National Institute for Public Health
and the Environment
Ministry of Health, Welfare and Sport

Development of an experimental model for assessing adverse effects of juvenile chemical exposures

RIVM report 340701001/2011

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Colophon

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This investigation has been performed by order and for the account of nVWA, within the framework of KV 9.1.32.

Abstract

Development of an experimental model for assessing adverse effects of juvenile chemical exposures

Exposures to chemicals may have larger risks in children than in adults. Test methods for chemical hazards do not take sufficient account of these differences. Therefore, RIVM has carried out several animal studies to design an optimal protocol for the assessment of hazards of chemicals for children. The preliminary conclusion indicates that exposure between postnatal days 10 and 50 in the rat is most relevant for hazard assessment. Effects on physical and immune system development are most prominent in this period.

The protocol was designed by order of the ministry of health, welfare and sports. Neurologic diseases and immune system malfunction, ranging from ADHD to allergies and diabetes, may or may not be caused by chemical exposures early in life.

Physical and behavioural differences
Variability in risks can be explained by physical and behavioural differences between children and adults. Children will more often put objects in their mouth (toys, sand), which may result in possible exposures to hazardous chemicals. Incomplete organ development in children may reduce excretion of chemicals from the body. In addition, incomplete development of the brain and immune system may render these tissues more susceptible to adverse effects of chemicals. Continued studies are needed for a final judgment. It is important to also consider the neural system in this research.

International perspective
Internationally, the results of this research have been discussed in the test guidelines programme of OECD (Organisation for Economic Cooperation and Development). This programme provides worldwide consensus of test guidelines. In addition, a scientific symposium was organised on the subject.

Keywords:
children, risk assessment, development, immune system, chemicals

Rapport in het kort

Ontwikkeling van een experimenteel model voor het vaststellen van schadelijke effecten van blootstelling aan stoffen op jonge leeftijd

Als kinderen aan chemische stoffen worden blootgesteld, kan dat grotere risico's hebben voor hun gezondheid dan bij volwassenen. Testmethoden voor gevaren van chemische stoffen houden onvoldoende rekening met deze verschillen. Het RIVM heeft daarom enkele dierstudies uitgevoerd om een optimaal protocol op te stellen om schadelijke effecten van stoffen op jonge kinderen te kunnen vaststellen. De voorlopige conclusie is dat een blootstelling bij ratten vlak na de geboorte (tussen de 10 en 50 dagen) het meest geschikt is om de risico's in kaart te brengen. Daarnaast blijken de effecten het meest zichtbaar te zijn in de fysieke ontwikkeling en op het immuunsysteem van het kind.

Het protocol is in opdracht van het ministerie van VWS opgesteld. Vermoed wordt dat neurologische aandoeningen of afwijkingen in het immuunsysteem – van ADHD tot allergieën en diabetes – veroorzaakt kunnen worden door blootstellingen aan chemische stoffen op jonge leeftijd.

Fysieke en gedragsmatige verschillen
De uiteenlopende risico's zijn te verklaren vanuit fysieke en gedragsmatige verschillen tussen kinderen en volwassenen. Kinderen stoppen bijvoorbeeld veel in hun mond (speelgoed, zand), waardoor zij via deze 'route' blootgesteld kunnen worden aan giftige stoffen. Door de onvolgroeide organen bij kinderen worden stoffen bijvoorbeeld minder snel uit het lichaam verwijderd dan bij volwassenen. Ook wijkt de ontwikkeling van organen af; bij jonge kinderen zijn onder meer het immuunsysteem en de hersenen sterk in ontwikkeling, wat hen gevoeliger kan maken voor schadelijke effecten van stoffen. Vervolgstudies zijn nodig voor een beter oordeel. Het is belangrijk hierbij de gevoeligheid van het zich ontwikkelende zenuwstelsel te betrekken.

Internationale inbedding
De bevindingen van dit onderzoek zijn internationaal onder meer uitgedragen in het testrichtlijnenprogramma van de OESO (Organisatie voor Economische Ontwikkeling en Samenwerking). In dit programma worden testrichtlijnen wereldwijd afgestemd. Daarnaast is hierover een wetenschappelijk symposium georganiseerd.

Trefwoorden:
kinderen, risicoschatting, ontwikkeling, immuunsysteem, chemische stoffen

Contents

Summary

The specific risk of children after chemical exposures is a subject of continuous concern for governmental agencies. Usually, risk assessment is performed for the general population based on adult men. Children, however, differ substantially from adults, not only as they are smaller and their organs and tissues are still developing, but also their exposures are different. The latter is secondary to both differences in behaviour as well as physiological differences in metabolism and excretion. Therefore, risks for children may not always be covered acceptably by the current general risk assessment paradigm.

The current absence of tailored experimental study protocols for juvenile toxicity testing hampers dedicated risk assessment for children. To fill this gap in hazard assessment, novel protocols for juvenile toxicity testing are being developed. This report gives an overview of finished and ongoing studies in which several protocols are being tested for their applicability for juvenile toxicity testing. Studies that are described herein have included methylmercury, dioctyl tin chloride, ethanol, diethyl hexyl phthalate and nonylphenol as test compounds. These are known developmental toxicants after prenatal exposure; however, the possible effects of postnatal exposure are still largely unknown.

The main study design emanating from this work so far involves exposure between days 10 and 50 after birth in the rat. Using that protocol we show that the developing immune system is relatively sensitive as compared to developmental landmarks. This is especially obvious for functional immune parameters such as measured through an immune challenge with keyhole limpet haemagglutinin (KLH), showing that functional immune testing provides a more sensitive readout as compared to morphological immune parameters.

This work is being extensively communicated in relevant scientific and regulatory arenas, and receives much international support and agreement. We especially aim at involving international regulatory authorities such as OECD and EU in order to pave the way for international agreement on specific hazard and risk assessment methodologies for children. The current project provides important background information indicating that the developing individual may be more vulnerable to chemical exposures than the adult.

1 Introduction

There is increasing attention for possible specific sensitivity of children as compared to adults regarding adverse effects as a consequence of chemical exposures. As an example, in the Netherlands, the issue of phthalates used as plasticisers in children's toys has raised significant concern. This concern was generated by a combination of factors. First, phthalates are characterised by anti-androgenic hormonal activity, differing in potency dependent on carbon chain length. Second, the natural chewing behaviour of infants and toddlers can lead to relatively high oral exposure levels as compared to the adult situation. Third, infant endocrine as well as metabolic physiology is different from that in adults. Fourth, organ systems in children may be more vulnerable to adverse effects due to the fact that they are still in a stage of development as opposed to the mature organ stages in adults.

Current hazard and risk assessment methodologies largely ignore the differences between children and adults. There is no specific animal study with exposures restricted to the juvenile period. In addition, risk assessment usually considers the default situation of a 60kg adult. The Dutch National Food Authority has considered that this situation is undesirable, as it may result in unnoticed unacceptable hazards and risks for children. The same awareness emerged from the Public Health policy department of the Ministry of Health in the Netherlands. Therefore, RIVM was asked to investigate possibilities for designing specific hazard assessment methods dedicated to specific risk estimation for children. As a consequence, a series of experimental studies was initiated incorporating several exposure scenarios around the juvenile period. The rat was taken as the default standard experimental animal, both in view of a multitude of existing international guidelines utilising this species, as well as given the vast historic database on toxicity responses in this species. This background allows contemplating adaptation of existing guideline studies, whilst restricting animal use to the necessary minimum. It also allows findings to be compared with existing data, providing important information about life stage dependent sensitivity of organ systems.

One important question in designing a juvenile toxicity study protocol relates to the developing organ systems that can be anticipated to be relatively sensitive in childhood as compared to adulthood. In a now classical study, Cooper et al. (2006) published an extended one generation reproductive toxicity study protocol in which apart from developmental landmarks specific attention was proposed for the developing neural system and the developing immune system. These organ systems were deemed relatively sensitive in the juvenile period in view of continued development and maturation after birth and up to adulthood. In addition, increasing current disease prevalences for early onset neural related conditions such as schizophrenia, bipolar neuropathy, autism and ADHD, and immune related diseases such as asthma, allergies and autoimmune diseases in the human population have prompted enhanced attention for a possible but as yet, largely unproven association with early life chemical exposures. As to the neurodevelopmental system, the OECD TG 426 guideline for developmental neurotoxicity testing already incorporates a series of relevant end points for the neural system. Developmental immunotoxicity was still an area of research and debate which had not yet achieved regulatory implementation into international guidelines. The inclusion of both types of end points in a reproductive toxicity

study design was novel and provided the basis for the OECD TG 443 extended one generation study guideline that was officially accepted by OECD in July 2011.

Although the OECD TG 443 incorporated these novel end points of anticipated specific juvenile sensitivity, its exposure protocol extended throughout life, starting in adult parents and continuing in offspring after weaning and until final necropsy at adulthood. This extended exposure duration limits the possibility of determining which of the life stages exposed is actually the more vulnerable. In order to allow for a dedicated conclusion on the specific sensitivity of the juvenile stage, this stage should be exposed exclusively and effects should be compared with those observed in other stages also after exclusive exposure. The current RIVM report describes a series of studies with varying exposure scenarios to enable comparison of lengthy exposure designs such as in the OECD TG 443 with more dedicated ones such as juvenile only and adult only. In this report we have especially focused on the developing immune system. The project was carried out in collaboration with TNO QOL, where specific attention was given to neurodevelopmental parameters. The results of the latter parameters are still under study and will not be reported here.

In the following, the RIVM studies that have been performed and are being carried out are reported up to their current state of execution. Some of them have already been published in the scientific literature (Tonk et al., 2010, 2011a,b). We will focus on the highlights as regards parameter specificity and sensitivity and the impact of the outcomes on study design for a general juvenile toxicity study protocol. In the discussion, we provide a preliminary proposal for a juvenile toxicity study protocol. Ongoing studies as well as several studies planned as a follow-up in the coming years will provide additional information that will be useful for fine-tuning of the protocol. In addition, these results and proposals are being fed into the international scientific and regulatory arenas for further expert scrutiny. The final aim of this project is the establishment and international acceptance of a protocol for juvenile toxicity testing. Such a protocol should alleviate the concerns generated by the current gap in hazard and risk assessment when it comes to specific risks of chemical exposures for children.

2 Reproductive and juvenile toxicity studies

Figure 1 provides a schematic representation of the various exposure scenarios that were applied in the different studies within this project up to the present time, incorporating five different chemicals that are relevant in view of their known or suspected stage-specific developmental toxicity. Scenarios included a pre- and postnatal exposure period (MeHg). In addition, two OECDTG 443 exposure scenarios with two adjunct PND10 and onward scenarios for comparison were carried out (DOTC and EtOH). Finally, a juvenile (PND10-50) protocol was compared with an adult (PND50-90) protocol and these were tested for two compounds (DEHP and NP). Figure 1 also indicates the route of exposure, which was chosen differently either in view of the nature of the compound tested or in view of the specific research question underlying the study. In the following, each compound-related set of studies is presented separately, focusing on highlights relevant for the determination of an optimal study design for juvenile toxicity testing. References are made to scientific publications to enable the reader to more fully appreciate the study results when desired.

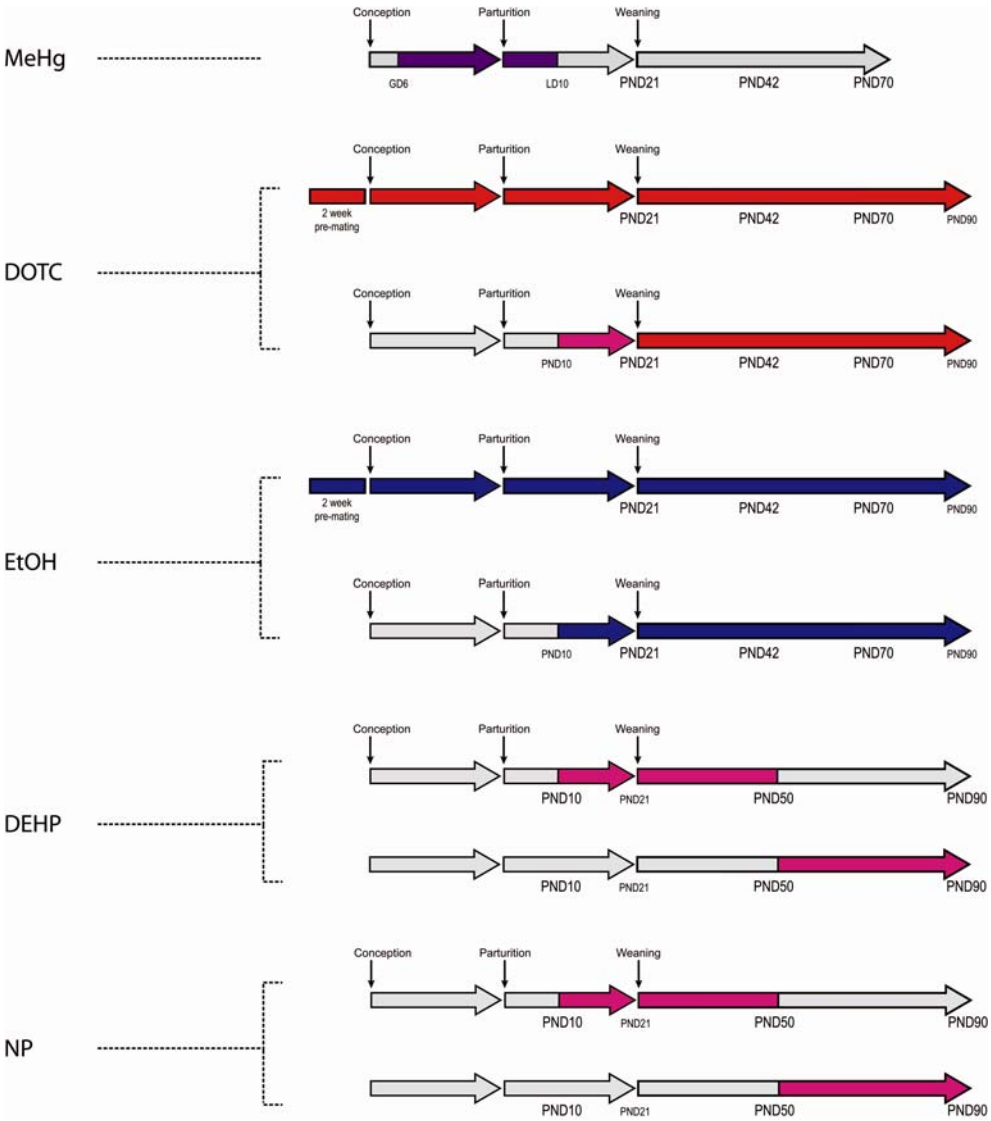

Figure 1. Designs for the studies reported in the current report. Arrows indicate the time line with from left to right the pregnancy, lactation and postweaning periods, respectively. Coloured sections indicate exposure periods. Dark purple (MeHg only) indicates maternal gavage, brown indicates dietary exposure, purple indicates direct gavage exposure and blue indicates drinking water exposure. MeHg=methylmercury; DOTC=dioctyl tin chloride; EtOH=ethanol; DEHP=diethyl hexyl phthalate; NP=nonylphenol; GD=gestation day; LD=lactation day; PND=postnatal day.

3 Methyl mercury

Compound and study design
Methylmercury (MeHg) is a widespread environmental and food contaminant that is commonly identified as a developmental neurotoxicant (Risher et al., 2002). However, MeHg is also a well-known immunotoxicant (Descotes, 1986), and there are indications that the immune system might be at least as sensitive as the developing brain (Dourson et al., 2001; Haggqvist et al., 2005). An epidemiological study in a maritime population in Canada showed that newborns from pregnant women exposed to polychlorinated biphenyls (PCBs) and MeHg via seafood consumption presented a decrease in the proportion of $CD4^+CD45RA^+$ cells and IgM levels in cord blood as compared with infants with lower prenatal exposures (Belles-Isles et al., 2002). In rodents, MeHg has been shown to suppress humeral immunity (Blakley et al., 1980). Indirect perinatal exposure to MeHg leads to a reduction in natural killer (NK) cell activity (Ilback et al., 1991) and alterations in the proliferative response of lymphocytes to mitogens (Ilback et al., 1991; Ortega et al., 1997; Wild et al., 1997).
MeHg was tested in an exposure protocol resembling the test protocol for developmental neurotoxicity testing proposed by the U.S. Environmental Protection Agency (test guideline U.S. EPA OPPTS 870.6300) (U.S. EPA, 1998). Reproduction and developmental parameters were selected from those proposed in the extended one-generation protocol (Cooper et al., 2006) and in addition, a wide range of structural and functional neurological and immune parameters were included. The results of the behavioural and neuropathological survey performed in this study will be published elsewhere.

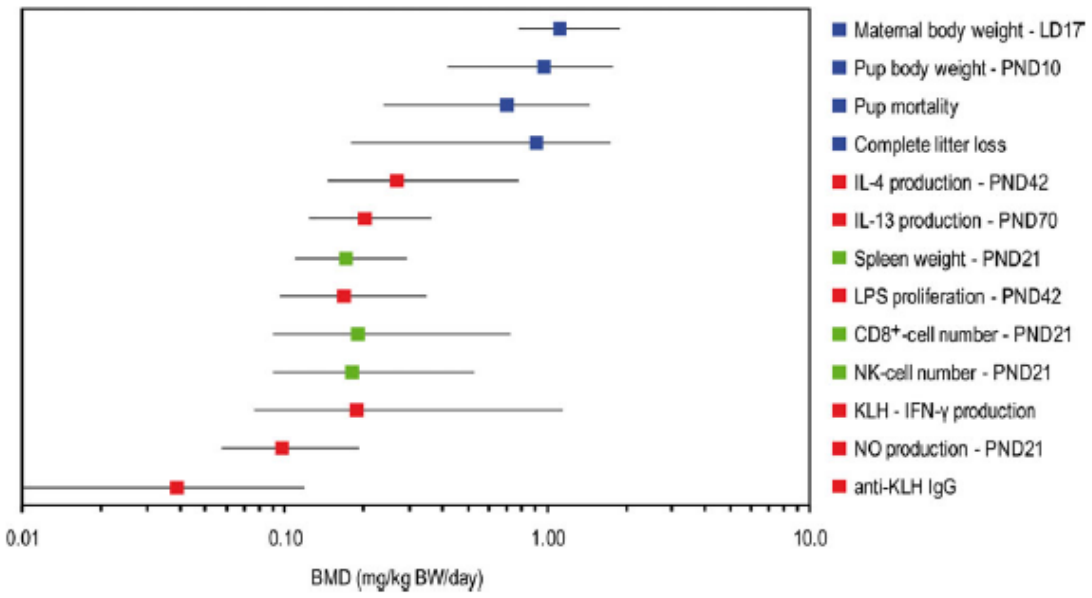

Figure 2. Overview of the BMDs and their confidence intervals of the most sensitive immune and developmental parameters (red, functional immune parameter; green, structural immune parameter; blue, developmental parameter).

Results
Methylmercury induced effects on developmental parameters, such as growth parameters and pup mortality (figure 2). However, effects on a series of immune parameters were found at doses without observed developmental toxicity. Immune effects differed with the age at assessment (PND 21, 42 or 70) and consisted mainly of effects on functional parameters. The parameter with the lowest 5% lower confidence bound of the Benchmark dose (BMD) (BMDL) was the primary KLH-specific IgG antibody response, which showed a dose-dependent decrease with a BMD of 0.039 mg/kg BW/day (CI 0.010–0.12).

Discussion
The exposure protocol and route of this study was agreed between RIVM and TNO to allow for comparison of this study with existing data at TNO for the same compound. Therefore, this study did not allow full comparison with either the OECD TG 443 design or a juvenile exposure only design. However, the direct comparison of developmental parameters and landmarks with immune parameters in the same study clearly showed that after this pre-and postnatal exposure scenario the immune related parameters were substantially the more sensitive category. These data thereby illustrate the relevance of considering testing immune parameters in reproductive and developmental toxicity testing protocols. It is additionally of significance to realise that especially functional parameters were most sensitive. This is in line with the consideration that the unchallenged immune system may not display abnormalities, but that it may need a challenge to display its reduced capacity to cope with functional demands. Therefore, addition of a functional challenge in the protocol should be seriously considered. This is in line with the OECD TG 443 protocol, which also includes a KLH challenge.

4 Dioctyl tin chloride

Compound and study design
Di-n-octyltin dichloride (DOTC) is used as a stabiliser in polyvinyl chloride plastics and was 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 our first DOTC 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.

In our second DOTC study, exposure was done from PND10 onwards, via gavage until weaning and after weaning via the diet. In this study, a wide range of immunological parameters were included in a juvenile toxicity protocol to investigate the utility of different assays for the evaluation of developmental immunotoxicity. The animals were exposed to Di-*n*-octyltin dichloride (DOTC) from postnatal day (PND) 10 and immune assessments were performed on PNDs 21, 42, and 70 to evaluate the age-dependency of the effects found. A subset of animals was used to evaluate the T-cell dependent antibody response (TDAR) following subcutaneous immunisations on PNDs 21 and 35.

Results
In the pre- and postnatal exposure DOTC study, the T cell-dependent antibody response to Keyhole Limpet hemocyanin (KLH) was assessed following subcutaneous immunisations with KLH on PNDs 21 and 35 and the delayed-type hypersensitivity measured 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.

In the postnatal exposure protocol, DOTC induced thymus atrophy and dysfunction in the T-lymphocyte-mediated immune response. Figure 3 shows a compilation of the fitted dose response curves for the most sensitive parameters along with an overview of the corresponding BMDs and their confidence intervals. Parameters depicted have a BMDL below the lowest dose (3 mg/kg). Only relative parameters (corrected for body weight/organ weight) are included and the model shown is the optimal model resulting in the lowest BMDL. The $CD4^{-}8^{-}$ thymocyte subset (PND 42), KLH primary IgG response, and the $CD4^{-}8^{+}$ thymocyte subset (PND 42) have a BMD below the lowest dose (3 mg/kg). The NO production (PND 21) and KLH-specific proliferation (PND 63) have BMDs surrounded by confidence intervals with a higher bound (BMDU) below the lowest dose (3 mg/kg). The most sensitive immune parameters affected included TDAR parameters, thymocyte subsets and LPS-stimulated

NO-production by adherent splenocytes. BMDLs observed for these parameters were below the, in literature reported, NOAEL for DOTC. Assessment at PNDs 21 and 42 revealed the most pronounced effects as opposed to PND 70.

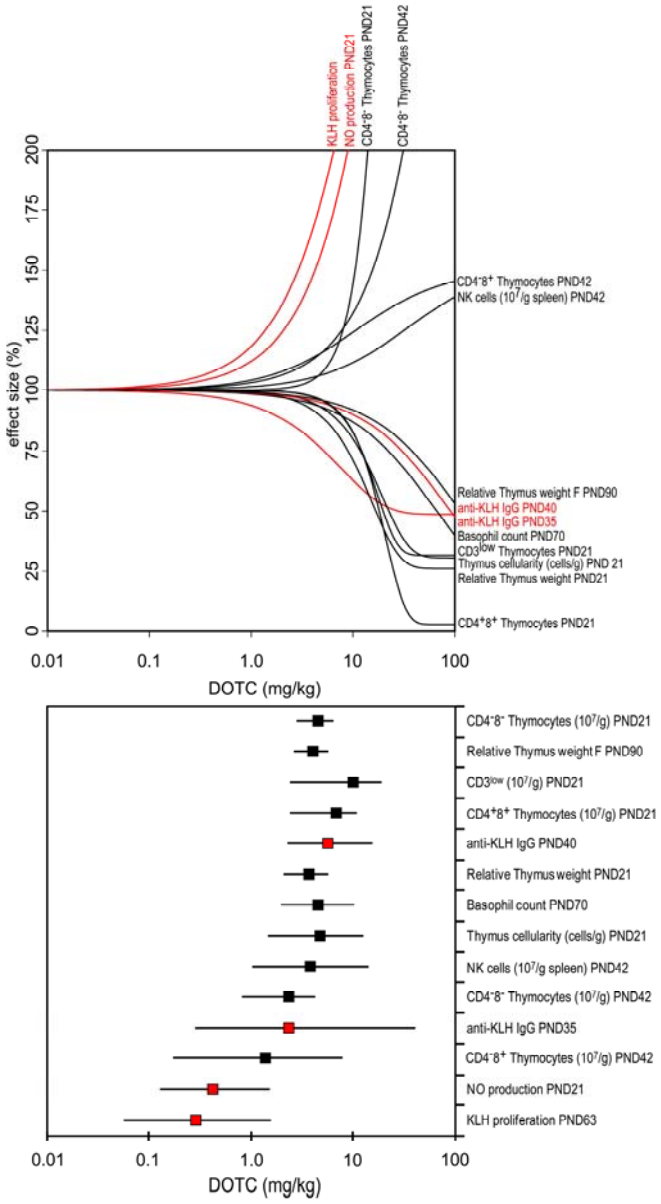

Figure 3. Compilation of fitted dose-response curves for the most sensitive parameters and overview of related BMDs and their confidence intervals in the postnatal DOTC study.

Discussion

The reported no adverse effect level (NOAEL) for immunotoxicity for DOTC is 0.23 mg/kg bw/day, based on a decreased thymus weight after a 3 months exposure period (Dobson and Howe, 2006) (EFSA TDI 0.25 µg/kg bw/day). The BMDL for decreased relative thymus weight in this study was 0.11 mg/kg bw/day (PND 21). However, certain functional immune parameters and thymocyte subset parameters were found to be more sensitive than relative thymus weight, resulting in BMDLs ranging from 0.06 to 0.28 mg/kg feed, equivalent to 0.003 to

0.014 mg/kg bw/day, respectively, which are 14 to 77x lower than the NOAEL reported in literature. This indicates that the juvenile period is a window of high susceptibility for the immune system when exposed to DOTC and illustrates the relevance of additional functional immune parameters and, especially for DOTC, thymocyte subset parameters.
The TDAR, with its different parameters, was one of the most sensitive parameter sets affected in this study. This supports the notion that the TDAR is appropriate for inclusion in developmental toxicity studies, even though we used early time points for immunisation (PNDs 21 and 35), resulting in suboptimal anti-KLH IgM and IgG responses compared with adults.

Other sensitive parameters included the NO production by adherent cells and thymocyte subsets. DOTC selectively affects the thymocyte maturation in the thymus resulting in thymus atrophy and altered T-lymphocyte subpopulations in thymus and spleen. Spleen cellularity and splenocyte subset parameters are more commonly included in reproductive and developmental protocols. However, while effects were found on spleen and splenocyte subpopulation parameters, thymus and thymocyte subpopulation parameters evaluated in this study were more sensitive.
The most sensitive developmental parameter affected was body weight with a BMD of 57.5 mg/kg (CI 30.2-63.9 mg/kg). The most sensitive immune parameters have BMDLs surrounded by confidence intervals with a BMDU below the lowest dose (3 mg/kg) illustrating the selective immunotoxic potential of DOTC in the present study design.

When comparing both DOTC study designs employed it is remarkable that the juvenile exposure study shows an overall NOAEL two orders of magnitude below that of the pre- and postnatal study. We speculate that there may be at least two explanations for this finding. First, the prenatal exposure may have produced tolerance to the compound by enhancing metabolism and excretion due to prolonged exposure. A second and probably more important explanation could be the oral exposure that we applied for pups in the juvenile protocol as opposed to maternal dietary exposure during pre-weaning in the pre-and postnatal protocol. This has probably resulted in higher pup exposure levels during critical windows of development in the juvenile study, which could explain the stronger effects observed in this protocol. This finding gives reason to consider preferring direct oral exposure to pups over dietary exposure in a juvenile toxicity study.

5 Ethanol

Compound and study design
Ethanol is a well-known neurodevelopmental and immunotoxic compound (Riley et al., 2011; Waldschmidt et al., 2008) in experimental animals as well as in man. Being a substance of abuse in man it does reach internal dose levels that are actually detrimental to health. Both of the above classes of effect parameters have been observed to be affected in man, which allows comparison with experimental animal test outcomes. We have tested alcohol in an extended one-generation protocol versus a PND10 and onwards exposure protocol, dosing Wistar rats with alcohol via drinking water exposition. The results of these studies are still being analysed at the time of writing this report. Therefore, we have to restrict our reporting here to preliminary outcomes.

Results
In the EOGRTS protocol, most sensitive parameters were lymphoproliferative response to ConA and NO/TNFα production by adherent splenocytes. In the juvenile exposure study, KLH-specific parameters (anti-KLH IgG and IgM antibody responses) were most sensitive, followed by NO production by adherent splenocytes and thymocyte subpopulations. Comparison of these studies shows that the juvenile study has an overall LOAEL more than one order of magnitude lower than the EOGRTS study based on immune parameters.

Discussion
It is remarkable that developmental immunotoxicity parameters also appear to be more sensitive than general developmental parameters in the case of ethanol exposure. In addition, juvenile exposure appears to show effects at lower doses than the EOGRTS protocol, even though the latter is a longer exposure scenario. This time, the pre-weaning exposure routes were not different. Therefore, in contrast to differences in pre-weaning exposure being a possible explanation in the DOTC studies, the ethanol studies show that prenatal exposure may reduce the severity of effects caused by juvenile exposure. It should be realised that ethanol readily passes from the maternal circulation into the milk, causing significant pup exposure even in the absence of direct oral dosing of pups. Of course, the examples of DOTC and ethanol should not be generalised throughout the chemical domain. However, these studies illustrate the importance of the exposure design in terms of the period in the life cycle in which exposure is given. Finally, definitive statements on the ethanol study outcomes await completion of the analyses.

Table 1. Overview of most sensitive parameters based on preliminary results of ethanol studies

BMD %EtOH	Parameter identification			day
	EOGRTS EtOH Study			
0,49	Proliferation	ConA	Spleen	PN21
0,68	KLH	Antibody	IgM40	
0,77	KLH	Proliferation		
0,53	Haematology	White differential	Lympho	PN42
0,58	Haematology	White differential	WBC	PN42
0,69	Adherent cells	TNF-a	dif	PN70
0,69	Adherent cells	NO	dif	PN70
1,24	organ weights	cellularitySpleen		PN42
1,44	Adherent cells	NO	dif	PN21
	Juvenile EtOH Study			
0,02	KLH	Antibody	IgG35	
0,04	KLH	Antibody	IgG40	
0,04	Adherent cells	NO	dif	PN42
0,52	Luminex	ConA	IL10	PN42
0,54	thymus	total4+8+(107/g)		PN70
0,55	organ weights	cell/gThymus		PN70
1,62	thymus	immat3(107/g)		PN70
0,26	KLH	Antibody	IgM40	
0,42	Adherent cells	NO	dif	PN70
0,41	KLH	Antibody	IgM26	
0,73	Haematology	White differential	Neutro	PN21
1,30	Haematology	White differential	Mono	PN70

6 Diethyl hexyl phthalate

Compound and study design
Phthalates are industrial chemicals used as additives in polyvinyl plastics, lubricants, solvents, cosmetics, and pharmaceuticals. DEHP is a high production volume chemical used as a plasticiser to increase the flexibility and durability of polyvinylchloride (PVC) products. It is used in a wide range of consumer products and can easily migrate out from PVC-containing items because it is not covalently bound to the plastic matrix, making it one of the phthalates most abundantly found in the environment. DEHP is a known testicular toxicant in rodents, disrupting Sertoli cells and Leydig cells, thereby affecting spermatogenesis and testosterone production (Jones, 1993, Heindel and Powell, 1992, Akingbemi et al., 2003). Developing, prepubertal rats are considered more sensitive to DEHP exposure than adult rats (Gray and Butterworth, 1980). Effects of DEHP in the foetal male have been of special interest due to the sensitivity of the male reproductive tract for phthalate induced malformations after gestational exposure. When DEHP is administered post-weaning (from PND 22), the onset of puberty, as indicated by preputial separation, is delayed and androgen-dependent organ weights are reduced (Noriega et al., 2009). Some findings in human populations are consistent with animal data suggesting that phthalates exposure can produce toxic effects in the human reproductive system (Main et al., 2006, Pan et al., 2006, Swan et al., 2005), however, epidemiological studies on effects of phthalate exposures in humans have remained controversial due to limitations of the study designs.

For phthalates, and DEHP in particular, the research focus has been on potential adverse reproductive and carcinogenic effects. However, phthalates have also been suggested as modulators of the immune system. Epidemiological studies indicate an association between indicators of phthalates exposure and allergic symptoms such as asthma or wheezing, rhinitis and eczema (Bornehag et al., 2004; Kolarik et al., 2008), though the implication of these data is limited by a lack of objective exposure information (Jaakkola and Knight, 2008). Experimental studies investigating the effects of phthalates exposure on immune responses in experimental animals have yielded conflicting results. Studies to examine the potential of phthalates to display adjuvant properties have shown both immunosuppressive and adjuvant effects (Larsen et al., 2001a). For DEHP, intraperitoneal and subcutaneous injection in combination with the reference allergen Ovalbumine (OVA) resulted in significantly increased IgG1 anti-OVA antibody levels in BALB/c strain mice, without affecting specific IgE responses (Larsen and Nielsen, 2008; Larsen et al., 2001b). However, oral exposure of BALB/c strain mice to DEHP was reported not to affect the anti-KLH IgG_1 or IgE antibody levels after subcutaneous immunisation with Keyhole limpet hemocyanin (KLH) (Kimber and Dearman, 2010). As for more general immune parameters, Badr et al. (Badr et al., 2007) found dietary DEHP exposure to shift the Th_1/Th_2 cytokine balance in the liver towards a Th2 dominated phenotype , while Piepenbrink et al. (Piepenbrink et al., 2005) found lymphoid organ weights, thymus histology and antibody levels to be unaffected by oral administration of DEHP.

This present study was performed to evaluate the relative sensitivities for DEHP induced effects in juvenile and adult male rats using a 40-day exposure regimen. Male wistar rats were dosed with corn oil or DEHP by gavage from postnatal day (PND) 10-50 or PND 50-90 at doses between 1 and 1000 mg/kg/day. To evaluate the potential immunomodulatory properties of DEHP, assessment of a wide range of immune parameters was included.

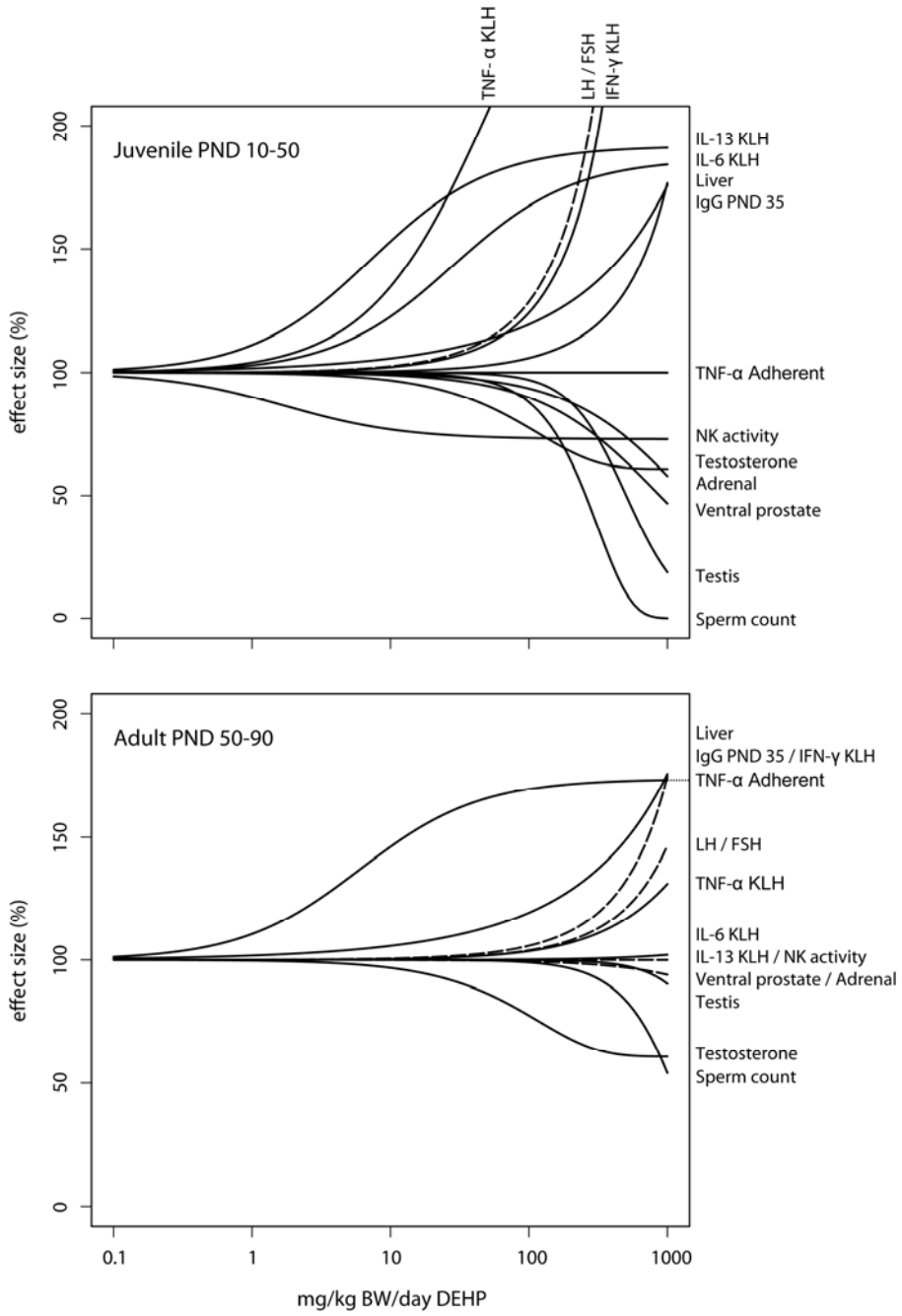

Figure 4. Compilation of the fitted dose response models for continuous data, scaled to the response level in the controls in the DEHP study.

Results
Androgen-dependent organ weights showed effects at lower dose levels in juvenile versus adult animals. Immune parameters affected included TDAR parameters in both age groups, NK activity in juvenile animals and TNF-α production by adherent splenocytes in adult animals. Immune parameters were affected at lower dose levels compared to developmental parameters. Overall, more immune parameters were affected in juvenile animals compared to adult animals and effects were observed at lower dose levels (Figure 4). The results of this study show a relatively higher sensitivity of juvenile versus adult rats. Furthermore, they illustrate the relative sensitivity of the developing immune system in juvenile animals as compared to general toxicity and developmental parameters. This study therefore provides further argumentation for performing dedicated developmental immune toxicity testing as a default in regulatory toxicology.

Discussion
The results of this study illustrate the age-dependency of DEHP toxicity, the relative sensitivity of the developing immune system as compared to general developmental parameters and the relative sensitivity of functional versus structural immune parameters. Based on these findings, it may be postulated that DEHP immunotoxicity during maturation of the immune system may play a role in the expression of allergic symptoms such as asthma or wheezing, rhinitis and eczema, as has been observed in epidemiological studies. In more general terms, this study supports the notion of a role for immunotoxicity during maturation of the immune system in the increasing prevalences of early-onset immune-related diseases in man so that currently, immune-based diseases may affect as many as 25% of children in certain developed countries (Dietert and Zelikoff, 2009). Whereas these diseases and associated immune-based conditions later in life cause a significant lifetime burden, we still have limited knowledge on the potential contribution of exposures to chemical agents to these trends. Therefore, this study underscores the importance of including parameters of the developing immune system within reproductive toxicology test protocols. The inclusion of parameters to assess developmental immunotoxicity in the recently accepted extended one-generation reproduction toxicity guideline (OECD TG 443, 2011) is an important step forward. Continued research is needed to optimise the developmental immunotoxicity parameter set for chemical safety testing.

7 Nonylphenol

Compound and study design
Nonylphenol is a surfactant metabolite with low potency estrogenic activity that has been shown to interfere with reproduction in wildlife species. It has been shown also to affect the immune system in fish species (Razia et al., 2005; Jin et al., 2010). It is a compound of interest in view of endocrine disruption and related regulatory issues. In particular, the safety of endocrine active compounds in relation to childhood exposure has been questioned. Therefore, nonylphenol is considered a relevant compound for the present project. Nonylphenol juvenile (PND10-50) and adult (PND50-90) exposure protocols were used similar to the DEHP study design. This study is being carried out in the autumn of 2011 and will be reported in due course.

8 General discussion

In the present report we provide an overview of the state of the art of experimental studies carried out with the aim to collect background data for the derivation of a standardised guideline for assessing juvenile toxicity. Such a guideline should enhance the hazard and risk assessment of chemical exposures specifically for children. Given the knowledge that children are very different from adults as regards their behaviour, exposures, internal fate of compounds (absorption, distribution, metabolism, excretion) and sensitivity of developing organ systems, specific hazard and risk assessment for children is highly warranted. This has already been realised in the pharmaceutical area, where ongoing international debates specifically address hazards of pharmaceutical exposures to children. In that area, risk-benefit considerations play an important role, but also the relative pharmacological effectiveness may differ with age, requiring different dosing regimens in different age groups. Juvenile toxicity studies have been discussed in the pharma field over the last decade (Hurtt et al., 2004). For chemicals, no specific international guidelines exist at the OECD level.

It is remarkable that all life stages but the juvenile period have their own specific toxicity test guideline for chemical hazard assessment. It is only the juvenile period that is not separately tested, but is incorporated in the reproductive toxicity generation studies (see figure 5).

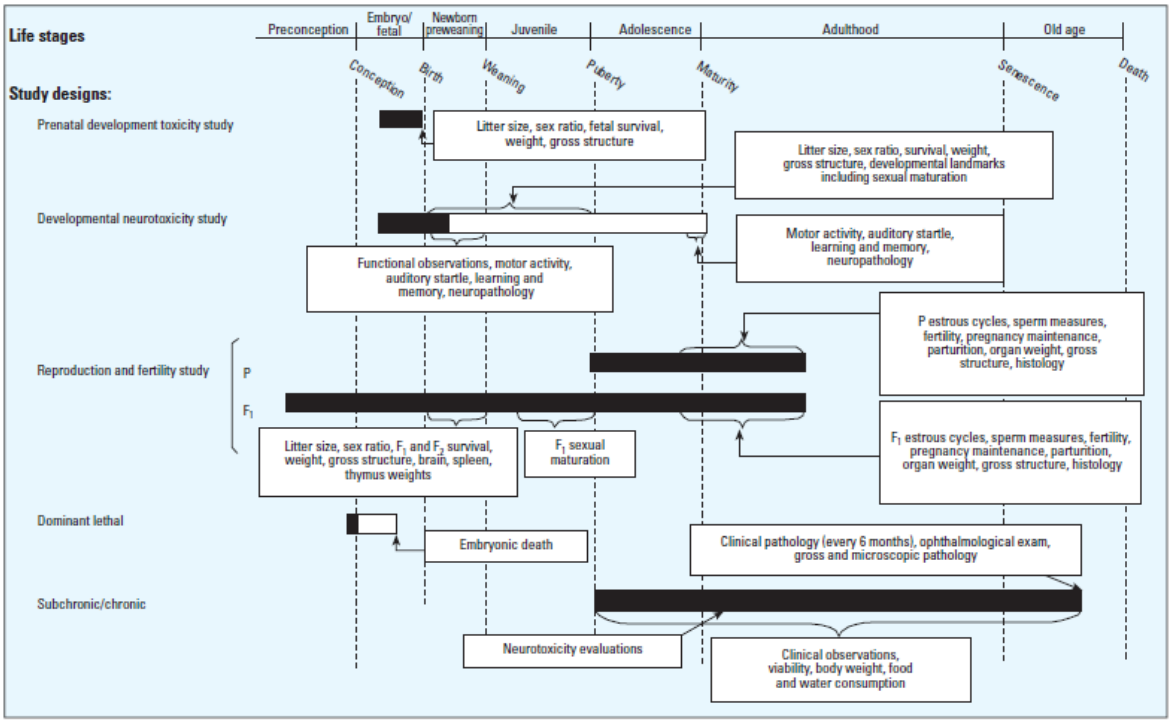

Figure 5. Overview of exposure periods in chemical toxicity guideline tests (Daston et al., 2004).

These generation studies have been in place since the early 1980s and have extensively been used to assess adverse effects on reproductive and fertility parameters in experimental animals. The primary animal species of choice for testing has been the rat, in view of its size which is easy to handle, its short generation time and its relatively large litter size, which provide a relatively practical framework for reproductive toxicity studies. With many hundreds of studies performed over the past 30 years, a large database exists providing historic background information against which new test results can be compared (Piersma et al., 2011; Rorije et al., 2011).

Generation studies provide information about the most sensitive parameter(s) studied, but it is not always straightforward to pinpoint the period of highest sensitivity within the reproductive cycle, since exposure has been given throughout the cycle. Moreover, when exposure is restricted to a certain life stage, sensitivity may be different as compared to long-term exposure because adaptation of the metabolic system with time may potentially occur, through enhancing metabolism and excretion and thus decreasing compound dose level at the target organ(s). Therefore, it is important to compare different exposure scenarios in order to establish the most relevant study design for assessing juvenile toxicity.

Apart from exposure timing, the choice of end points addressed is another critical item of study design. The juvenile period may express specific sensitivities in view of the immature status of developing organ systems. The expert group designing the relatively novel OECD TG 443 extended one generation reproductive toxicity study (Cooper et al., 2006) has specifically earmarked the developing neural system and the developing immune system as possibly relatively sensitive in the early postnatal period of life. Cohorts dedicated to these parameter classes have been incorporated in the OECD TG 443 protocol, which was accepted as a guideline by OECD in July 2011. It is nothing but logical to also study these parameters when designing a juvenile toxicity study protocol.

In the studies reported herein, we have tested and are testing five different environmental chemicals which had been shown to induce developmental toxicity in various test protocols. The exposure period varied from pre- and perinatal (MeHg), premating to offspring adulthood (DOTC and EtOH), PND10 into adulthood (DOTC, EtOH), PND10-50 (DEHP and NP), to PND50-90 (DEHP and NP). The comparisons of these various scenarios have provided important lessons, which will feed into the definition of a preferred juvenile toxicity testing protocol.

The scenario employed in the MeHg study showed us that the developing immune system was relatively sensitive as compared to general growth and development as well as developmental landmarks of the maturation of external genitalia. Comparison of this outcome with a study performed in the past (Hessel et al., 1996) in which we gave a single exposure to cyclophosphamide during pregnancy, shows the importance of exposure timing. In that study, severe malformations were induced as observed at necropsy one day before parturition, whereas immune parameters of organ cellularities and cellular subset analysis where unaffected. This result is therefore entirely opposite to the current findings. We should consider that besides the possible role of different exposure scenarios, the test compound was also different, which may have played a role in the differences in outcomes.

The DOTC studies also showed a relatively sensitive immune system, extending similar findings in the MeHg study and stressing the importance of testing these parameters in juvenile animals. These DOTC study designs showed a relatively low sensitivity of tested immune parameters in the OECD TG 443 exposure design as compared to the PND10 to adult exposure design. Here we envisage two possible explanations. First, long term exposure may cause tolerance by inducing metabolic enzymes in the liver, enhancing detoxification and excretion of the compound. This may secondarily cause lower target organ concentrations of the test compound. A second and perhaps more important factor causing differences in parameter sensitivities may have been the difference in exposure route in the PND10-21 period. In the OECD TG 443 design the pups were solely exposed via the mother and in increasing amount by their own dietary exposure through feed consumption. In contrast, in the PND10 and onward exposure scenario we exposed pre-weaning pups directly by gavage to the test compound, which has likely resulted in higher exposures than in the same developmental period in the OECD TG 443 protocol. Given the higher sensitivity of immune parameters in the latter shorter exposure scenario, it may be concluded that the pre-weaning period seems especially sensitive to adverse effects on the developing immune system.

The ethanol studies were carried out using the same exposure scenarios as for DOTC, with the difference that ethanol was provided in both studies through drinking water only throughout the entire exposure duration. Remarkably, given the same exposure routes, the shorter exposure scenario again showed the highest sensitivity and again immune parameters provided the overall lowest effective dose. Again, tolerance induction in the prenatal period may have played a role, and this may therefore mask the sensitivity of these parameters after acute exposure during the weanling period. These findings also provide argumentation for a specific juvenile exposure and effect study protocol. Although these data already show important information in line with earlier studies, a final assessment needs to await a full analysis of the results of this study. This analysis is currently underway.

The DEHP and NP study designs deviated from those in the earlier studies. The rationale for these designs was several-fold. First, we wanted to be able to discriminate early effects from effects caused later in life. Second, we wondered what would be the relative sensitivity of immune parameters after the same exposure duration in juvenile versus adult animals. This knowledge again would be informative for the optimal exposure scenario as well as the critical parameters to be addressed in juvenile toxicity testing. To these ends, we compared exposure from PND10-50 with PND 50-90, in both cases keeping to a 40-day exposure period, and performed parameter assessment at various ages. The general outcome of these studies clearly showed that the juvenile period was more sensitive than the adult period tested and in addition, showed that immune parameters were more sensitive than general developmental parameters and developmental landmark development. These results again support the need for dedicated juvenile toxicity testing with immune parameters being important in view of their relative sensitivity. In view of the apparent importance of the juvenile only exposure period, we decided to repeat the study with a second compound, nonylphenol, to increase the database and weight of evidence for a proposed study protocol. This study is currently ongoing and will be analysed and reported in the coming year.

Developmental immune parameters proved crucial for determining overall lowest adverse effect levels in each of the studies reviewed above. They appeared more sensitive than, e.g., growth characteristics, organ weights, organ cellularities, and developmental landmarks such as eye opening, preputial separation and vaginal opening. Additional information from parallel studies of neurodevelopmental parameters either a showed lower sensitivity of neurodevelopmental parameters (MeHg) or this information is not available (DOTC and ethanol) for analysis at the present time. Therefore, neurodevelopmental parameters may still prove important and may change overall conclusions. This discussion is based on available information as discussed above. As to the most sensitive immune parameters, it appears overall that functional parameters were more sensitive than morphological parameters. This is reminiscent of the notion that in a rest situation, an organ system may not appear different from control, whereas under stress challenge the organ may appear to be less able to cope than control, showing an adverse effect. In our studies functional parameters such as lymphoproliferative responses to ConA and NO/TNFα production by adherent splenocytes, as well as KLH-specific parameters (anti-KLH IgG and IgM antibody responses) were oftentimes most sensitive. In the presence of these functional parameters, structural parameters such as immune organ weights, cellularities and subset cell fractions could show differences but only at higher dose levels.

Protocols for developmental immunotoxicity have been extensively contemplated by expert groups over the years, as evidenced by a wide body of scientific publications (Smialowicz et al., 2002; Luster et al., 2003; Holsapple et al., 2004, 2005; Dietert and Holsapple, 2007). We have also published pleas ourselves for increased attention in regulatory toxicology for these important parameters (van Loveren et al., 2002; van Loveren and Piersma, 2004). The overall conclusions as to the relative sensitivity of functional immune parameters after an immune challenge and the ideal exposure protocol largely concur with our preliminary findings. It is interesting to note that the current research project, aiming at an optimal juvenile toxicity testing protocol, ends up leading to similar preliminary conclusions as those emanating from parallel discussions from the perspective of developmental immunotoxicity testing. Our focus on immune parameters has clearly facilitated this congruency, but surely given that both activities support each other’s findings this provides further assurance that the route taken so far is relevant and sound. The formulation of a consensus study design for juvenile toxicity testing can only be preliminary at the present time. The postnatal juvenile period, e.g., PND 10-50 in the rat, seems a logical exposure window for such studies, both in view of relative parameter sensitivity and relevance of this period for human childhood. However, actual experimental evidence currently comes from a very limited number of chemicals, the choice of which has been biased by existing knowledge about the developmental toxicity and/or immune toxicity potential of these compounds. Clearly, a wider array of compounds needs to be tested and the continuation of the project with nVWA will allow for such studies in the coming years. As to the exposure route, direct oral exposure needs to be contemplated for compounds with little lactational transfer, in order to achieve meaningful exposure of suckling pups. Otherwise, dietary exposure may suffice. As to the end points of choice, given current information it seems logical to include both structural as well as functional immune parameters to allow the most sensitive end points to be assessed as well as to collect a comprehensive picture of immune status. This should be accompanied with detailed assessment of developmental parameters such as body weight, eye opening, preputial separation and vaginal opening in order to be able to judge

possible interdependency of general developmental delay with effects on the immune parameters tested.

The international embedding of this project is carried out primarily through presentations and publications in the scientific arena. Furthermore, regulatory implementation is being facilitated by involving OECD in the discussion about a possible novel juvenile toxicity testing guideline. In November 2010, at an OECD National Coordinators meeting in Paris, France, we brought the idea forward, illustrated, among other things, by Figure 5 in this report. Clearly, the argumentation was considered persuasive to the extent that participants concluded that this would be a logical priority area of future activities in the OECD test guidelines programme. A second international activity emanating from our project was the organisation of a satellite symposium to the European Teratology Society Meeting and J&J Juvenile Toxicity Workshop in Ghent, Belgium in September 2011. The symposium was chaired by Prof. Aldert Piersma and Prof. Henk van Loveren, both from RIVM. Our project was presented there by Ms. Ilse Tonk M.Sc. (RIVM), with the various studies addressed as in this current report. International experts on developmental immune toxicity (Dr Rodney Dietert, Cornell University) and neurodevelopmental toxicity (Dr Susan Makris, US Environmental Protection Agency) gave lectures, as well as a representative of the Dutch Public Health Ministry (Dr Wieke Tas, Dutch Ministry of Public Health, Welfare and Sports) explaining the regulatory importance of the subject. A lively general discussion followed, showing that there is clear momentum in the scientific as well as regulatory fields as to the further development of a test protocol for juvenile toxicity testing, with the aim to improve hazard and risk assessment specifically for exposures in children.

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