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# Developmental immunotoxicity of di-*n*-octyltin dichloride (DOTC) in an extended one-generation reproductive toxicity study

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ABSTRACT

Developmental immunotoxicity assessment is considered ready for inclusion in developmental toxicity studies. Further evaluation of proposed and additional assays is needed to determine their utility in assessing developmental immunotoxicity. In this study, a wide range of immunological parameters was included in an extended one-generation reproductive toxicity protocol. $F_0$ Wistar rats were exposed to DOTC via the feed (0, 3, 10, and 30 mg/kg) during pre-mating, mating, gestation and lactation and subsequently $F_1$ were exposed from weaning until sacrifice. Immune assessments by several immune parameters were performed at PNDs 21, 42 and 70. The T cell-dependent antibody response to Keyhole Limpet hemocyanin (KLH) was assessed following subcutaneous immunizations with KLH on PNDs 21 and 35 and the delayed-type hypersensitivity response (DTH) against KLH was evaluated at PND 49.

No effects were found on PND 21. While effects on lymphocyte subpopulations in the thymus were only observed in the 30 mg/kg group on PND 42, effects on lymphocyte subpopulations in the spleen were found in the 30 mg/kg group on both PNDs 42 and 70. The DTH response already showed an effect at 3 mg/kg and was the overall critical endpoint. The results from this study support the inclusion of splenocyte subpopulation parameters in developmental toxicity studies and identified the DTH response as an important functional parameter.

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## 1. Introduction

Over the last decades, there has been an increasing interest in the specific sensitivity of early life stages to effects of chemical exposures (Daston et al., 2004; Landrigan et al., 2004). The OECD is currently in the process of adopting a test guideline (OECD EOGRTS) based on the international Life Science Institute (ILSI)-Health and Environmental Sciences Institute (HESI), Agricultural Chemical Safety Assessment (ACSA) Technical Committee proposal for a life stage $F_1$ extended one generation reproductive study as published by Cooper et al. (2006). This extended one-generation reproductive toxicity study was designed to evaluate a specific combination of life stages and parameters not covered by existing regulatory toxicity studies. In this study protocol, chemicals are tested for effects resulting from of pre- and postnatal exposure, including effects on sexual maturation, reproductive organ integrity and function, neurological and behavioural endpoints, and immune system related endpoints.

The developmental immunotoxicity assessment included in the OECD extended one-generation reproductive toxicity study protocol comprises of the weighing of the spleen, thymus and adrenal glands, determination of bone marrow cellularity, and splenic lymphocyte subpopulation analysis and evaluation of the T cell Dependent Antibody Response (TDAR) using sheep red blood cells or Keyhole Limpet Hemocyanin (KLH) (OECD). These assays are considered ready for inclusion in reproductive toxicity studies (Holsapple et al., 2005, 2007). However, inclusion of a TDAR, lymphocyte subpopulation analysis, organ weights and histopathology of lymphoid organs as included in the extended one-generation reproductive toxicity protocol might not allow complete assessment of developmental immunotoxicity. The most sensitive tests may vary for different chemicals and therefore, further evaluation of different assays is needed to determine their utility in assessing developmental immunotoxicity. This pertains to among others

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an evaluation of cell-mediated immune (CMI) responses which are currently not included in the extended one-generation reproductive toxicity protocol, even though there are some examples (i.e., lead and dioxin) of preferential alteration of the CMI versus humoral immunity (Bunn et al., 2001a,b; Gehrs and Smialowicz, 1999; Miller et al., 1998). In addition, the time course of the development of the immune system is important. Different assays may possibly have a different optimal time point at which assessment should be carried out (Holsapple et al., 2005).

Di-*n*-octyltin dichloride (DOTC) is used as a stabilizer in polyvinyl chloride plastics and is shown to cause thymus atrophy and suppression of thymus-dependent immune responses in the rat (Miller et al., 1986; Seinen and Penninks, 1979; Seinen and Willems, 1976). In the present study, we examined the effects of DOTC after pre- and postnatal exposure with emphasis on the immune system of rats. Immune assessments were performed at postnatal days (PNDs) 21, 42, and 70 to investigate the utility of the different assays at the different ages. In addition, effects on the T-cell dependent antibody responses were evaluated in a subset of animals.

## 2. Materials and methods

### 2.1. Animals

The *in vivo* part of the study was carried out at the Animal Facilities of TNO, Zeist, The Netherlands. Animal care and use were in accordance with Directive 86/609/RRC, which established the general principles of governing the use of animals in experiments of the European Communities and with Dutch-specific legislation (The Experiments on Animals Act, 1997).

Ninety-two female (9–10 weeks old) and 46 male (11–12 weeks old) rats, Wistar outbred (Crl:(WI)WU BR), were purchased from Charles River Deutschland (Sulzfeld, Germany). Animals were housed in groups of four per sex in the animal facility with a 12:12-h light:dark cycle and maintained at 22 ± 3 °C and 30–70% relative humidity. Tap water was provided *at libitum*. The diet was provided as a powder in stainless-steel cans covered by a perforated stainless-steel plate that served to prevent spillage.

### 2.2. Test material

Di-*n*-octyltin dichloride, CAS no. 3542-36-7, was obtained from ABCR GmbH &Co.

### 2.3. Diets

During the acclimatization period of 12 days, the rats were fed a commercial rodent diet (Rat & Mouse No. 3 Breeding diet, RM3, SDS Special Diet Services, UK). After the acclimatization period, rats were fed the same diet supplemented with DOTC at concentrations of 0, 3, 10 or 30 mg/kg, respectively. Fresh diets were prepared at least every 4–6 weeks and stored at −20 °C until use.

### 2.4. Study design

Rats were randomly assigned to the treatment groups and received the test diets with 0, 3, 10 or 30 mg/kg DOTC during the premating period, mating, gestation and lactation and subsequently $F_1$ were exposed from weaning onwards. The dose levels were selected based on in house dose range finding studies (data not shown). At the end of the two-week pre-mating period, rats were mated at a ratio of 2 females: 1 male. The day of sperm detection in the vaginal smear was considered day 0 of gestation and the mated $F_0$ females were housed individually.

The morning after birth was considered postnatal day (PND) 1. Litters were not standardized and pups were weaned on PND 21. Evaluation of sexual maturation was performed using 1 pup/sex/litter.

### 2.5. Observations

Throughout the study, all animals were checked daily for clinical signs and abnormal behavior. The body weights of all males and females were recorded weekly during the premating period, and the body weights of the males weekly thereafter. Mated females were weighed on gestational days (GD) 0, 6, 14, and 21 and during lactation on days 1, 4, 8, 10, 13, 17, and 21. Pup body weights were recorded at PNDs 1, 4, 8, 10, 13, 17, and 21 and weekly from weaning.

During the premating period, food consumption was measured weekly for each cage by weighing the feeders. Individual food consumption of all mated females was recorded from GD 0–6, 6–14, and 14–21 and for all females with live pups from postpartum days 1–4, 4–8, 8–10, and 10–13. $F_1$ food consumption was recorded weekly from weaning.

### 2.6. Immune assessments

Subsets of $F_1$ male rats ($n$ = 8/dose) originating from different litters, were evaluated at PNDs 21, 42, and 70 for changes in immune function. Terminal body weights were recorded, and the following organs were weighed: liver, thymus, spleen, kidneys, adrenals, heart, and testes. EDTA blood was collected and two femoral shafts were flushed with 4 ml Impulse Cytophotometer (ICP) solution (only on PND 21 and 70), containing 3.22 g/l trisodium–citrate–dihydrate, 3.4 g/l sodium–dihydrogenphophate–dihydrate, 3.87 g/l disodium–hydrogenphophate–dihydrate, 1.17 g/l citric-acid-monohydrate, 3.65 g/l dextrose, 4.96 g/l sodiumchloride in demineralized water, pH 7.4 at 20 °C. The resulting cell suspension and the collected EDTA blood were kept at 4 °C until automated analysis in an ADVIA 120 (Siemens, Deerfield, IL) within 4 h. Spleen and thymus single cell suspensions were prepared and used for determination of immune parameters as described previously (Tonk et al., 2010). In short, spleen and thymus single cell suspensions were examined for lymphocyte subpopulation distribution using three-color flow cytometry. Allophycocyanin (APO)-conjugated mouse anti-rat CD3 (clone 1F4); R-Phycoerythin (R-PE)-conjugated mouse anti-rat CD8a (clone OX-8); fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat CD4 (clone OX-35); cy-chrome (Cyc)-conjugated mouse anti-rat CD45 (clone OX-1); FITC-conjugated mouse anti-rat CD45RA (clone OX-33); and R-PE-conjugated mouse anti-rat NKR-P1A (clone 10/78) (Pharmingen, San Diego, CA) were used for splenic assessment at predetermined saturation levels. For thymus, only anti-CD3, anti-CD8a, and anti-CD4 monoclonal antibodies were used. Splenic natural killer (NK) cell activity was assessed using an *in vitro* $^{51}$Cr-release assay. Adherent splenocytes were stimulated with lipopolysaccharide (LPS) (Sigma) for 24 h and the supernatants were used to measure the nitric oxide (NO) production using the Griess reaction (Green et al., 1982) and tumor necrosis factor (TNF)-α production. Splenic lymphoproliferative responses to concavalin (Con) A and LPS and the thymocyte lymphoproliferative response to ConA were determined using *in vitro* $^{3}$H-thymidine incorporation. In addition, supernatants of ConA stimulated splenocytes were used for the determination of interleukin (IL)-4, IL-10, Il-13, and interferon (IFN)-γ using a MILLIPLEX Map Kit (Millipore).

A separate group of rats ($n$ = 8 males/dose) were immunized with a subcutaneous injection of 0.2 ml of 5 mg/ml Keyhole Limpet Hemocyanin (KLH) (ThermoScientific) at PND 21 and PND 35. The primary IgM (PND 26), primary IgG (PND 35), and the secondary IgM and IgG (PND 40) response to KLH were determined using an anti-KLH IgM- or IgG-specific ELISA. At PND 49, the Delayed Type Hypersensitivity (DTH) response to KLH was assessed using a challenge with 10 mg/ml KLH in 0.01 ml of saline injected into one ear and an equivalent volume of saline injected in the other ear. The DTH response was measured 24 h later using a digital caliper. The KLH-immunized rats were euthanized at PND 63, spleens were removed aseptically and single spleen cell suspensions were prepared. KLH-induced cell proliferation was determined using *in vitro* $^{3}$H-thymidine incorporation and supernatants of KLH-stimulated splenocytes were used for the determination of IL-4, IL-10, Il-13, and IFN-γ using a MILLIPLEX Map Kit (Millipore).

### 2.7. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA), with post hoc analysis using Dunnett's multiple comparison $t$-test. When either the normality or equal variances prerequisite was violated, data were analyzed using Kruskal–Wallis followed by a Mann–Whitney $U$ in combination with the Benjamini–Hochberg procedure to correct for multiple testing. Fisher's exact probability test was used to evaluate the numbers of mated and pregnant females, females with liveborn pups, females with (all) stillborn pups, pups lost at various stages, pups surviving 21 days, and male pups on PNDs 1 and 21.

## 3. Results

### 3.1. Parental assessment

Parental animals showed no adverse behavior or clinical signs. No statistically significant effects of DOTC on the body weights of the $F_0$ rats were observed, except for the $F_0$ females during lactation. On lactation days 4, 8, 10, 17, and 21 the $F_0$ females in the mid and high dose groups (on day 8 only in the high dose) showed a slight (approximately 5%), but statistically significantly increased body weight when compared to controls (Fig. 1). There were no effects of DOTC on the food consumption of the $F_0$ females during gestation or lactation. The substance intake for the treated F0 females was 0.17–0.21, 0.56–0.71, 1.7–2.1 mg/kg bw/day during gestation and 0.27–0.55, 1.0–1.9, 2.9–5.2 mg/kg bw/day during

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