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Developmental immunotoxicity in male rats after juvenile exposure to ethanol

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ABSTRACT

The aim of the present study was to determine the sensitivity of the developing immune system to ethanol (EtOH) after exposure from postnatal day (PND) 10 onward. Adult Wistar dams and litters were exposed to EtOH via drinking water (0, 0.25, 1.5, 2.75, 4, 5.25, or 6.5% (w/v) EtOH ad libitum) and drinking water exposure of the F_1 was continued from weaning until sacrifice. Immune assessments were performed at postnatal days (PNDs) 21, 42, and 70. Furthermore, Keyhole Limpet Hemocyanin (KLH) specific immune responses were evaluated following subcutaneous immunizations on PNDs 21 and 35. EtOH exposure affected innate immune responses, such as LPS-induced NO-production by adherent splenocytes, as well as adaptive immune responses as represented by KLH-specific parameters. The most sensitive developmental parameters included effects on maternal and pup bodyweight with calculated BMDs of 4.0% and 4.3% EtOH, respectively. The most sensitive immune parameters were affected at dose levels lower than those affecting developmental parameters and included KLH-specific immune responses, LPS-induced NO production by adherent splenocytes, and IL-10 production by ConA stimulated splenocytes. Calculated BMDs for these parameters were between 0.01% and 0.1% EtOH. A comparison of the results of this juvenile study with an extended one-generation reproductive toxicity study revealed that the juvenile study design may result in a higher sensitivity related to differences in the exposure design.

These findings demonstrate the relative sensitivity of the developing immune system for EtOH exposure, the additional value of assessing functional immune parameters, and the importance of the juvenile window in developmental immunotoxicity testing.

1. Introduction

Fetal and early postnatal life represent critical periods in the development of the vertebrate immune system (Dietert et al., 2000). The developing immune system may be more susceptible to disruption by chemical agents than the adult immune system (Luebke et al., 2006; van Loveren et al., 2003) and the various developmental stages all have their specific sensitivity to toxic insult. Therefore, adult immunotoxicity testing does not suffice for the prediction of developmental immunotoxicity (DIT). An appropriate selection of exposure windows and sensitive parameters in developmental toxicity testing protocols is essential for the identification of DIT.

In regulatory pharmaceutical safety evaluations, preclinical juvenile toxicity testing is applied to assure safety for the pediatric population (EMA, 2008). The study design is not specified in the regulation and can be tailored to 'case-by-case' needs. Chemical safety testing protocols include regulatory guidelines for developmental toxicity testing during the prenatal window and lifetime studies, such as the extended one-generation study, which includes pre as well as postnatal exposures (OECD, 2012). Numerous studies have demonstrated chemical-induced DIT using lifetime exposure protocols. However, this study design does not allow conclusions as to which part of the exposure window is most sensitive for perturbation of the developing immune system. Some studies have shown that pups were more affected after early postnatal or lactational exposure versus gestational exposure (Barrow et al., 2006; Smialowicz et al., 1988). This may be a consequence of differences in target exposure level due to variations in transfer from dam to pup during gestation and lactation. However, higher sensitivity of early postnatal developmental windows may also play a role. It is therefore important to study the relevance of the juvenile exposure window for chemical safety testing. In a recent study, we have studied the effects of EtOH in an extended one-generation study protocol with exposure starting in the premating period and continued in offspring. We observed specific effects on the developing immune system (Tonk et al., 2012a). In the current study, for comparison, we studied EtOH effects in a juvenile exposure

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protocol starting exposure on PND 10. This comparison sheds light on the impact of exposure timing and duration on developmental immune parameters as compared to general parameters of growth and development. Furthermore, findings inform about the possible need for a juvenile exposure study for hazard and risk assessment.

Ethanol (EtOH) exposure has been shown to have immunomodulatory properties (Szabo and Mandrekar, 2009) and gestational exposure has been associated with impaired immunity in offspring (Chiappelli and Taylor, 1995; Johnson et al., 1981; Zhang et al., 2005). Focussing on early postnatal exposure, Giberson and Blakley (1994) reported greater effects of maternal EtOH exposure on the expression of differentiation antigens of murine splenic lymphocytes in offspring after lactational exposure versus gestational exposure. Seelig et al. (1996) examined the effects of maternal EtOH exposure on antigen-driven responses in the neonate using the *Trichinella spiralis* model. Exposure throughout gestation, lactation and from weaning onwards had the greatest effect on most immune parameters tested. However, exposure during lactation only also demonstrated significant effects with some of the effects being more profound compared to the effects after pre- and postnatal exposure.

Juvenile rats were exposed from postnatal day (PND) 10 onwards based on comparative age categories between species as described by Coogan (2009). We compared the status of a series of immune parameters at different ages, on PNDs 21, 42 and 70. In a previous study with ethanol exposure (Tonk et al., 2012a), we observed effects on T cell functioning and on adaptive immune responses. The endpoints assessed in this study included lymphoproliferative responses and KLH-specific parameters, such as the T cell dependent antibody response (TDAR). Effects of EtOH on innate immunity were described in literature as not typically observed in animal studies (Zhang et al., 2005). However, in our previous study we observed effects on NO and TNF-α production by adherent splenocytes, as an indicator of macrophage function, and on NK cell activity (Tonk et al., 2012a); and these parameter are also included in the current study.

2. Materials and methods

The in vivo part of the study was carried out at the Animal Facilities of NVI, Bilthoven, The Netherlands. Animal care and use were in accordance with Directive 86/609/RRC, which established the general principles of governing the use of animals in experiments of the European Communities and with Dutch-specific legislation (The experiments on Animals Act). Parental F_0 animals, 84 female (9–10 weeks old) and 42 male (11–12 weeks old) rats, Wistar outbred (Crl:(WI)WU BR), were obtained from Charles River Deutschland (Sulzfeld, Germany). F_0 females were housed individually for the birth and rearing of their young (F_1) while F_1 animals were housed in groups of 4 per sex in the animal facility with a 12:12-h light:dark cycle, maintained at 22 ± 3 °C and 30–70% relative humidity and fed a commercial rodent diet (Rat Mouse No. 3 Breeding diet, RM3, SDS Special Diet Services, UK) ad libitum. At the end of the 2-week acclimatization period, F_0 animals were mated at a ratio of 2 females:1 male. The morning after birth was considered postnatal day (PND) 1. Litters were not standardized and pups were weaned on PND 21.

Dams were randomly assigned to the treatment groups and received tap water with ethanol concentrations of 0, 0.25, 1.5, 2.75, 4, 5.25, or 6.5% (w/v) EtOH ad libitum. Dose levels were selected based on a previous study with ethanol exposure using an extended one-generation reproductive toxicity study exposure protocol (Tonk et al., 2012a). Dams assigned to the EtOH groups were gradually introduced to EtOH exposure starting on PND 8 with 0% EtOH for the 0.25% and 1.5% EtOH exposure groups and 1.5% for the 2.75%, 4%, 5.25%, and 6.5% exposure groups. On PND 10 dams assigned to the 0.25%, 1.5%, 2.75%, and 4% EtOH exposure groups were given ultimate exposure levels while the 5.25% and 6.5% exposure groups received 4% EtOH in drinking water. From PND 12 all dams received their ultimate EtOH exposure levels and exposure of F_1 animals was continued from weaning onwards.

Throughout the study, all animals were checked daily for clinical signs and abnormal behaviour. Pup body weights were recorded at PNDs 8, 10, 13, 17, and 21 and weekly from weaning onwards. Individual water consumption of F_0 females with live pups was recorded for postpartum days 8–10, 10–12, 12–17, and 17–21. F_1 water consumption was recorded twice weekly from weaning onwards. Juvenile males were examined daily for the onset of preputial separation, indicating the onset of puberty, from PND 35 until complete separation.

2.1. Necropsy

Subsets of F_1 male rats (n = 8–10/dose group) were evaluated for the effects of EtOH exposure. During necropsy, terminal body weights were determined and on PNDs 21 and 90 and liver, kidneys, adrenals, spleen, thymus, heart, brain, testes and epididymis were weighed. Immune assessments were performed on PNDs 21, 42 and 70. EDTA blood was collected and two femoral shafts (PNDs 21 and 70 only) were flushed with 4 ml impulse cytophotometer solution, containing 3.22 g/l trisodium-citrate-dihydrate, 3.4 g/l sodium-dihydrogenphosphate-dihydrate, 3.87 g/l disodium-hydrogenphosphate-dihydrate, 1.17 g/l citric-acid-monohydrate, 3.65 g/l dextrose, 4.96 g/l sodium chloride in demineralized water, pH 7.4 at 20 °C. The resulting cell suspension and the collected EDTA blood were kept at 4 °C until automated analysis in an ADVIA 120 (Siemens) within 4 h. Spleen and thymus were collected en weighed and part of the spleen and thymus were used to prepare single-cell suspensions by pressing through 70 μm sterile nylon mesh (Falcon). The cells were washed twice in RPMI 1640 (Gibco) with 5% heat-inactivated fetal bovine serum (FBS) (Integro BV), 100 U/ml penicillin, and 100 μg/ml streptomycin (Gibco) (standard medium) and resuspended in standard medium containing 10% FBS. Cellularities of the splenic and thymic cell suspensions were determined using a Coulter Counter Z2 (Coulter Electronics). Spleen single cell suspensions were used for immune assessments as described previously (Tonk et al., 2010).

2.2. Immune assessments

2.2.1. Lymphocyte subpopulations

Single spleen and thymus cell suspensions were examined for lymphocyte subpopulation distribution using four-color flow cytometry. For spleen, the following monoclonal antibodies were used at predetermined saturation levels: Allophycocyanin (APC)-conjugated mouse anti-rat CD3 (clone 1F4); R-Phycoerythin (R-PE)-conjugated mouse anti-rat CD8a (clone OX-8); fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat CD4 (clone OX-35); cy-chrome (Cyc)-conjugated mouse anti-rat CD45 (clone OX-1); FITC-conjugated mouse anti-rat CD45RA (clone OX-33); and R-PE-conjugated mouse anti-rat NKR-P1A (clone 10/78) (Pharmingen, San Diego, CA) at predetermined saturation levels. For thymus, only anti-CD3, anti-CD8a, and anti-CD4 monoclonal antibodies were used for assessment. Erythrocytes in the cell suspensions were lysed with BD Pharma Lyse™, cells were washed with wash buffer (PBS containing 1% bovine serum albumin), resuspended in PBS containing 0.1% paraformaldehyde and analyzed on a FACS Calibur flow cytometer (BD Biosciences).

2.2.2. Natural killer cell activity

Splenic natural killer (NK) cell activity was assessed using an in vitro ^{51}Cr-release assay. Spleen single cell suspensions were incubated with ^{51}Cr-labeled YAC-1 lymphoma cells. The amount of ^{51}Cr released from the YAC cells after incubation for 4 h was used as a measure of natural killer cell activity. NK activity was only determined on PNDs 42 and 70.

2.2.3. NO/TNF-α production by adherent splenocytes

Adherent splenocytes were stimulated with lipopolysaccharide (LPS) (Sigma) for 24 h and the supernatants were used to measure the nitric oxide (NO) production using the Griess reaction (Green et al., 1982) and tumor necrosis factor (TNF)-α production using an ELISA kit (eBioscience).

2.2.4. Lymphoproliferative responses

Splenic lymphoproliferative responses to concanavalin (Con) A and LPS and thymic lymphoproliferative response to ConA were determined using in vitro ^{3}H-thymidine incorporation. In addition, supernatants of ConA stimulated splenocytes were used for the determination of interleukin (IL)-4, IL-10, IL-13, and interferon (IFN)-γ using a MILLIPLEX Map Kit (Millipore).

2.2.5. KLH-specific parameters

A separate group of F_1 male rats (n = 4–8 males/dose group) were immunized with a subcutaneous injection of 0.2 ml of 5 mg/ml KLH (ThermoScientific) at PNDs 21 and 35 to evaluate T dependent antibody response (TDAR) parameters. The primary IgM (PND 26), primary IgG (PND 35), and the secondary IgM and IgG (PND 40) response to KLH were determined using an anti-KLH IgM- or IgG-specific ELISA. At PND 49, the Delayed Type Hypersensitivity (DTH) measured response to KLH was assessed using a challenge with 10 mg/ml KLH in 0.01 ml of saline injected into one ear and an equivalent volume of saline injected in the other ear. The DTH response was measured 24 h later using a digital caliper. The KLH-immunized rats were euthanized at PND 63, spleens were removed aseptically and single spleen cell suspensions were prepared. KLH-induced cell proliferation was determined using in vitro ^{3}H-thymidine incorporation and supernatants of KLH-stimulated splenocytes were used for the determination of IL-4, IL-10, IL-13, and IFN-γ using a MILLIPLEX Map Kit (Millipore).