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Critical Reviews in Toxicology

ISSN: 1040-8444 (Print) 1547-6898 (Online) Journal homepage: http://www.tandfonline.com/loi/itxc20

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To cite this article: Ellen V.S. Hessel, Elisa C. M. Tonk, Peter M.J. Bos, Henk van Loveren & Aldert H. Piersma (2015) Developmental immunotoxicity of chemicals in rodents and its possible regulatory impact, Critical Reviews in Toxicology, 45:1, 68-82, DOI: 10.3109/10408444.2014.959163

To link to this article: http://dx.doi.org/10.3109/10408444.2014.959163

Published online: 05 Nov 2014.

Critical Reviews in Toxicology

http://informahealthcare.com/txc
ISSN: 1040-8444 (print), 1547-6898 (electronic)

Crit Rev Toxicol, 2015; 45(1): 68–82
 DOI: 10.3109/10408444.2014.959163

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REVIEW ARTICLE

Developmental immunotoxicity of chemicals in rodents and its possible regulatory impact

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Abstract

Around 25% of the children in developed countries are affected with immune-based diseases. Juvenile onset diseases such as allergic, inflammatory and autoimmune diseases have shown increasing prevalences in the last decades. The role of chemical exposures in these phenomena is unclear. It is thought that the developmental immune system is more susceptible to toxicants than the mature situation. Developmental immunotoxicity (DIT) testing is nowadays not or minimally included in regulatory toxicology requirements. We reviewed whether developmental immune parameters in rodents would provide relatively sensitive endpoints of toxicity, whose inclusion in regulatory toxicity testing might improve hazard identification and risk assessment of chemicals. For each of the nine reviewed toxicants, the developing immune system was found to be at least as sensitive or more sensitive than the general (developmental) toxicity parameters. Functional immune (antigen-challenged) parameters appear more affected than structural (non-challenged) immune parameters. Especially, antibody responses to immune challenges with keyhole limpet hemocyanine or sheep red blood cells and delayed-type hypersensitivity responses appear to provide sensitive parameters of developmental immune toxicity. Comparison with current tolerable daily intakes (TDI) and their underlying overall no observed adverse effect levels showed that for some of the compounds reviewed, the TDI may need reconsideration based on developmental immune parameters. From these data, it can be concluded that the developing immune system is very sensitive to the disruption of toxicants independent of study design. Consideration of including functional DIT parameters in current hazard identification guidelines and wider application of relevant study protocols is warranted.

Abbreviations: *2,4-D* 2,4-Dichloroacetic acid, *ADI* Acceptable Daily Intake, *ATSDR* Agency for Toxic Substances and Disease Registry (USA), *BMD* Benchmark Dose, *BMDL* Lower confidence limit on the BMD, *BSA* Bovine Serum Albumin, *ConA* Concanavalin A, *CPF* Chlorpyrifos, *DEHP* Di Ethyl Hexyl Phthalate, *DIT* Developmental Immune Toxicity, *DMTC* Di Methyl Tin Chloride, *DNT* Developmental NeuroToxicity, *DOTC* Di Octyl Tin Chloride, *DTH* Delayed-Type Hypersensitivity, *EFSA* European Food Safety Agency, *EOGRTS* Extended One Generation Reproductive Toxicity Study, *EtOH* Ethanol, *FAS* Fetal Alcohol Syndrome, *FASD* Fetal Alcohol Spectrum Disorders, *H* Heptachlor, *Ig* Immonoglobulin, *IL* Interleukin, *INF* Interferon, *IPCS* International Program for Chemical Safety (WHO), *ITER* International Toxicity Estimates for Risk, *JECFA* Joint FAO/WHO Expert Committee on Food Additives (WHO/FAO), *JMPR* Joint Meeting on Pesticides Residues (WHO/FAO), *KLH* Keyhole Limpet Hemocyanine, *LOAEL* Lowest Observed Adverse Effect Level, *LPS* Lipopolysaccharide, MeHg Methylmercury, *MXC* Methoxychlor, *NK* cell Natural Killer cell, NO Nitrous Oxide, *NOAEL* No Observed Adverse Effect Level, *NRC* National Research Council (USA), *OECD* Organisation for Economic Cooperation and Development, *PFC* Plaque Forming Cells, *PHA* phytohaemagglutinin, *PND* Postnatal Day, *pTDI* Provisional Tolerable Daily Intake, *pTMI* Provisional Tolerable Monthly Intake, *PVC* Poly Vinyl Chloride, *PWM* Pokeweed Mitogen, *REACH* European legislation for Registration, Evaluation, and Acceptance of CHemicals, *RIVM* National Institute for Public Health and the Environment (NL), *SRBC* Sheep Red Blood Cells, *TBP* 2,4,6-Tribromophenol, *TBTO* Tri Butyl Tin Oxide, *TCDD* 2,3,7,8-TetraChloroDibenzo-*p*-Dioxin, *TDAR* T-cell Dependent Antibody Response, *TDI* Tolerable Daily Intake, *TNF* Tumor Necrosis Factor, *TG* Test Guideline, WHO World Health Organization

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Keywords

allergy, developmental immunotoxicity, DOTC, DEHP, Ethanol, EOGRTS, immune system, juvenile, MeHg, OECD 443, rat

History

Received 4 April 2014
Revised 14 July 2014
Accepted 24 August 2014

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Introduction

In recent decades, there has been an increased focus on chemical risk assessment in children as a specific subpopulation. Children are not small adults and hazard and risk assessment in adults need not be protective for children (Holsapple et al. 2004). Developing organs may be more susceptible to chemical-induced toxicity than mature adult organs. The nervous system, immune system and reproductive system are more vulnerable to toxicants during the developmental period (NRC 1993, Smialowicz et al. 2001). In this review, we will focus on the effect of toxicants on the (developing) immune system in rodents.

The primary focus of the immune system is to protect the host against invading pathogenic microorganisms and arising neoplasms. Many xenobiotic compounds are known to have toxic effects on the immune system and this may result in reduced immune response and lead to enhanced incidence and/or severity of infections. The development of the immune system starts *in utero* and completes around puberty. Immaturity of the immune system leads to a higher susceptibility to infections and makes the immune system more sensitive to chemical perturbations (Holsapple et al. 2004). Immune-based diseases in the developed countries affect around 25% of the children (Dietert 2009). Early onset diseases related to malfunctioning of the immune system show high prevalence in children in western societies, including recurrent otitis media (18–26%), asthma (15–26%), atopic dermatitis (15%) and allergic rhinitis (8–12%) (Dietert 2009). Moreover, juvenile onset allergic, inflammatory and autoimmune diseases have shown increasing prevalences in recent decades (IPCS 1999, 2006). The underlying causes are probably multifactorial, and a possible causative role of chemical exposures during pre- and postnatal development of the immune system cannot be excluded. On the other hand, direct evidence for a relation with chemical exposure is scarce. Part of this lack of knowledge is the current virtual absence of developmental immunotoxicity testing in the regulatory requirements of chemical hazard assessment. This situation warrants an investigation into the relative sensitivity of the developmental immune system in experimental settings, in order to determine whether regular testing of developmental immune parameters might provide relatively sensitive parameters of toxicity and whether their inclusion in regulatory toxicological testing might improve chemical safety for children. It should be noted that available parameters for the viability and responses of the developing immune system to chemical exposures cannot always directly be extrapolated to human disease states such as autoimmunity and allergy. On the other hand, a compromised immune system should be considered more prone to escape homeostasis, enhancing risk for disease development.

Guidelines for the evaluation of reproductive and developmental toxicity include the prenatal developmental toxicity study (OECD-TG-414 2001), the one- and two-generation reproductive toxicity studies (OECD-415 1983, OECD-416 2001), and developmental neurotoxicity study (OECD-426 2007). These protocols do not contain parameters for evaluation of developmental immunotoxicity (DIT). Parameters indicating possible immune toxicity such as spleen and thymus weight, differential blood cell counts and immunohistopathology parameters could easily be incorporated into these reproductive toxicity studies. Moreover, functional assays may provide additional sensitivity to DIT testing (Cooper et al. 2006, Luster et al. 1992, 1993). In 2011, OECD adopted the guideline for the extended-one-generation reproduction toxicity study (EOGRTS), with dosing from the premating period to offspring adulthood, which now includes structural and functional endpoints for developmental neurotoxicity and DIT (OECD-TG-443 2011). Besides the above-mentioned structural immune parameters, a T-cell-dependent antibody response assay, that is the primary IgM antibody response to a T-cell-dependent antigen, such as sheep red blood cells (SRBC) or keyhole limpet hemocyanin (KLH) is mandatory. This cohort of DIT testing is thought to improve current developmental hazard identification and risk assessment.

The acceptance of the OECD TG 443, including specific attention for developmental immunotoxicity parameters, has been a significant hallmark. It recognizes the importance of assessing developmental immune parameters in regulatory toxicology. However, this study is only applied for a subset of chemicals of high tonnage under the European legislation for chemical safety (REACH). DIT testing could perhaps be more practical in other study designs. In the past, several research groups have embarked on defining exposure protocols considering windows of sensitivity of the developing immune system (reviews: Boverhof et al. 2014, Burns-Naas et al. 2008, Dietert 2009, 2011, Holsapple and O'Lone 2012, Luebke et al. 2006, Smialowicz 2002). Luebke et al. (2006) reviewed five different compounds and concluded that the developing immune system was more susceptible to these toxicants than the adult immune system. Smialowicz (2002) reviewed the effect of three different classes of environmental chemicals after exposure during gestational, lactational, and/or juvenile development on a battery of immune function assays, confirming the potential of the rat as a model for DIT testing. Boverhof et al. (2014) summarized a recent workshop on study designs and guidelines for DIT testing (Boverhof et al. 2014). Many studies have included DIT parameters in juvenile animal models. Some recent examples are chlorpyrifos (CPF) exposure from gestation to puberty in Swiss Albino mice (Singh et al. 2013); 2,4-dichlorophenoxyacetic (2,4-D) exposure in the EOGRTS model (Marty et al. 2013); imidacloprid exposure during gestation, lactation and puberty in Wistar Rats (Gawade et al. 2013);

and dimethyl tin dichloride (DMTC) exposure from gestation to weaning in rats (DeWitt et al. 2007). In recent years, we have added a series of developmental immunotoxicity studies in the rat to this list (Tonk et al. 2010, 2011a, 2011b, 2012, 2013a, 2013b).We have published a series of studies with methylmercury (MeHg), di-*n*-octyl tin chloride (DOTC), ethanol (EtOH) and di-(2-ethylhexyl)-phthalate (DEHP) in rats using various study designs (Figure 1). The developmental exposure windows were based on existing protocols, the developmental neurotoxicity (DNT) protocol (Tonk et al. 2010), the EOGRTS protocol (Tonk et al. 2011a, 2013a), a juvenile exposure protocol (Tonk et al. 2011b) and an adult exposure protocol (Tonk et al. 2012). Compounds were administered via oral intake in the drinking water or food.

Studies on MeHg were performed to compare the relative sensitivities of developmental and immune parameters in a pre- and postnatal exposure design (DNT exposure protocol). MeHg is a widespread environmental and food contaminant that was identified as a developmental neurotoxicant (Risher et al. 2002). Furthermore, MeHg is a well-known immunotoxicant (Descotes 1986) and there are indications that the susceptibilities of the immune system and the developing brain to MeHg are similar as to lowest effective doses (Belles-Isles et al. 2002, Haggqvist et al. 2005). DOTC is used as a stabilizer in polyvinylchloride (PVC) plastics. It was shown to cause thymus atrophy and suppression of T-cell-dependent antibody response in the rat (Miller et al. 1986, Seinen and Penninks 1979, Seinen and Willems 1976). EtOH is a well-known neurodevelopmental and immunotoxic compound in experimental animals as well as in humans (Riley et al. 2011, Szabo and Mandrekar 2009). EtOH and DOTC were both tested in the EOGRTS and juvenile exposure protocols (Figure 1). By testing DIT in both exposure protocols, the effect of EtOH and DOTC on pre- and postnatal development with different exposure windows could be compared. DEHP is a plasticizer used to increase flexibility and durability of PVC plastics. It is a known testicular toxicant, which affects spermatogenesis and testosterone production (Akingbemi et al. 2004, Heindel and Powell 1992, Jones et al. 1993). Phthalates have also been suggested as modulators of the immune system; however, experimental studies investigating effects on the immune response in animals have yielded conflicting results (Piepenbrink et al. 2005, Tonk et al. 2012). By studying DEHP in the juvenile and adult model, we can compare the effect of DEHP exposure on the immune system in juvenile and adult rats.

In this review, we give an overview of experimental studies designed in such a way that they allow an analysis of the relative sensitivity of structural and functional parameters of the developing immune system as compared to regularly tested parameters of general developmental toxicity and discuss the potential of the implementation of DIT parameters in different exposure scenarios. Furthermore, we address the question of whether the additional consideration of developmental immune parameters in the reviewed studies might have changed the derivation of overall no observed adverse effect levels (NOAEL), which in turn could have impacted on the risk assessment of the compounds under study. Our findings provide further support for the inclusion of developmental immune toxicity parameters at an early stage of chemical hazard identification.

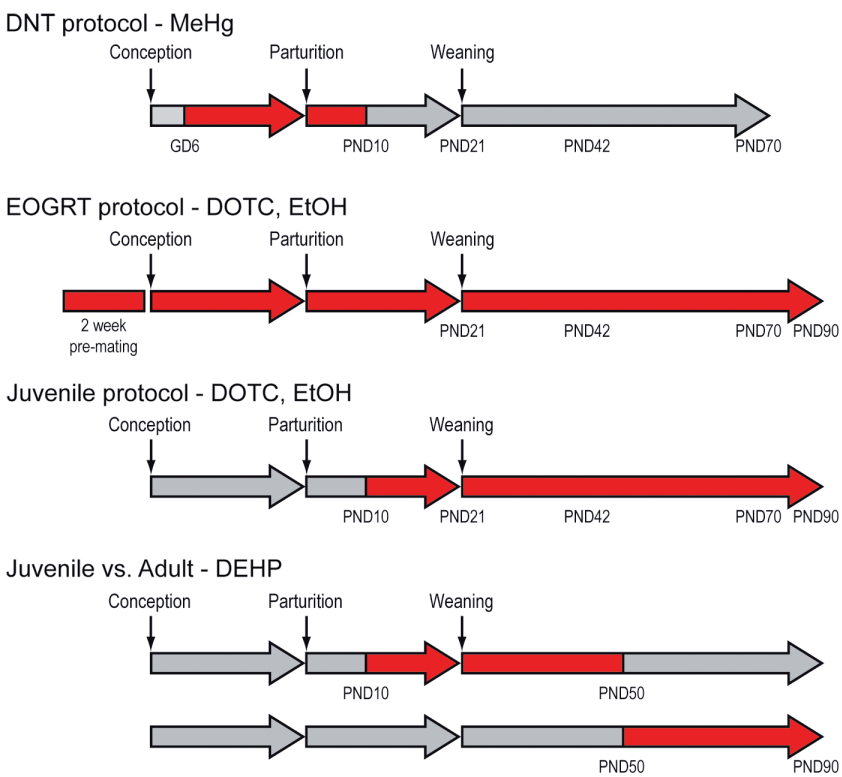

Figure 1. Overview of the rat models and compounds tested at RIVM: DNT: developmental neurotoxicity protocol; EOGRTS: extended one-generation reproductive toxicity study: *MeHg* methylmercury, *DOTC* di-*n*-octyl tin chloride, *EtOH* ethanol, *DEHP* di-2(ethylhexyl)phthalate. Exposure periods are indicated in *red*.

Data collection and analysis of relative parameter sensitivity

Classical developmental toxicity parameters assessed included maternal body weight and water consumption, pup body weight and water consumption, stillborns, mortality, complete litter loss, organ weights (excluded thymus and spleen weight) and hematology (differential cell counting red and white blood cells) (Tonk et al. 2010). The immune parameters selected for our DIT testing were based on the immune parameters included in the EOGRTS protocol (Cooper et al. 2006, OECD-TG-443 2011) complemented with some additional parameters. In addition to lymphoid organ weights, these parameter sets consisted of phenotypic analysis of lymphocyte subpopulations and cellularity in the spleen and thymus as structural immune parameters. As suggested in the OECD TG 443 guideline, in addition to developmental and structural immune parameters, functional DIT testing may provide additional endpoints that involve characterization of the cellular and functional status of the immune system (functional parameters) (Boverhof et al. 2014, OECD-TG-443 2011). The OECD TG 443 prescribes including serum IgM antibody titers (sensitization to SRBC or KLH) and testing the T-cell-dependent antibody response (TDAR). In the studies of Tonk, these functional DIT parameters included: 1) natural killer cell activity assessment in splenocytes (NK cell activity), 2) evaluation of lipopolysaccharide (LPS)-stimulated nitric oxide (NO) and tumor necrosis factor (TNF)-α production by adherent splenocytes (adherent cells), 3) assessment of mitogen (LPS or ConA) –stimulated lymphoproliferative (LP) responses in splenocytes and thymocytes (proliferation): evaluating proliferation and cytokine production. Furthermore, 4) KLH-specific responses including: a) T-cell-dependent antibody response (TDAR). Using KLH as a T-cell-dependent antigen, immunized rats were assessed for both primary and secondary anti-KLH IgM and anti-KLH IgG responses; b) delayed type hypersensitivity (DTH) measured response to an ear challenge with KLH and c) KLH-specific LP response: proliferation and cytokines (Tonk et al. 2010). Tonk et al. (2010) describe the details of the different parameter tests. We compiled these studies, which were preferably analyzed using a benchmark dose (BMD) approach wherever possible. The lower confidence limit of the BMD (BMDL) was used as the indicator of parameter sensitivity. For the studies that did not use a sufficient number of dosages to allow a meaningful BMD analysis, the lowest observed adverse effect levels (LOAEL) of the parameters and parameter groups tested were determined (e.g. DOTC exposure in the EOGRTS model) (Tonk et al. 2011a). We ranked the parameters by BMDL/LOAEL within one parameter group and determined the parameter with the lowest BMDL, which was collected into Table 1 for each of the studies analyzed. The lowest BMDL of the different groups within each study listed in Table 1 is represented in bold. In addition to our own studies, we summarized literature developmental toxicity studies in which both general developmental as well as immune toxicity parameters were measured and in which differences were found in the LOAELs between the different parameter groups (Table 2). As these studies invariably used the classical NOAEL approach, LOAEL within the studies reviewed were determined. If within one parameter group more than one parameter was affected at the NOAEL, they were all added to the table. Data were presented in three groups of parameters, together covering the entire study, and defined as (1) general and developmental toxicity parameters, (2) structural immune parameters, and (3) functional immune parameters, respectively. Within each parameter group, the lowest BMDL (Table 1) or LOAEL was derived (Table 2). This approach allowed us to analyze the relative sensitivity among these parameter groups in terms of lowest effective dose, to establish the contribution of the immune parameters to the overall NOAEL or LOAEL in each study. For comparison, acceptable daily intakes (ADIs) and/or tolerable daily intakes (TDIs) for the respective chemicals as published were collected. ADIs and TDIs published by official advisory boards, governments and legislation agencies were preferred (e.g., EFSA, JMPR, WHO/IPCS, RIVM). The ITER database (International Toxicity Estimates for Risk) was accessed since this database provides an overview of evaluations by many organizations. ADIs were published for the pesticides, chlorpyrifos, heptachlor and methoxychlor. TDIs were retrieved for the other compounds. Wherever possible, the rationales for the ADIs and TDIs were verified in the evaluating documents or opinions. The respective sources are given in Table 3.

Developmental immune toxicity studies

MeHg

Rodents have shown suppression of humoral immunity after MeHg exposure (Blakley et al. 1980). Furthermore, perinatal exposure of MeHg can lead to reduced NK cell activity and alteration in the proliferation response of lymphocytes to mitogens (Ilback et al. 1991, Ortega et al. 1997, Wild et al. 1997). Children exposed to MeHg *in utero* via maternal seafood consumption presented a decrease in the proportion of $CD4^+CD45RA^+$ cells and IgM levels in cord blood. These studies suggest that the developing immune system is sensitive to MeHg. This was confirmed by the study of Tonk et al. (2010). They showed that MeHg exposure (from gestation day (GD) 6 to postnatal day (PND) 10) affected developmental parameters, such as litter loss, pup mortality and body weight (Tonk et al. 2010). Furthermore, immune parameters were affected at lower dose levels which were without observed developmental toxicity. Functional immune parameters appeared even more sensitive with a BMDL of 0.01 mg/kg-day for the primary anti-KLH IgG response on PND 35 as the most sensitive parameter (Table 1) (Tonk et al. 2010). Comparisons between the developmental parameters and the developmental immune parameters clearly showed a relatively high sensitivity of the DIT parameters. This was the first study using the benchmark approach that determined the relative sensitivity of the developing immune system for MeHg. A TDI of 0.0001 mg/kg has been derived for MeHg based on a developmental NOAEL in humans of 0.0013 mg/kg (Baars et al. 2001). The NOAEL was calculated from the mercury concentration in hair taken at parturition from the highest exposure group; no (neuro) developmental effects were observed in this group (ATSDR 1999). The BMDL of 0.01 mg/kg derived from the study by Tonk et al. is a factor of 100 higher than this TDI, which might be considered as a sufficiently large margin. However, in rats,

Table 1. Overview of the most sensitive parameters from RIVM studies.

Compound	Exposure period	Dose mg/kg	Species	Developmental parameter BMD (BMDL-BMDU)/ LOAEL	Structural DIT parameter BMD (BMDL-BMDU)/ LOAEL	Functional DIT parameter BMD (BMDL-BMDU)/ LOAEL	References
MeHG *Methylmercury*	GD6 - PND10	0; 0,1; 0,4; 0,7; 1,0; 1,5; 2,0	Wistar Rats	Liver weight (g) (PND 21) 0.38 (0.20–1.72)	NK cell number (10^7) (Spleen; PND 21)** 0.18 (0.09–0.52)	**anti-KLH IgG response (PND 35) (ng/ml) 0.04 (0.01–0.12)**	Tonk et al. (2010)
DOTC *Di-n-octylin dichloride*	EOGRTS	0; 3; 10; 30	Wistar Rats	Liver weight (g) (PND 70) 3 mg/kg	Thymus weight (g) & cellulairity; Different spleen & thymus cell subtypes (PND 42 and/or 70) 30 mg/kg	**KLH specific antibody response-DTH (PND 49) 3 mg/kg**	Tonk et al. (2011a)
	PND10 - PND90	0; 0,15; 0,3; 0,5; 1,0; 1,5; 3,0; 5,0	Wistar Rats	Liver weight (g) (PND 21) 2.27 (0.31–9.51)	Number CD4-CD8 + cells (10^7/g) (Thymus; PND 42)** 1.37 (0.18–7.89)	**KLH - Proliferation response (cpm) (PND 63) 0.29 (0.06–1.57)**	Tonk et al.; (2011b)
EtOH *Ethanol*	EOGRTS	0; 1.5; 4.0; 6.5; 9,0; 11.5; 14%	Wistar Rats	**Water consumption 0.2 (0.09–0.37)**	Number CD4-CD8- cells (10^7/g) (Thymus; PND 42)** 0.42 (0.23–0.69)	ConA proliferation Spleen (cpm) (PND 21) 0.49 (0.20–1.53)	Tonk et al. (2013a)
	PND10 - PND90	0; 0.25; 1.5; 2.75; 4.0; 5.25; 6.5%	Wistar Rats	Water consumption 0.97 (0.62–1.66)	rel Number CD4 + CD8+ cells (10^7/g) (Thymus; PND 70)** 0.54 (0.07–10.72)	**anti-KLH IgG (PND 40) (ng/ml) 0.04 (0.004–0.16)**	Tonk et al. (2013b)
DEHP *Di(2-ethylhexyl) phthalate*	PND10 - PND50	0; 1; 3; 10; 30; 100; 300; 1000	Wistar Rats	rel Liver weight (g/100g bw) (PND 50 + 90) 11.70 (4.42–26.30)	T/B ratio** (PND 50) 172.32 (105.82–435.03)	**KLH- stimulated IL-13 (pg/ml) (PND 50) 0.53 (0.09–2.30)**	Tonk et al. (2012)
	PND50 - PND90	0; 1; 3; 10; 30; 100; 300; 1000	Wistar Rats	rel Liver weight (g/100g bw) (PND 50 + 90) 11.70 (4.42–26.30)	**CD3 + cell count (Spleen)** (PND 90) 0.88 (0.04–79.23)**	LPS- induced TNF-α production of adherent cells (PND 90) 0.49 (0.12–2.23)	Tonk et al. (2012)

**Lymphocyte subpopulations.

DOI 10.3109/10408444.2014.959163

Table 2. Overview of the most sensitive parameters from literature studies.

Compound	Exposure period	Dose	Species	Developmental parameter LOAEL	Structural DIT parameter LOAEL	Functional DIT parameter LOAEL	References
Heptachlor	G12–PND42	0, 0.03, 0.3, 3 mg/kg	Spraque-Dawley rats	Body weight pup (g) (PND 1) & Liver weight (g) (PND 46) 3 mg/kg/day	not significantly affected	**anti SRBC-IgM antibody response and secundairy IgG repsonse(males; 8 wks and 26 weeks respectively) 0.03 mg/kg/day**	Smialowicz et al. (2001)
TBTO Tribytyltin oxide	PND3–PND24	0; 2.5; 5; 10 mg/kg/dosis dosis 3x a week, total 10 dosages	Rat, F344	Body weight pup (g) 10 mg/kg/dosis	Spleen and Thymus weight (g) (age 26 days) 10 mg/kg/dosis	**NK cell activity/ConA, PHA and PWM proliferation response Spleen (age 26 days) 5 mg/kg/dosis (= 1,1 mg/kg/day)**	Smialowicz et al. (1989) Luebke et al. (2006)
	from PND21 up to 5,5–6 months from PND21 up to 15–16,5 months	0; 0,5; 5; 50 mg/kg/diet	Wistar rats	not significantly affected	Thymus weight (g) 50 mg/kg-diet	**IgE antibody response to T. spiralis infection 5 mg/kg-diet (= 0,025 mg/kg/day)**	Vos et al. (1990)
TCDD 2,3,7,8-Tetrachlorodibenzo-pdioxin	G14	0; 0,1; 0,3; 1; 3 ug/kg	Rat, F344	not significantly affected	Thymus atrophy 3 ug/kg	**DTH response to BSA (14 months old) 0.1 ug TCDD/kg/day**	Gehrs et al. (1997, 1999), Smialowicz (2002) Luebke et al. (2006)
	PND0–PND21	0; 1,8; 18 ng TCDD/L drinking water	Mouse: C57BL/6J	not significantly affected	CD4 + cells thymus; age 3 wks 11,0 ng/kg	**TNF-α expression after infection (age 3 wks) 1.1 ng/kg/day**	Sugita-Konishi et al. (2003), Smialowicz (2002)
Methoxychlor (MXC)	GD14–PND42 from day 7 direct dosing	0; 5; 50; 150 mg/kg	Spraque-Dawley rats	Body weight PND 35–42 males and females 50 mg/kg/day	Thymus and Spleen weight (g) males age 8–9 wks 50 mg/kg	**Primary antibody response to SRBCs (males only; 9 wks) 5 mg/kg**	Chapin et al. (1997), Smialowicz (2002)
Chlorpyrifos (CPF)	GD12–PND42 from day 7 direct dosing	0, 0.3 and 3 mg/kg	Swiss albino mice	not significantly affected	**Spleen CD8+; T-cells; CD4+/CD8 + ratio & T/B cell ratio (females, age 7 wk)** 0,3 mg/kg**	**anti SRBC- IgM antibody response (age 7 wks) 0.3 mg/kg**	Singh et al. (2013)

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Table 3. Comparison of the current norm in man and its NOAEL with the affected dosage found in developmental immunotoxicity (DIT) studies.

Compound	NOAEL/LOAEL/ BMDL DIT study	ADI/TDI	Current NOAEL used to determine ADI/TDI	Species	Study design	Critical effect	References	Source
MeHG	40 ug/kg (BMDL)	0,1 ug/kg	1,3 ug/kg	human	Diet (fish)	Developmental	ATSDR (1999)	RIVM
Methylmercury								
*DOTC**	0,06 mg/kg (BMDL)	0,0021 mg/kg	0,23 mg/kg			**Immunosuppresive**		IPCS
Di-n-octylin dichloride								
DEHP	0,09 mg/kg (BMDL)	0,004 mg/kg	4,8 mg/kg	rat	multigeneration	Testicular effects	Wolfe (2003)	EU RAR
Di(2-ethylhexyl) phthalate								
Heptachlor	0,03 mg/kg (LOAEL) effect size: ± 10%	0,0001 mg/kg ADI	0,025 mg/kg	dog	Diet (2 years)	liver tox	Wazeter et al. (1971)	JMPR, 1992
TBTO	0,025 mg/kg (NOAEL)	0,00025 mg/kg	0,025 mg/kg	rat	Diet (18 m)	**Immunosuppresive**	Vos et al. (1990)	RIVM
Tribytyltin oxide								
TCDD	1,1 ng/kg (LOAEL) effect size: ± 10%	0,002 ng/kg	13–19 ng/kg	rat	single dose	Reproductive effects	Ohsako et al. (2001)	RIVM
2,3,7,8-Tetrachlorodibenzo-p-dioxin								
Methoxychlor (MXC)	5 mg/kg (LOAEL) effect size ± 40%	0,10 mg/kg ADI	10 mg/kg	rat	Diet (2y)	Growth reduction	Hodge et al. (1952)	RIVM
Chlorpyrifos (CPF)	0,3 mg/kg (LOAEL) effect size: 40–60%	0,01 mg/kg ADI	1 mg/kg	rat/dog/mouse	Diet (2y) rat/dog; mouse gavage (dev)	Brain Ach inhibition (dog, rat)	Rat: Young and Grandjean (1988) Dog: McCollister et al. (1971)	
			0,1 mg/kg	human	tablet oral	Maternal tox (mouse)		

the immune parameters appeared as a more sensitive endpoint than the developmental parameters, indicating the importance of routinely assessing developmental immune parameters in regulatory toxicity studies.

DOTC

Most toxicology studies on DOTC exposure were performed in adult animal models and these studies were performed using high dose levels ($\geq$ 50 mg/kg diet). Effects in adult rats have been identified on thymus atrophy, decreased numbers of circulating lymphocytes and dysfunction of the T lymphocyte-mediated immune response (Miller et al. 1986, Seinen and Penninks 1979). To determine the effect of DOTC on the developing immune system, Fischer 344 rats were exposed to DOTC by gavage (20, 30, 40 or 50 mg/kg) during the prenatal and/or postnatal period (Smialowicz 2002, Smialowicz et al. 1988). The pups showed no consistent alteration in immune function. This was determined by studying the *in* vitro LP response to T- and B-cell mitogens or by the *in* vitro NK cell ^{51}Cr release assay. Interestingly, direct dosing of the pups three times a week with DOTC (5, 10 or 15 mg/kg) from PNDs 3–24 (10 dosages in total) resulted in suppression of the LP responses up to PND 70, after which LP responses returned to control levels. In comparison, young adults [8 weeks old rats dosed by gavage (10 or 20 mg DOTC/kg, 3 times per week for a total of 10 dosages)] show no effect on LP response 1 week after the last exposure (Smialowicz 2002, Smialowicz et al. 1988). In conclusion, these studies showed that the developing immune system was more susceptible to orally administered DOTC than the mature immune system.

Additionally, the effects of DOTC in the EOGRTS and juvenile exposure model have been studied. In the juvenile DOTC study, immune effects were most pronounced on PNDs 21 and 42 and observed at lower dose levels than effects on developmental parameters (Table 1). The most sensitive immune parameters included TDAR parameters, NO production by adherent splenocytes and thymocyte subpopulations. Most of these DIT parameters were more sensitive (lower BMDL) than the developmental parameters measured in these experiments. In the EOGRTS model, assessments at later time points revealed effects of DOTC exposure (30 mg/kg) on thymus weight, cellularity and subpopulations on PND 42; in the spleen, an effect was found on splenocyte subpopulations on PNDs 42 and 70 but not on cellularity or spleen weight. The DTH-measured response was identified as an important functional parameter that was affected at lower concentrations (3 mg/kg) than any other parameters (Table 1). This showed that, also in the EOGRTS model, high sensitivity of DIT parameters to DOTC exposure was identified. Consistent with the results of Smialowicz et al. (1988), on PND 21, a lower dosage effect was found in the direct-dosed juvenile model in comparison to the EOGRTS model in which the rats were exposed to DOTC via the placenta and milk. The lowest $BMDL_{05}$s in the juvenile exposure study were 0.06 mg/kg for KLH – proliferation response (cpm) (PND 63) and 0.13 mg/kg for NO stimulation of adherent cells. A provisional TDI of 0.0021 mg/kg was calculated for DOTC based on a NOAEL of 0.23 mg/kg, derived for DOTC for thymus lymphoma in a 2-year study in rats, calculated from a 65:35 mixture of

monooctyl tin trichloride (MOTC) and DOTC (Dobson et al. 2006). The $BMDL_{05}$s are therefore approximately a factor of 4 and 2, respectively, lower than the NOAEL underlying the provisional TDI. This indicates that developmental immune parameters provide sensitive endpoints for DOTC that may affect the derivation of the TDI. This further illustrates the relative sensitivity of the developing immune system for DOTC and the importance of assessing functional immune parameters (Tonk et al. 2011b).

EtOH

Prenatal ethanol exposure can cause physical and mental defects in children, known as the fetal alcohol syndrome (FAS) or fetal alcohol spectrum disorders (FASD) (Riley et al. 2011). Furthermore, prenatal ethanol exposure is associated with impairments in immune competence in humans and rodents (Chiappelli and Taylor 1995, Szabo and Mandrekar 2009, Zhang et al. 2005). In the juvenile exposure model, immune parameter effects were observed at lower exposure levels (BMDLs) than effects on developmental parameters (Tonk et al. 2013b). Functional immune parameters were found to be most sensitive, in particular the KLH-specific immune response, LPS-induced NO production by adherent cells, and IL-10 production by ConA stimulated splenocytes. Anti-KLH IgG response showed the lowest BMDL (Table 1). In addition, in the EOGRTS model, functional immune parameters were also affected at lower exposure levels than general reproductive and developmental parameters. The most sensitive parameter was eosinophil counts and the most sensitive functional immune parameter, the ConA-stimulated splenocyte proliferation (Table 1). These data confirm the importance of testing the effect of toxicants on the developing structural and functional immune parameters and demonstrate the relative sensitivity of functional immune parameters. Comparing ethanol exposure in the two models showed that effects on thymus subpopulations, T cell function reflected by ConA-stimulated splenocyte proliferation and innate immunity were found in both studies. The KLH-specific functional immune parameters were found to be differentially affected. In the EOGRTS exposure, design exposure to EtOH resulted in a decreased anti-KLH IgM production and KLH-specific proliferation, while in the juvenile model, anti-KLH antibody responses were increased and DTH decreased. These findings suggest that the exposure window can influence the nature and extent of the toxic insult (Tonk et al. 2013a).

The exposure window of the juvenile model was shorter than the ethanol exposure in the EOGRTS model (Figure 1). However, the effects observed in the juvenile model occurred at lower exposure levels. The lowest BMDL in the EOGRTS model was 0.13% EtOH for eosinophil concentrations in blood (excluding the BMDL on water consumption (0.09%) probably due to taste aversion (Table 1)), whereas in the juvenile model, the lowest BMDL was observed for anti-KLH IgG (0.004%), IL-10 production by ConA-stimulated splenocytes (0.007%) and NO production by adherent splenocytes (0.009%) (Tonk et al. 2013a). There is no official acceptable exposure limit for ethanol intake during conception, pregnancy and breast-feeding, since there is no accepted safe level of alcohol exposure in pregnancy (Gezondheidsraad 2005). The EOGRTS and juvenile studies illustrate this by showing that developmental and immune toxicity occurs at very low exposures (down to a BMDL of 0.004%) with the immunological parameters appearing to be the most sensitive.

DEHP

DEHP is the most abundant phthalate in the environment and perinatal exposure affects testosterone production and male sexual differentiation (Akingbemi et al. 2004, Heindel and Powell 1992, Jones et al. 1993). DEHP exposure post-weaning in male rats showed that the onset of puberty is delayed and male sex organ weights are reduced (Noriega et al. 2009). Furthermore, epidemiology studies suggest that DEHP affects the immune system and the expression of allergy in human, such as asthma and eczema (Bornehag et al. 2004, Kolarik et al. 2008); however, these findings remain controversial due to limitations in study design (Tonk et al. 2012). Studies in rodents on the effect of DEHP on the (developing) immune system have yielded conflicting results, probably due to the difference in experimental design of the studies (Piepenbrink et al. 2005, Tonk et al. 2012). In one study, rats were exposed to DEHP (1–1000 mg/kg) during development (PNDs 10–50, 40 days exposure) and a separate group was exposed in adulthood (PNDs 50–90, 40 days exposure). These parallel study designs enabled to directly compare the developing and the adult immune system as to the effects of DEHP. Exposure of DEHP showed effects on testosterone in juvenile and adult animals, as expected (Tonk et al. 2012). The most sensitive parameters affected in the juvenile exposed group (PNDs 10–50) were KLH-stimulated cytokine production (IL-13 with a BMDL of 0.09 mg/kg (Table 1); IL-6 with a BMDL of 0.50 mg/kg; IL-4 with a BMDL of 1.10 mg/kg) and NK activity (BMDL of 0.361 mg/kg). In the adult exposed group (PNDs 50–90), the most sensitive parameter was TNF-α production by adherent splenocytes [BMDL of 0.12 mg/kg; however, the levels of TNF-α were low and close to the detection limit (Table 1)]. Furthermore, the results of this study show a relatively higher sensitivity of the developing immune system in juvenile animals as compared to general toxicity and developmental parameters. In addition, the effects of DEHP on the immune system was more extensive in juvenile animals in comparison to adult animals, since more immune parameters were statistically significantly affected in the juvenile exposed animals (Tonk et al. 2012). A TDI of 0.05 mg/kg was derived based on a NOAEL of 4.8 mg/kg (for testicular toxicity and developmental toxicity) determined in a multigeneration reproductive toxicity rat study (AFC 2005, Wolfe 2003). The lowest BMDL of 0.09 mg/kg for the most sensitive effect in the DIT study (KLH-stimulated IL-13) is below this NOAEL and even close to the TDI. Also, the $BMDL_{05}$s for KLH-stimulated IL-6 and IL-4 and for NK activity are below the NOAEL from the multigeneration study. Although the interleukin effects occur at very low dosages, it cannot be excluded that they represent non-adverse physiological responses to a toxicant in combination with antigen exposure. Therefore, these findings need not warrant reconsideration of the current NOAEL and TDI. On the other hand, it must be taken into consideration that the asthma response is based on a cytokine response (including IL-4 and IL-13) after exposure to an allergen (Harper and Zeki

2014), which underlines the importance of interleukin levels as indicators of disease susceptibility.

Compilation of relevant data from literature

No DIT studies were found in the literature that used the benchmark approach for dose–response analysis. Moreover, single dose studies were not included in this review, since they do not allow conclusions about differences in sensitivity between the measured parameters. Examples include cyclosporin A 10 mg/kg (Barrow et al. 2006); atrazine 35 mg/kg/day (Rowe et al. 2006). atrazine 35 mg/kg; bromocryptine 0.2 mg/kg and propylthiouracil 2 mg/kg (Rooney et al. 2003), cocaine and ethanol (Figliomeni and Turkall 1997). Some other studies show no effect on (functional) immune parameters but did show an effect on developmental parameters. Examples are DMTC in the juvenile and adult model (DeWitt et al. 2007); 2,4-dichlorophenoxyacetic acid in the EOGRTS model (Marty et al. 2013); dibutyl tin dichloride (DBTC) exposure from GD6-PND 21 (DeWitt et al. 2006); and phenol in the two-generation reproduction study (Ryan et al. 2001). Finally, another group of studies showed that at the lowest dosage tested, both developmental and DIT parameters were affected. These studies concluded that the compounds tested were developmental immunotoxicants; however, we cannot include these studies to investigate if DIT parameters were more susceptible than developmental parameters. Examples are vinclozolin in the EOGRTS model (Schneider et al. 2011); cyclosporin A administered from G6–G21 (Hussain et al. 2005); dexamethasone exposure from G6–21 (Dietert et al. 2003); 2,4,6-tribromophenol (TBP) (Lyubimov et al. 1998); and imidarcloprid (Gawade et al. 2013). To conclude about relative sensitivity of DIT parameters lower dosages would have to be tested. Below, we would like to summarize studies identified that did allow an analysis of relative parameter sensitivity.

Heptachlor

Heptachlor (H) is a chlorinated cyclodiene pesticide used primarily as an agricultural and domestic insecticide from the mid-1960s to the early 1980s. Time pregnant Sprague–Dawley rats were dosed by gavage with H (0, 0.03, 0.3, 3.0 mg/kg) from GD 12 to PND 7 and following direct pup dosing with H through PND 42 (Smialowicz et al. 2001). Absolute body weights of pups at birth were reduced at 3.0 mg/kg. Female and male absolute and relative liver weights were increased in the 3.0 mg/kg dosage group. Exposure did not affect spleen and thymus weight or cellularity at 8 weeks of age nor did it affect immune function tests (e.g. NK cell activity, LP response to mitogens, ConA or PHA proliferation). In 8-week-old male pups, but not in females, the primary IgM antibody response to SRBCs was decreased at 0.03 mg/kg. The IgM antibody response to SRBC was decreased in 0.3 and 3 mg/kg dosages as well. The IgM response in 21-week-old male rats was decreased in the 0.3 dosed group only, and in the same animals the secondary IgG response 4 weeks later was decreased in all the male groups but not in de females at 26 weeks of age. The percentage of B-lymphocytes ($OX12^{+}OX19^{-}$) in the spleen was also reduced in the high-dose males. DTH response was not affected in both genders, but the authors do discuss a non-significant decrease in DTH response. They discussed that other studies focusing on perinatal exposure to organochlorine chemicals do affect immune suppression of T-cell-mediated response in males (Chapin et al. 1997, Gehrs et al. 1997;Gehrs and Smialowicz 1999). The T-cell response to SRBCs has been demonstrated to be one of the most commonly affected and sensitive functional parameters in animals exposed to chemical immunosuppressants (Luster et al. 1992, Smialowicz 2002).

The current ADI (0.0001 mg/kg) is based on a NOAEL of 0.025 mg/kg for liver effects in dogs (JMPR 1992, Wazeter et al. 1971). The study of Smialowicz et al. (2001) described above determined a LOAEL of 0.03 mg/kg on anti-IgM primary antibody response (circa 8% response magnitude) and anti-IgG secondary antibody response in males (10–20% response magnitude) (Table 2). Thus, several immune parameters were affected at 0.03 mg/kg/day, suggesting that this LOAEL is based on a relevant DIT effect. Although not significant, a trend was found for DTH response as well in males. Gender differences should be taken seriously for functional parameters, since this is more commonly found for immune responses, like for methoxychlor (Chapin et al. 1997). The NOAEL that underlies the ADI is in the same order as the LOAEL (0.03 mg/kg) of this DIT study (Smialowicz et al. 2001). Considering the low magnitude of responses in the developmental immune parameters at the LOAEL, adjustment of the ADI might not be necessary. It does show, however, that these DIT parameters are relatively sensitive endpoints for H.

Tribytyl tin oxide

One of the most widely used organo tin compounds is tribytyl tin oxide (TBTO), commonly used as a wood preservative, insecticide and fungicide (Smialowicz et al. 1989). The aim of the study of Smialowicz et al. (1989) was to compare the difference in immune response to TBTO exposure in adult rats (9 weeks old; 0; 1.25; 2.5; 5; 10 mg/kg/doses) vs rat pup rat (3–24 days old; 2.5; 5; 10 mg/kg, total of 10 dosages, 3 × per week). Here, we will only summarize the pup results. Body weight was reduced in the pups treated with 10 mg/kg and no effect was found on body weight in the other treated groups. In the 5 and 10 mg/kg group, thymus weight was reduced, and spleen weight was increased in the 10 mg/kg group. However, the cellularity of these spleens in the 10 mg/kg group was decreased. NK cell activity was reduced at 5 and 10 mg/kg. The 5 and 10 mg/kg group showed a reduction in the LP response to ConA, PHA and PWM (Smialowicz et al. 1989). The 2.5 mg/kg dosage (3 × per week) is equivalent to = 1.1 mg/kg/day. These data suggest that TBTO might have an effect at lower dosages on the developing immune system (e.g. NK cell activity, LP response) than on regular developmental endpoints (body weight). Vos et al. studied the effect of TBTO on different immune parameters in young and adult exposed rats (Vos et al. 1990). Different dosages (0; 0.5; 5 and 50 mg/kg diet) and immune parameters (DTH response, antibody response to SRBC; ovalbumin and T. Spiralis) were tested. They identified an effect on IgE antibody response after T. Spiralis exposure at 5 mg/kg diet. This effect was found in juvenile long-term and short-term exposed animals, but in adult exposed animals

this immune parameter was only affected at 50 mg/kg diet. Based on these DIT studies, they determined a NOAEL of 0.5 mg TBTO/kg diet (estimated dose of 0.025 mg/kg) (Vos et al. 1990). RIVM (Tiesjema and Baars 2009) has derived a TDI for TBTO of 0.00025 mg/kg-day, based on a NOAEL of 0.025 mg/kg for depression of IgE titers in rats (immunosuppression) (Vos et al. 1990). Thus, interestingly, this TDI is already based on functional developmental immune parameters that appear to be the most sensitive endpoint for TBTO.

2,3,7,8-Tetrachlorodibenzo-p-dioxin

A well-studied developmental immunotoxicant is 2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) (Smialowicz et al. 2002). Studies in different strains and species have been performed showing that the immune system is relatively sensitive to TCDD (Smialowicz 2002). TCDD produces more severe effects when exposure occurs during development (Gehrs et al. 1997, Smialowicz 2002, Vos et al. 1974). In rats, after developmental exposure, TCDD affects T cell proliferation and DTH responsiveness (Faith and Moore 1977, Gehrs et al. 1997, Gehrs and Smialowicz 1999, Vos et al. 1974). Furthermore, mouse thymocyte subpopulations were affected (Fine et al. 1989, Holladay et al. 1991). Smialowicz et al. applied exposure on GD 14 to different TCDD dosages (ranging from 0.1 to 3.0 ug TCDD/kg) and studied effects on the developmental immune system (DTH response to KLH and/or BSA, thymus weight, cellularity, LP response to T- and B-cell mitogens and the plaque forming cells (PFC) antibody response to SRBCs). The results of these three studies (Gehrs et al. 1997, Gehrs and Smialowicz 1997, 1999) indicate that suppression of the DTH response to BSA associated with perinatal TCDD exposure occurs at a low dose (i.e., 0.1 ug TCDD/kg) and is more pronounced in males than females (in females the effect is found in the 0.3 ug TCDD group) (Smialowicz 2002). Developmental parameters like body weight and pup mortality were not affected at 1 ug TCCD/kg or lower TCCD dosages (Gehrs et al. 1997, Gehrs and Smialowicz 1997, Smialowicz 2002).

The effect of TCDD on the developing immune system was further studied in C57BL/6J mice (Sugita-Konishi et al. 2003). Mice pups were exposed to TCDD (0; 1.8; 18 ng TCDD/L in the drinking water of the dams, dosages of 0; 1.1 and 11.1 ng/kg bw, respectively) from birth to weaning, via the milk. The exposure had no effect on developmental parameters like body weight. No significant effect was found on weight of immune organs and the spleen and thymus cell populations (at the highest dose percentage of CD4-positive cells in the thymus were increased). Enhanced TNF-α and IFN-γ levels in the serum after Listeria infection were found at the lowest dosage. These data suggest that the developing immune system was affected by TCDD exposure in mice, at doses at which general developmental parameters, such as body weight were not affected (Sugita-Konishi et al. 2003).

The Scientific Committee on Food of the European Union (SCF 2000, 2001) derived a provisional tolerable weekly (pTDI) of 14 pg/kg bw, whereas JECFA derived a comparable provisional tolerable monthly intake (pTMI) of 70 pg/kg bw (JECFA 2002, Tiesjema and Baars 2009). Both these values were based on a LOAEL for the reproductive system of male offspring from a study in which dams were treated subcutaneously before and throughout mating, pregnancy and lactation and on a NOAEL for the same endpoint in a study in which dams received a single oral dose on GD15 (Ohsako et al. 2001). The maternal body-burden LOAEL and NOAEL were estimated by PBPK modeling to be 25–39 ng/kg and 13–19 ng/kg, respectively. Based on these evaluations, RIVM derived a pTDI of 2 pg/kg for pragmatic reasons (Tiesjema and Baars 2009). The LOAEL of the DIT study was 1.1 ng/kg/day for TNF-α levels after infection in males and females (8% response magnitude) (Sugita-Konishi et al. 2003). This LOAEL is a factor of 10 and 20 lower than the calculated maternal body burdens for the NOAEL and LOAEL, respectively, for the reproductive system of male offspring in rat. Based on the parameter affected, cytokine production, and the limited magnitude of the effect, it is uncertain whether these findings represent adverse or physiological responses at the lowest dosage (1.1 ng/kg/day). Furthermore, the critical functional immune parameter, the number of bacteria in whole spleen, related to cytokine production after Listeria infection, was not affected at this dosage. Therefore, these data provide insufficient evidence that the developing immune system is adversely affected by TCDD exposure, as was also concluded previously by the JECFA and RIVM (JECFA 2002, Tiesjema and Baars 2009).

Methoxychlor

Methoxychlor (MXC) is an organochlorine pesticide that is widely used for insect and larval control (Chapin et al. 1997, Smialowicz 2002). Time-bred Spraque–Dawley rats were dosed by gavage (GD 14–PND 8) with 5, 50 or 150 mg MXC/kg. From PND 7, the pups were dosed until PND 42. Developmental parameters, like live pups/litter and body weights were affected at 150 mg/kg. Absolute and relative thymus weights were reduced in the PND 46 males at 50 and 150 mg/kg. Immune function was measured by splenocyte mitogen LPS response, NK cell activity, plaque-forming cells antibody response to SRBCs, and flow cytometry phenotypic analysis of splenic lymphocytes, at 8–9 weeks of age. Of these parameters, only the plaque-forming cell (PFC) primary antibody response to SRBCs was suppressed 3 weeks after the final exposure to MXC in males (5 mg MXC/kg) but not in females (Smialowicz 2002). At these dosages, no overall developmental parameters were affected like body weight gain of dams or pups or the number of pups born. These data suggest that this effect of T-cell-dependent antibody response to SRBCs was affected in males at 5 mg/kg and probably at lower dosages without an effect on overall developmental toxicology parameters (Table 2).

The present ADI for MXC is 0.10 mg/kg based on a NOAEL of 10 mg/kg for growth reduction in a 2-year diet study in rats (Hodge et al. 1952). The LOAEL in the DIT study (5 mg/kg MXC for T-cell-dependent antibody response to SRBCs in males only with an effect size of 35%) is a factor of 2 lower than the NOAEL underlying the ADI. Given the magnitude of this effect and the functionality of the parameter, it seems warranted to reconsider whether the present ADI is sufficiently protective.

Chlorpyrifos

Pregnant Swiss albino mouse dams were exposed to chlorpyrifos (0, 0.3 and 3 mg CPF/kg) from GD 12 to PND 7 and then pups were directly dosed until PND 42 (Fine et al. 1989). One week after the final dose, a series of parameters were assessed (including organ weights, splenic and thymic lymphocytic subpopulation, regulatory T-cell staining, LP response, cytokine analysis, IgM assay, DTH response). This study design allowed time for recovery after dosing and before assessment, which may affect measured parameter sensitivity. There were no effects found on overall developmental parameters in all the CPF-dosed groups meaning that pup body and organ weights, pup number and maternal body weight were not affected in all CRF-treated groups. Spleen and thymus weight were also not affected. In the thymus, no effect was found on lymphocytic sub-populations in the treated groups. In the spleen, percentage of CD8 + and T-cells and T-/B-cell ratios were significantly increased in 7-week-old females treated with both dosages of CPF and CD4+/CD8 + was significantly decreased. In males, CD4+/CD8 + ratio was affected at 3.0 mg CFP, the other parameters were not affected. The T-cell LP responses to ConA and PHA were significantly suppressed in males and females in the 3.0 mg CFP/kg group. The B-cell LPS response was significantly suppressed in both dosages in males and in the 3.0 mg CPF/kg group in the females, involving the cytokines IFN- , TNF-α and IL-6. The primary IgM antibody response to SRBC was decreased in both CPF-treated groups and both genders at a dose of 0.3 mg/kg. No significant effect on DTH response was found in all the groups. This study showed that developmental immune parameters were more susceptible to CPF exposure than overall developmental toxicity parameters (Singh et al. 2013) (Table 2).

The present ADI for CPF is 0.01 mg/kg based on a NOAEL of 0.1 mg/kg for erythrocyte acetyl cholinesterase (Ach) inhibition in humans. In addition, a NOAEL for ACh inhibition was observed in 2-year diet studies in rats and dogs and in a developmental study in mice, which confirmed the ADI (McCollister et al. 1971, Young and Grandjean 1988). The LOAEL in the DIT study was 0.3 mg/kg CPF for developmental immunotoxicity in mice. The affected parameters at 0.3 mg/kg were increased percentages of splenic T-cells and B-lymphocyte subpopulations in females and a decrease in T-cell-dependent primary (IgM) antibody responses in both genders, with approximately 60–90% and 40–60% response magnitude, respectively. An increase in T-cell number would be expected to lead to an increased IgM response, whereas the opposite was the case in this study, suggesting an adverse effect on the DIT parameters. This LOAEL is only a factor 3 higher than the current ADI indicating the immune parameters are a sensitive endpoint for CPF. These data suggest that reconsideration of the ADI may be warranted.

Discussion

In the present review, we identified nine compounds for which extensive developmental immunotoxicity testing has been performed. The underlying investigations used different experimental designs, varying from generation exposure to juvenile exposure, but all included the early postnatal developmental phase. Most importantly, the experimental designs employed allowed comparison of relative parameter sensitivity within each study. Ideally, but not in all cases, a BMD design was used, allowing derivation of a BMD defining the threshold dose of adversity for each parameter tested, derived from dose–response fitting using all available data for each parameter (Slob 2002). A 5% effect magnitude is taken as the default threshold for BMD derivation, in line with EFSA policy (EFSA 2009). This approach is superior to the classical NOAEL approach in that the BMD is independent of tested dose levels and integrates all dose–response information. The lower confidence limit on BMD (i.e. the BMDL) is taken as the critical parameter for comparison, thus including the uncertainty in the BMD. Parameter sensitivity is defined here as the lowest dose at which a biologically relevant effect is observed. The EFSA policy to use the 5% effect size as a general default for BMD derivation can be subject to criticism. Especially for non-challenge immune parameters such as blood cytokine levels and cellularities of lymphoid organs, a 5% change may or may not indicate adversity. However, for immune responses after an antigen challenge, we consider any compound-related effect as indicative of adversity. This is most relevant in the present study that shows that responses to KLH and SRBC as well as DTH are often among the most sensitive parameters.

The overall picture emerging from this study is that for each of the nine compounds in this review, selected based on the availability of studies of sufficient design and quality for the analysis at hand, developmental immune parameters were among the most sensitive parameters. Moreover, for DOTC, MXC and CPF, the current TDI or ADI might need reconsideration based on the developmental immune toxicity observed. In most cases, functional immune parameters tested after challenging the immune system were most sensitive, appearing in some cases at more than two orders of magnitude lower doses than general developmental parameters. Among the general developmental parameters tested in these studies, body and organ weights usually were most sensitive. This review did not address the relative sensitivity of immune parameters in young versus adult animals.

As for structural (non-challenged) immune parameters, all reviewed studies included thymus and spleen weight. Subpopulations of lymphocytes in these organs were generally affected at lower dosages than the organ weights (Singh et al. 2013). Luster et al. (1992, 1993) already suggested that in particular enumeration of lymphocyte subpopulations was relevant for the determination of immunotoxicity. In the current OECD-TG-443 guidelines, the spleen and thymus weight and subpopulations were included to measure pre- and postnatally induce dimmunotoxic effects (OECD-TG-443 2011). Functional immune parameters appear to be more sensitive than structural immune parameters (Tables 1 and 2). This is again in line with previous studies done by Luster et al. (1992, 1993). Throughout literature, a variety of immune challenges and functional parameters has been measured at different time points. Instead of KLH, other antigens have been used like SRBC or BSA. Luster et al. 1993 concluded that quantification of T-dependent antibody response to an antigen-like KLH would be valuable for the determination of immunotoxicology (Luster et al. 1993, 1992). Indeed, in our review, for 5 (MeHg, EtOH, Methoxychlor, Heptachlor and Chlorpyrifos) out of 9 compounds, the T-cell-dependent antibody response (primary

IgM or secondary IgG) was the most susceptible parameter. For DOTC, the KLH-induced proliferative response was the most vulnerable (BMDL 0.06 mg/kg bw/day); however, the anti-KLH IgG response at PND 35 showed a BMDL of 0.3 mg/kg, showing that TDAR is also a sensitive parameter after juvenile DOTC exposure. In the DOTC exposure in the EOGRTS model, TDAR was not significantly affected in all dosages, which could be due to the inability to administer DOTC via the placenta and milk, which occurs in the EOGRTS model until PND 21, whereas TDAR was measured at PNDs 26, 35 and 40. For DEHP exposure in juvenile and adult animals, the most sensitive TDAR parameter was the primary anti-KLH IgG response with a BMD of 99.03 mg/kg bw/day at PND 35 or 75. For TCDD, the TDAR was not significantly affected in the highest doses in rats; however, DTH response was an important significantly affected parameter in all TCDD-dosed animals. The lack of TDAR response after TCDD exposure was already reviewed by Burns-Naas et al., in 2008. They discussed the issue of only including TDAR (humoral response) in the current guidelines and not DTH response (cellular and humoral responses) (Burns-Naas et al. 2008). Our studies also confirmed that DTH response to KLH or another antigen may be a worthwhile parameter to include since this is the most significantly affected parameter in two of the compounds (TCDD and DOTC in the EOGRTS model). The TBTO antibody response to SRBC and DTH response were not significantly regulated at the lowest dosages.

These data suggest consideration of including other functional parameters into the DIT test guidelines. NK cell activity or LPS-induced NO and TNF-α production by adherent splenocytes were also identified as susceptible parameters. These parameters are relatively simple to include, since single cell splenocytes suspensions are already isolated for the analysis of lymphocyte subpopulations and no extra animals are needed. This is in line with conclusions of Luster et al., who showed that none of the individual assays will give a 100% prediction of immunotoxicity of a toxicant, supporting the importance of including multiple parameters covering the structural (cellular) and functional (humoral) immune system (Luster et al. 1992, 1993). Overall, of the functional parameters tested, antigen (like KLH, SRBC or BSA) specific functional parameters including TDAR and DTH responses were most sensitive for eight of the nine reviewed toxicants. These overall conclusions were based on different study designs (e.g. route, window and duration of toxicant exposure). This stimulates the importance of including functional parameters, especially TDAR (humoral immunity) and DTH measured response (cellular and humoral immunity) in the current DIT protocols (Burns-Naas et al. 2008, Holsapple 2002; Holsapple et al. 2005).

These findings warrant serious consideration for expanding current perinatal exposure protocols with functional immune parameters. Especially, immunoglobulin levels in DTH and after challenge with KLH or SRBC appear to provide sensitive information about adverse effects on the functionality of the developing immune system. The only currently available OECD test guideline that contains perinatal exposure and prescribes testing structural as well as functional immune parameters is the OECD TG 443 EOGRTS. This study design has provided a significant step forward for testing immune and neurotoxicity parameters in a reproductive and developmental exposure design. This design was instrumental in our investigations of the developmental immune toxicity of DOTC and EtOH (Tonk et al. 2011a, 2013a). On the other hand, our juvenile onset exposure studies with DOTC and EtOH have shown a higher sensitivity of developmental immune parameters than the EOGRTS, indicating that exposure window does play an important role. In both study designs however, immune parameters proved most sensitive, showing that both designs are effective in detecting developmental immune toxicity.

It should be noted that the current data compilation is inherently biased toward compounds that have developmental immunotoxic properties. Nevertheless, given the general picture that immune parameters are among the most sensitive parameters of toxicity, these findings evoke questions about whether the current hazard and risk assessment strategies give sufficient attention to developmental immunotoxicity parameters. Evaluations of DIT began to be required as part of the US Environmental Protection Agency (EPA) Office of Prevention, Pesticide and Toxic Substances (OPPTS) testing guideline for the two-generation reproductive toxicity study in 1998 (USEPA 1998). Study design, conduct, and interpretation should be performed by trained immunotoxicologists. This guideline requires the collection of spleen and thymus weights in one pup/sex/litter in F1 and F2 weanlings (Collinge et al. 2012). Within the pharmaceutical industry, the FDA was the first to put forward recommendations regarding DIT. In its 2002 guidance for industry (FDA 2002), the Agency noted that all drugs that are immunotoxic in adult animals and are to be given to pregnant women should be evaluated for DIT (Collinge et al. 2012). The parameters they suggested to include for DIT testing were hematology and lymphoid organ weights. Collinge suggested that there might be an increased need to conduct non-clinical and clinical pediatric immunotoxicity assessments in the future. Introduction of yet another mandatory animal study protocol focusing on developmental immunotoxicity is clearly not acceptable given the generally accepted need to reduce animal testing in chemical hazard and risk assessment. However, the existing OECD TG 443 EOGRTS study provides an opportunity for developmental immunotoxicity testing. In line with current requirements for generation reproduction toxicity studies, this study is currently only carried out in specific cases at higher tonnage production levels under the REACH legislation, covering only a small minority of compounds. The current analysis, showing the relative prominence of adverse effects on the developing immune system, warrants reconsideration of whether this study should already be mandatory at a lower tonnage level, collecting this important piece of information for a far larger subset of compounds on the market. This would provide a higher level of protection, especially for the relatively sensitive group of the unborn and the developing child.

Declaration of interest

The authors are affiliated to the National Institute for Public Health and the Environment (RIVM), Bilthoven, The Netherlands. RIVM is a government agency under the Dutch Ministry of Health, Welfare and Sports. RIVM has fully sponsored the present piece of work. The design of the appraisal, the conduct of the appraisal and the conclusions drawn in the paper are the

exclusive work product of the authors and may not necessarily be those of their employer, RIVM.

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