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Developmental immunotoxicity testing of 4-methyl anisole

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ABSTRACT

The developmental immunotoxicity of 4-methyl anisole (4MA) was investigated in the rat. Four study designs were used, with either premating or post-weaning onset of exposure, continued to postnatal day 50, and with or without additional oral gavage of pups from postnatal day 10 onward. Reduced litter size (benchmark dose lower confidence limit (BMDL) 80 mg/kg bw/day) was the most sensitive developmental parameter, with pup relative organ weight effects observed at similar BMDLs, in the absence of maternal toxicity. Eosinophil numbers were reduced at lower doses (BMDL 16 mg/kg bw/day). KLH challenge resulted in increased IL-13 and TNF-α responses, and variably reduced IgG production (BMDL 27 mg/kg bw/day). T_4 levels were reduced by 11% at maximum with a BMDL of 73 mg/kg bw/day. Differences between exposure cohorts were limited and were considered to be without biological significance. This study shows that 4MA induces developmental immunotoxicity at doses below those inducing developmental and general toxicity. These observations being independent of the study designs applied suggest that the post-weaning period, included in all designs, is the most relevant sensitive period for inducing 4MA mediated developmental immunotoxicity. Moreover, this study stresses the importance of including developmental immunotoxicity testing by default in regulatory toxicology.

1. Introduction

Developmental immunotoxicology is a relatively neglected area in regulatory toxicity testing. The recent acceptance of the OECD TG 443 Extended One generation Reproductive Toxicity Study (EOGRTS), including a specific cohort for immune testing provides a significant step forward (OECD, 2011, TG 443), but this test may only be required at high tonnage levels under the European REACH regulation for chemical safety. Some structural immune parameters such as lymphoid organ weights and subset organ cellularities are also monitored in regular acute and subchronic toxicity testing. However, the developing immune system may be more vulnerable to toxic insults, which warrants specific testing in developmental phases. In addition, structural immune system parameters, as opposed to functional immune parameters, may appear normal in the presence of affected immune responsiveness to an immunological challenge. The realization that developmental immune toxicity testing may be important in chemical hazard identification and risk assessment is further supported by increasing trends of immune-related diseases in man that have an early onset, such as allergies, asthma and a variety of autoimmune diseases. Earlier studies have indicated that the developing immune system may have a specific sensitivity to compound exposure (Miller et al., 1998; Chapin et al., 1997; Gehrs et al., 1997; Smialowicz et al., 1988). We have previously tested several compounds in several developmental exposure designs, studying general, developmental and immunotoxic effects (Tonk et al., 2011a,b, 2010, 2013a,b, 2012). Overall, it appeared that developmental immune parameters were or were among the most sensitive parameters studied. As a consequence, developmental immune parameters often (co-)determined the overall NOAEL, and, if tested in a regulatory setting, would have had an impact on the derivation of threshold levels for human exposure such as TDI or ADI.

A variety of relevant study designs can be envisaged for developmental immunotoxicity testing. Provisional comparisons of generational exposure from the premating phase to offspring adulthood versus juvenile exposure studies indicate that both exposure scenarios have their advantages in detecting developmental immunotoxicity (Tonk et al., 2011a,b, 2013a,b). As generational exposure such as in the EOGRTS includes long term continuous exposure, effects caused during any phase of the entire developmental window can be detected. However, adaptation to exposure and prenatal programming can potentially influence postnatal sensitivity. A juvenile exposure design may show a higher sensitivity during early postnatal development but is also

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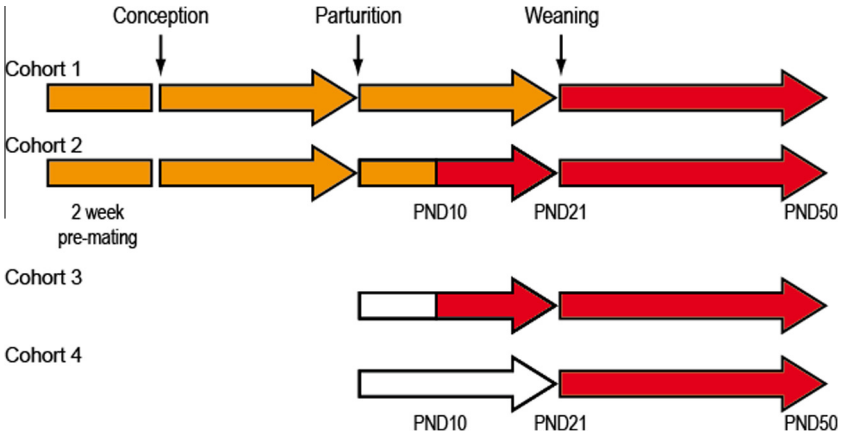

Fig. 1. Study design. Orange: maternal exposure via gavage; red: direct F_1 exposure via gavage. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

limited to this particular developmental window. The relative sensitivity of the juvenile period needs further study in order to clarify whether a specific testing protocol is warranted. Such a specific protocol may or may not need to include direct dosing of pups during lactation to ensure pup exposure, with or without direct exposure of the pregnant or lactating dams.

In the present study we assessed a series of developmental immune parameters in generational and juvenile exposure protocols with and without direct pup exposure during lactation using 4-methylanisol (4MA) as a test compound. 4MA is a food flavoring agent which naturally can be found in oil of ylang ylang used in fragrances. Currently 4MA is used a.o. in cleansing agents, air care products, biocidal products and scented toys. 4MA is soluble in apolar solvents with a Pow around 2.7, and after topical exposure 12% of the applied dose is excreted in urine, suggesting that systemic exposure does occur, possibly also via the oral route (ECHA). Therefore, lactational exposure may be a relevant route of weanling exposure. This compound has been shown to reduce spleen and thymus weight in a repeat dose toxicity study (OECD, 2008, TG 407), and caused pup mortality and reduced pup weight in a reproductive and developmental toxicity screening study (OECD, 1995, TG 421). Based upon the latter study, in Europe 4MA received an H361 classification for suspicion of damaging fertility or the unborn child (ECHA). The overall NOAEL was 100 mg/kg bw/day in both these studies. Thus, given that 4MA affected developmental as well as immune parameters in earlier studies, this compound was considered a relevant candidate for dedicated developmental immunotoxicity testing. Moreover, direct pup exposure via the oral route was included in this study in order to establish whether this developmental window might be particularly sensitive for 4MA induced effects.

2. Materials and methods

2.1. Animals

The animal experiment was carried out at Intravacc, Bilthoven, The Netherlands. Animal care and use were in accordance with the general principles of governing the use of animals in experiments of the European communities (Directive 86/609/RRC) and with Dutch-specific legislation (The Experiments on Animals Act). Parental (F_0) Wistar outbred rats were obtained from Harlan, The Netherlands. Animals were given a two week acclimatization period before the start of the experiment and housed in groups in macrolon cages with 12:12 h light:dark cycle, maintained at 22 ± 2 °C, 45–90% humidity on a commercial rodent diet (Rat & Mouse No. 3 breeding diet, RM3, SDS Special Diets Services, Witham, England). F_0 animals were mated at a ratio of 2 females:1 male. The day of sperm detection in the vaginal smear was considered day 0 of gestation and mated F_0 females were housed individually for the birth and rearing of their young. The morning after birth was considered postnatal day (PND) 1, litters were not standardized and pups were weaned on PND 21.

2.2. Test compound and exposure

4-Methylanisole (4MA), CAS 104-93-8, with a labeled purity of 99% was purchased from Aldrich and dissolved in laboratory-grade corn oil (CAS 8001-30-7, MP Biomedicals). The maximum period for which each preparation was used was 7 days. During exposure, animals were administered a daily oral dose of 0 (vehicle control), 8, 16, 32, 64, 125, or 250 mg/kg bw/day 4MA in 5 ml vehicle per kg bw except for juvenile animals (PND 10–21) for which a dosing volume of 10 ml per kg bw was used. Bodyweight were monitored twice weekly in F_0 animals and in F_1 animals after weaning, in pregnant dams on gestation day (GD) 0, 4, 7, 10, 13, 17, 21, lactation day (LD) 1, 4, 7, 10, 13, 17, 21 and pups on PND 1, 4, 7, 10, 13, 17, and 21.

A schematic diagram of the study design is shown in Fig. 1. In cohort 1 F_0 females are exposed from 2 weeks pre-mating, during mating, gestation and lactation and pups receive a vehicle from PND 10 to 21 and are individually exposed from PND 21 (weaning). Cohort 2 are exposed similarly as cohort 1 except maternal exposure stops at LD10 and F_1 pups are directly exposed from PND 10. In cohort 3 and 4 animals there is no maternal exposure, cohort 3 F_1 is exposed directly from PND 10 while cohort F_1 animals are directly exposed from PND 21 (receiving a vehicle from PND 10 to 21).

2.3. Effects assessment

F_1 males were examined daily for the onset of preputial separation (PPS) as an indicator for the onset of puberty from PND 31 until complete separation or necropsy (PND 50).

Subsets of F_1 males (n = 6/dose group) originating from different litters, were evaluated for the effects of 4MA exposure on PND 50. Terminal body weight were recorded, EDTA blood was collected and one femoral shaft was flushed with 4 ml Impulse Cytophotometer (ICP) solution Tonk et al., 2010. The bone marrow cell suspension and EDTA blood were kept at 4 °C until automated analysis using an ADVIA 120 Hematology System (Siemens) within 4 h. Liver, kidneys, spleen, thymus, adrenals, testes, heart and brain were removed and weighed. The right testis was frozen on dry ice and used for testis spermatid head count. The tunica alba was