functions examined in this study are only additive. It is likely that most environmental mixtures of xenobiotics have similarly interactive effects on other biological systems, including reproduction, development, and immune function. In terms of the importance of understanding chemical interactions with HAHs, most of our concerns about xenobiotics over the last few decades have been related to Ahr-active structures because of their potent pathobiological effects.

Whether or not mixtures of TBT and PCB-126 affect immune function in channel catfish following dietary or sediment-borne exposures obviously needs to be determined, and those studies are currently underway. Furthermore, it is important to realize that the modulation of antibody responses and phagocyte oxidative burst activity may be only a physiological marker of exposure and not necessarily one of toxicological effect. Beyond modulation of phagocyte physiology and humoral immune responses, other responses, such as lysozyme, nonspecific cytotoxic cell activity, neuroendocrine-immune interactions, and other aspects of immune function, need to be examined to fully evaluate the effects of mixtures of organotins and HAHs on immunity in fish.

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