An Organotin Mixture Found in Polyvinyl Chloride (PVC) Pipe is not Immunotoxic to Adult Sprague-Dawley Rats

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Organotin compounds used in polyvinyl chloride (PVC) pipe production are of concern to the U.S. Environmental Protection Agency (EPA) because they leach from supply pipes into drinking water and are reported multisystem toxicants. Immune function was assessed in male Sprague-Dawley rats exposed to the mixture of organotins used in PVC pipe production. Although several of these organotins are reported immunotoxicants, their immunotoxicity as a mixture when given by drinking water has not been evaluated. Adult male rats were given drinking water for 28 d containing a mixture of dibutyltin dichloride (DBTC), dimethyltin dichloride (DMTC), monobutyltin trichloride (MBT), and monomethyltin trichloride (MMT) in a 2:2:1:1 ratio, respectively, at 3 different concentrations (5:5:2.5:2.5, 10:10:5:5, or 20:20:10:10 mg organotin/L), MMT alone (20 or 40 mg MMT/L), or plain water as a control. Delayed-type hypersensitivity, antibody synthesis, and natural killer cell cytotoxicity were evaluated in separate endpoint groups (*n* = 8/dose; 24/endpoint) immediately after exposure ended. The evaluated immune functions were not affected by the mixture or by MMT alone. Our data suggest that immunotoxicity is unlikely to result from the concentration of organotins present in drinking water delivered via PVC pipes, as the concentrations used were several orders of magnitude higher than those expected to leach from PVC pipes.

Organotin compounds are listed on the US Environmental Protection Agency (EPA) Candidate Contaminant List 2 (CCL2) because they are known or anticipated to be present in public water systems and because the health effects of human exposure through drinking water are unknown. As members of the CCL2, organotin compounds used in polyvinyl chloride (PVC) pipe production are being evaluated as drinking water contaminants through the U.S. EPA risk assessment process. Part of this assessment includes the evaluation of immunotoxicity as several organotin compounds are known immunotoxicants (Penninks et al., 1990). The current risk assessment being performed for these chemicals takes a mixtures approach and assumes additivity among the individual chemicals (Lipscomb & Lambert, 2005). While several of these compounds were evaluated individually for immunotoxicity, the immunomodulatory potential of a mixture in drinking water is unknown. Because exposure through drinking water carried via PVC pipes is to a mixture of organotin compounds, assessment of their potential immunotoxicity when administered together will improve the quality of the database for the risk assessment. This study therefore determined whether immune function was affected by organotins given as a mixture.

The organotin compounds found in PVC pipe are added during production as heat and light stabilizers and include dibutyltin dichloride (DBTC), dimethyltin dichloride (DMTC), monobutyltin trichloride (MBT), and monomethyltin trichloride (MMT). PVC pipes may be used in drinking water delivery systems, and all four organotin compounds used in PVC pipe production have been reported to leach into drinking water carried through PVC pipes (Sadiki et al., 1996; Sadiki & Williams, 1999). Individual organotins have

been detected in drinking water at concentrations between 28.5 and 291 ng tin/L (Sadiki & Williams, 1999). Of the organotins used in PVC pipe production, DBTC has been the most extensively studied. DBTC is an ultimate breakdown product of the antifouling agent tributyltin oxide (TBTO) and is the potential immunotoxic principle of TBTO (Ueno et al., 2003). In rodent models, exposure to DBTC resulted in decreased lymphoid organ weights, thymus atrophy (a hallmark of organotin immunotoxicity), altered humoral and cell-mediated immune function in rodent models (Penninks et al., 1990), and reduced cytotoxicity by human natural killer cells (Whalen et al., 1999). It was previously reported that when given to rats in drinking water for 28 d at concentrations nearly 6 orders of magnitude higher than anticipated human exposure, neither DBTC nor DMTC affected T-cell-mediated antibody synthesis, delayed-type hypersensitivity, or natural killer cell activity (DeWitt et al., 2005, 2007). Neither MBT nor MMT has been evaluated extensively for immunotoxicity.

This article describes our evaluation of immune functions in adult, male Sprague-Dawley rats, a model sensitive to the immunomodulatory effects of organotin compounds. Rats were exposed to a mixture of organotins, or to MMT alone, via drinking water. Immune function was evaluated by monitoring antibody responses to sheep red blood cells (SRBCs), delayed-type hypersensitivity (DTH) responses to bovine serum albumin (BSA), and natural killer (NK) cell cytotoxicity. This study therefore provides information critical to the overall evaluation of organotins as immunotoxicants delivered via drinking water.

MATERIALS AND METHODS

Chemicals

Dibutyltin dichloride (DBTC, 96% purity, batch 02211AO), dimethyltin dichloride (DMTC, 97% purity, batch BA14015HS), monobutyltin trichloride (MBT, 95% purity, batch 05427LU), and monomethyltin trichloride (MMT, 97% purity, batch 12609CD) were purchased from Aldrich Chemical Company (Milwaukee, WI). Alkamuls EL-620 (Alkamuls; ethoxylated castor oil, an emulsifier) was obtained from Rhodia North American Chemicals (Cranbury, NJ).

Animals

Male Sprague-Dawley rats (6–7 wk old, weighing approximately 275 g, $n = 72$) were purchased from Charles River Laboratories (Raleigh, NC). Animals were housed individually in polycarbonate cages with pine shavings, provided a 12-h light (0600 h) to dark (1800 h) cycle, maintained at 22.3 ± 1.1°C and 50 ± 10% humidity, and given access to both food (PMI 5001, PMI Nutrition International, Richmond, IN) and water ad libitum. Animals were acclimated for at least 10 d before dosing began. All procedures employed in this study were approved in advance by the Institutional Animal Care and Use Committee of the National Health and Environmental Effects Research Laboratory, U.S. EPA.

Dose Selection and Preparation

Doses were chosen based on water palatability determined from previous studies, potential toxicity, and the proportion of each compound expected to occur in drinking water. In previous studies with DBTC and DMTC, although adult Sprague-Dawley rats would drink up to 25 mg DBTC/L of water or 40 mg DMTC/L of water and not show signs of overt toxicity, they drank less water relative to controls (DeWitt et al., 2005, 2007). Therefore, the concentrations of either of these chemicals used in previous studies were not exceeded. As the palatability of MBT or MMT was not evaluated, doses were chosen based on their proportion in drinking water relative to DBTC and DMTC and on their potential for toxicity. Sadiki and Williams (1999) reported that in sampled PVC pipes, organotin concentration was MMT > DMTC = DBTC > MBT and frequency was MMT = DMTC > DBTC = MBT. However, MMT was reported to be associated with neurotoxicity at concentrations of 40 and 120 mg Sn/L drinking water (Noland et al., 1982), and although these findings were not replicated in a study by Moser et al. (2006), doses were also chosen to minimize any potential complications from MMT neurotoxicity. The proportions of the dosing solutions were therefore 2:2:1:1 (DBTC:DMTC:MBT:MMT), at concentrations of 20:20:10:10 (high mix), 10:10:5:5 (medium mix), or 5:5:2.5:2.5 (low mix) mg organotins/L of water. The solutions of MMT alone contained 20 or 40 mg of MMT/L water. There was a total of five dose groups and one control group. Based on average body weight and average water consumption throughout the study, the calculated dosage (mg/kg body weight/d) of each of the chemicals were as follows: 20 mg MMT/L, 2.2 mg/kg/d; 40 mg MMT/L, 4.1 mg/kg/d; low mix, 1.3 mg/kg/d; medium mix, 2.5mg/kg/d; and high mix, 4.7 mg/kg/d.

Dosing solutions were prepared twice weekly in 25-L polycarbonate carboys and then transferred into individually labeled plastic drinking water bottles topped with double ball bearing sipper tubes. Although only DBTC and MBT are not miscible in water, all organotins were solubilized in Alkamuls prior to mixing with water. The final concentration of Alkamuls in all dosing preparations was 0.5% v/v. Dosing solutions were prepared twice weekly as previously described by DeWitt et al. (2005).

Experimental Design

Animals were randomly divided into 24 animals/endpoint and 8 animals/dose group. Dosing was via drinking water for 28 d. Animals were weighed twice weekly during the dosing period and also just prior to sacrifice. Dosing water was changed and water consumption was monitored twice weekly. Different immune functions were evaluated in separate groups of animals 1 d after dosing ended (Figure 1).
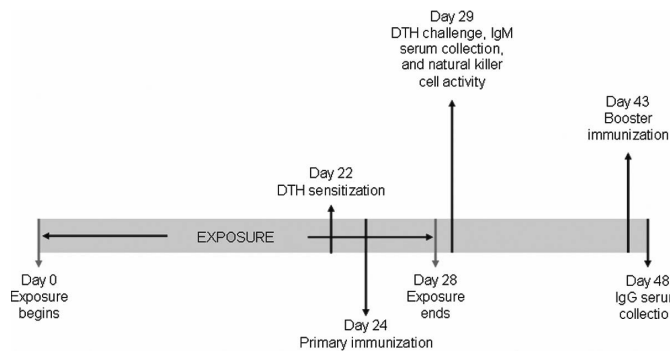
FIG. 1. Study design. Male Sprague-Dawley rats received a mixture of four organotins (DBTC:DMTC:MBT:MMT) or MMT alone in drinking water for 28 d.

Evaluation of Immune Function

Antibody responses. Eight animals per dose were used to measure T-cell-dependent antibody responses (immunoglobulin [Ig] M and IgG) to SRBC. Animals were immunized on d 24 of dosing by intravenous injection of 2×10^8 SRBCs in 0.5 ml sterile saline and were bled 5 d later by tail vein transection. Blood was held at room temperature for 30 min and centrifuged at 4°C to separate serum; serum was frozen at –80°C until analysis of SRBC-specific IgM. Two weeks after primary immunization, a booster immunization of SRBCs (2×10^8) was administered intravenously to the same animals. Blood from decapitated animals was collected 5 d after the booster immunization and processed as described above for later analysis of SRBC-specific IgG. The relative serum titers of SRBC-specific IgM and IgG antibodies were measured by enzyme-linked immunosorbent assay (ELISA) as described by DeWitt et al. (2005) and based on a method originally described by Temple et al. (1995). All ELISA procedures were optimized for differences in SRBC membrane preparations and each new lot of secondary antibody.

Delayed-type hypersensitivity responses (DTH). Eight animals per dose were used to measure the DTH responses to purified (Fraction V) bovine serum albumin (BSA; Sigma). BSA (1 mg/ml in sterile saline) was emulsified in Freund's complete adjuvant (CFA; Difco, Detroit, MI) at a 1:1 ratio. Animals anesthetized with isoflurane were sensitized on d 22 of dosing by injecting 0.1 ml BSA-CFA subcutaneously into the caudal tail fold. Seven days later, animals were anesthetized with isoflurane and challenged by injecting 0.1 ml heat-aggregated BSA into the right rear footpad. The left rear footpad was injected with the same volume of saline and served as the injection control. BSA was aggregated by heating 10 mg BSA/ml of sterile saline to 75°C for 1 h and removing excess saline by centrifuging for 10 min at $650 \times g$. After 24 h, footpad thickness (triplicate measurements) was determined in anesthetized animals with an electronic caliper designed and built in the

model shop at the U.S. EPA (Research Triangle Park, NC). The device applies very light, even, and reproducible pressure on the footpad for each measurement, thus increasing the accuracy of measurements. Standards of known thickness were measured before and after experimental measurements. Swelling was calculated by subtracting the mean saline-injected, left footpad thickness from the mean BSA-injected right footpad thickness.

Natural killer (NK) cell activity. Eight animals per dose were used to measure NK cell activity the day after dosing ended (d 29), as described by Smialowicz et al. (1991). Briefly, terminal body weight (BW) was recorded, the spleen and thymus were removed, and the weights of both organs were recorded. Splenocyte single cell suspensions were prepared and cultured with ^{51}Cr-labeled murine YAC-1 lymphoma target cells (100 µCi/1×10^6 cells) in round-bottomed microtiter plates (Costar, Corning Incorporated, Corning, NY) at effector to target cell ratios of 25:1, 50:1, or 100:1. After a 4-h incubation at 37°C, microtiter plates were centrifuged ($290 \times g$) and 25 µl of supernatant was transferred to a 96-well LumaPlate (Packard, Meriden, CT), covered, and read on a TopCount NXT (Packard) microplate scintillation counter. The data are presented as the average adjusted percent cytotoxicity for three replicates [(mean counts per min in supernatant – spontaneous release of ^{51}Cr) / (mean releasable counts from target cells – spontaneous release of ^{51}Cr) $\times 100$].

Statistics

All data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed with the SAS System (SAS Institute, Inc., Cary, NC). Analysis of variance (ANOVA) was used to analyze immune responses by dose and, when appropriate, linear regressions were used to determine dose response. When ANOVA indicated a statistically significant treatment effect, individual post hoc comparisons were made using Tukey's test and the least square means *t*-test with a Tukey's adjustment for controlling the familywise error rate. A repeated-measures ANOVA was used to analyze BW changes over time and dose. Statistical significance was determined using an alpha of .05.

RESULTS

Body Weight and Water Consumption

Water consumption was reduced by approximately 20–30% in all organotin mixture groups relative to controls (Figure 2). Although water consumption was decreased in the organotin mixture groups, BW did not differ between controls and dosed animals during any part of the dosing period nor when terminal weights were collected (data not shown). Exposure to MMT had no statistical effect on water consumption (Figure 2) or BW (data not shown).
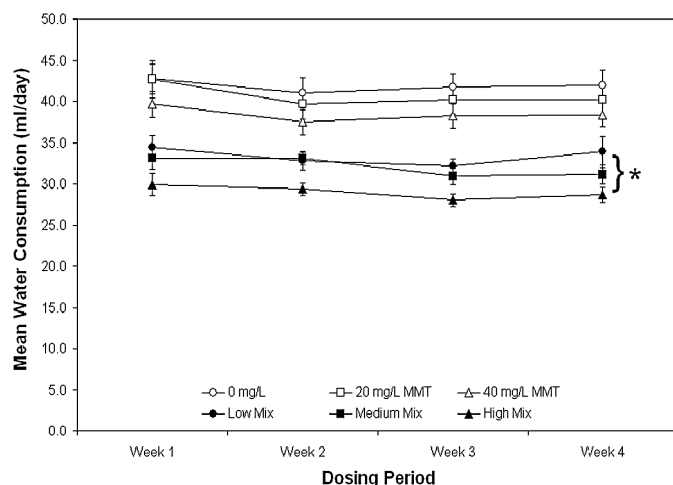
FIG. 2. Water consumption of male Sprague-Dawley rats exposed to one of three organotin mixtures or to two concentrations of MMT in drinking water for 28 d. Data are presented as mean ± SEM. N = 8 animals/dose. Animals from all organotin mixture dose groups consumed less water (asterisk indicates significant difference at $p < .05$) relative to controls. Animals from the high mix group consumed 30% less water than controls and animals from the medium and low mixes consumed ˜20% less water than controls. Low mix: 5:5:2.5:2.5 DBTC:DMTC:MBT:MMT; medium mix: 10:10:5:5 DBTC:DMTC:MBT:MMT; high mix: 20:20:10:10 DBTC:DMTC:MBT:MMT, all in mg/L drinking water.

FIG. 3. Mean SRBC-specific antibody titers of male Sprague-Dawley rats exposed to one of 3 organotin mixtures in drinking water for 28 d. Data are presented as mean ± SEM; $n = 8$ animals/dose. Low mix: 5:5:2.5:2.5 DBTC:DMTC:MBT:MMT; medium mix: 10:10:5:5 DBTC:DMTC:MBT:MMT; high mix: 20:20:10:10 DBTC:DMTC:MBT:MMT, all in mg/L drinking water.

FIG. 4. Mean delayed-type hypersensitivity responses (DTH) to bovine serum albumin of male Sprague-Dawley rats exposed to one of 3 organotin mixtures in drinking water for 28 d. Data are presented as mean ± SEM; $n = 8$ animals/dose. Low mix: 5:5:2.5:2.5 DBTC:DMTC:MBT:MMT; medium mix: 10:10:5:5 DBTC:DMTC:MBT:MMT; high mix: 20:20:10:10 DBTC:DMTC:MBT:MMT, all in mg/L drinking water.

Immune Functions

No statistical differences between control and organotin-exposed animals were detected for primary and recall antibody responses to sheep red blood cells (SRBCs; Figure 3), delayed-type hypersensitivity responses to bovine serum albumin (Figure 4), or natural killer cell cytotoxicity (Figure 5). The responses observed in this study were consistent with responses previously reported for adult Sprague-Dawley rats exposed to organotins or control water via drinking water (DeWitt et al., 2005, 2007). Spleen and thymus weights of animals used to evaluate natural killer cell activity did not differ between controls and dosed animals following exposure to the organotin mixture or to MMT alone (data not shown).

DISCUSSION

In this study, several adaptive and innate immune responses were evaluated in adult Sprague-Dawley rats following exposure to a mixture of organotin compounds likely to be found in drinking water carried through PVC pipes, or to MMT alone. Exposure to these organotin mixtures or to MMT alone did not statistically alter the adaptive (i.e., delayed-type hypersensitivity or T-cell-dependent antibody responses to SRBC) or the innate immune functions (i.e., natural killer cell cytotoxicity) examined. This particular combination of tests used to evaluate
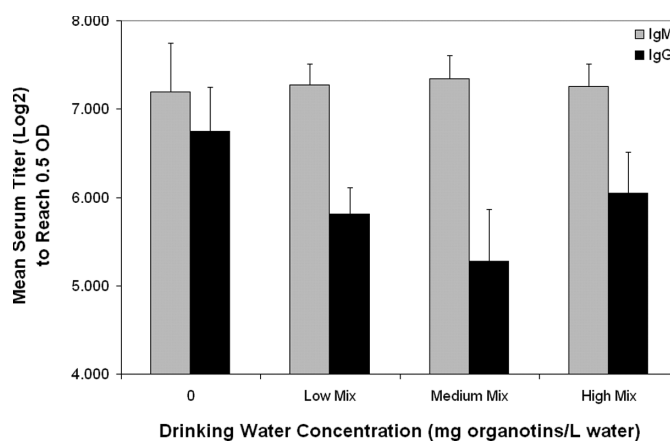
immunotoxicity of the organotins is a configuration that produces 100% concordance in predicting immunotoxic outcomes (Luster et al., 1992). Our data therefore suggest that immunotoxicity is unlikely to result from the concentration of organotins present in drinking water delivered via PVC pipe, as the concentrations used here were several orders of magnitude higher than those expected to leach from PVC pipe. It was also previously reported that when given individually to rats in drinking water for 28 d at concentrations nearly 6 orders of

FIG. 5. Mean natural killer cell (NK) cytoxicity of male Sprague-Dawley rats exposed to one of three organotin mixtures in drinking water for 28 d. Data are presented as mean ± SEM; *n* = 8 animals/dose. 25:1, 50:1, or 100:1 = effector to target cell ratio. Low mix: 5:5:2.5:2.5 DBTC:DMTC:MBT:MMT; medium mix: 10:10:5:5 DBTC:DMTC:MBT:MMT; high mix: 20:20:10:10 DBTC:DMTC:MBT:MMT, all in mg/L drinking water.
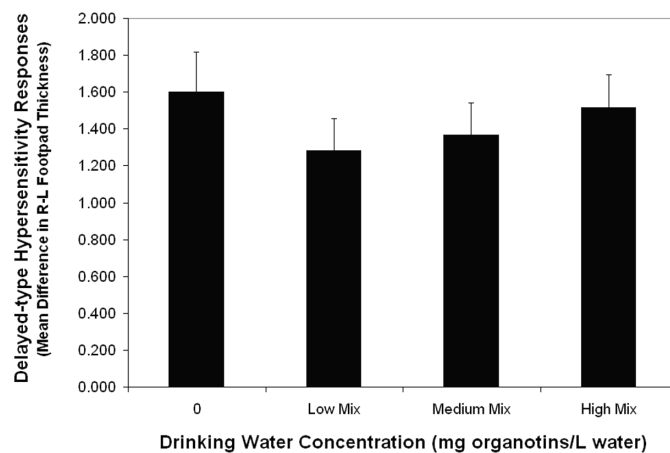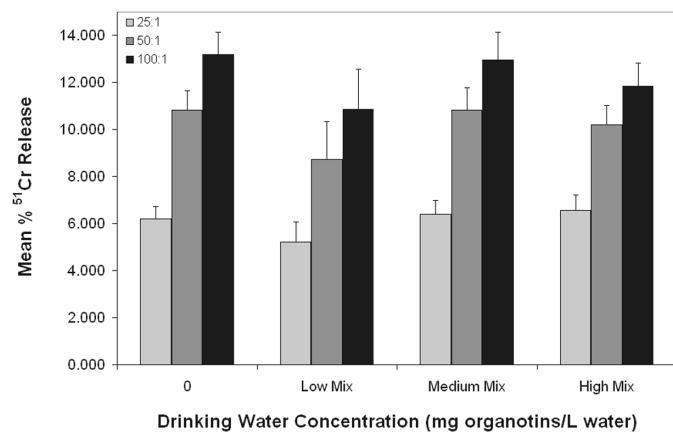
magnitude higher than anticipated human exposure, neither DBTC nor DMTC affected the same immune functions as evaluated in the current study (DeWitt et al., 2005, 2007).

Organotins are used in PVC pipe production because they slow or inhibit PVC degradation by dehydrochlorination (Forsyth et al., 1993). However, organotin compounds leach out of PVC pipes and into the water they carry (Forsyth et al., 1993; Forsyth & Jay, 1997; Quevauviller et al., 1991; Sadiki and Williams, 1999; Sadiki et al., 1996). As members of the U.S. EPA Candidate Contaminant List 2 (CCL2), organotins are currently unregulated by drinking water standards, but because they are present in drinking water and may be associated with negative health effects, they are being evaluated to determine if a guidance, health advisory, or regulation is warranted to protect public health (U.S. EPA, 2005). Our role in this effort was to evaluate the immunotoxicity of the organotin compounds found in PVC pipe. Several organotin compounds, including DBTC, were reported as immunotoxicants when given by gavage or in food; however, their immunotoxicity when given by drinking water had not been evaluated. When given to adult rats by drinking water, neither DBTC (DeWitt et al., 2005) nor DMTC (DeWitt et al., 2007) affected delayed-type hypersensitivity, T cell dependent antibody responses to SRBCs, or natural killer cell cytotoxicity. However, reports of suppressed immune function by an organotin mixture (Whalen et al., 1999) and the need for mixture data in the organotin risk assessment prompted this study.

Whalen et al. (1999) reported a synergistic effect of a mixture of butyltin compounds on human natural killer (NK) cell function in vitro. The cytotoxic ability of human NK cells was inhibited by nearly 80% after exposure to 0.846 mg MBT/L,

0.152 mg DBTC/L, or 0.0162 mg tributyltin chloride/L. The latter compound is used primarily as a wood preservative and as a disinfectant in industrial cooling waters. Whalen et al. (1999) also reported that alone, these compounds produced no or minimal inhibitory effects. Although the combined concentration of the organotin mixture used by Whalen et al. (1999) was lower than the lowest concentration of our organotin mixture (1.01 mg/L versus 15 mg/L), they used an idealized in vitro exposure scenario. In their study, NK cells were directly exposed to a serum-free mixture of organotins, whereas in our study, NK cells were indirectly exposed to an organotin mixture that was likely greatly reduced in concentration due to partitioning and elimination. The data by Whalen et al. (1999) suggest that the cytotoxic ability of human NK cells is sensitive to a mixture of organotins when NK cells are exposed directly; however, our data suggest that when exposure is in vivo, NK cell cytotoxicity is not sensitive to a mixture of organotins.

The immunotoxicity of dialkyltin compounds, especially DBTC at doses between 5 and 15 mg/kg BW, was demonstrated in many rat strains, with reported effects including thymus atrophy (Seinen & Willems, 1976; Seinen, 1980; Penninks et al., 1985; Snoeij et al., 1989) and immunosuppression (Seinen, 1980; Penninks & Seinen, 1984). Research over the past decade provided additional evidence that at least one of the organotins that leaches from PVC pipe (DBTC) is a potent thymotoxic agent that reduces thymus weight and induces thymic apoptosis and atrophy (Pieters et al., 1994; Gennari, et al., 1997, 2002). Although DMTC may also induce thymic atrophy, it is much less potent than DBTC. To our knowledge, the immunotoxicity of mono-substituted compounds has not been thoroughly evaluated. Emphasis on di-substituted compounds rather than mono-substituted compounds has likely been because they are breakdown products of the tri-substituted compounds (Appel, 2004) and the likely toxophores of organotins. However, thymotoxicity as measured by body-weight-adjusted thymus weight may not be the best predictor of a chemical's immunotoxicity; parameters such as lymphocyte enumeration or quantitation of the T-dependent antibody response are more effective predictors of immunotoxicity (Luster et al., 1992). Therefore, although a reduction in thymus weight following organotin exposure is suggestive of immunotoxicity, it is not necessarily predictive of compromised immune function.

Thymic atrophy, however, may be associated with changes in immune function. Seinen et al. (1977) reported that in rats exposed to 15 mg DBTC/kg BW for 4 wk, serum titers against SRBCs were reduced 10.5% relative to controls. At the same dose, but given as a single oral exposure, Snoeij et al. (1989) reported a statistical reduction in thymus weight, with a maximum reduction observed 4 d after exposure and recovery of thymus weight to control levels 9 d after exposure. Therefore, exposure to the most potent thymotoxic organotin reduces both thymus weight and T-dependent antibody responses.

A lack of effects on thymus weight in the current study is likely attributable to several factors noted previously (DeWitt et al., 2005, 2007), such as the dose, strain of rat used, and route of exposure. Our high mix dose contained 60 mg organotins/L, which resulted in an approximate dosage to the rats of 4.7 mg organotins/kg/d. However, as serum organotin concentrations were not measured in this study, it is not possible to know how the relative contribution of parent compounds, metabolites, and potential interactions among the organotins may have affected the thymotoxicity of one or more of the organotins within the mixture. The highest level of DBTC reported to leach out of PVC pipes is 197 ng DBTC/L (Forsyth & Jay, 1997), which is approximately 300,000 times lower than the concentration of the organotin mixture that was administered and likely results in a human dose lower than doses reported associated with immunotoxicity. For example, van Loveren et al. (1998) calculated that the dose of TBTO required to suppress human resistance to the nematode *Trichinella spiralis* was approximately 0.04–0.11 mg TBTO/kg, which is likely higher than an anticipated human dose for 197 ng DBTC/L, the immunotoxic principle of TBTO. In addition, Vos et al. (1990) reported a no-effect level of 0.025 mg TBTO/kg BW for immune effects in male rats, which is also likely higher than an anticipated human dose from exposure to organotins found in drinking water from PVC pipes alone. However, background concentrations for butyltin compounds (mono-, di-, and tributyltins) were reported between 1.8×10^4 and 2.5×10^4 ng/L in human blood samples (Kannan et al., 1999). Human exposure to organotin compounds may come from a variety of sources, which cannot be discounted in a comprehensive risk assessment. In conclusion, although our dosage was lower than 15 mg DBTC/kg reported to reduce both thymus weight and antibody responses, our data provide evidence that even when organotin concentrations in drinking water exceed expected human exposures from drinking water alone by nearly 300,000 times, negative impacts on functional immunity are unlikely.

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