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Developmental immunotoxicity of Diazepam in prenatally treated weanling Wistar rats

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Samenvatting

Een prenatale reproductietoxiciteitsstudie werd uitgevoerd bij ratten, die daartoe gedurende dag 14 tot dag 20 van de dracht behandeld werden met het geneesmiddel diazepam. In de literatuur is gerapporteerd dat diazepam immuunsuppressie bewerkstelligt bij nakomelingen van ratten die in het derde trimester van de dracht behandeld werden. Deze verbinding wordt in de kliniek toegepast bij zwangere vrouwen. Het doel van het onderzoek was te onderzoeken of opname van een aantal parameters voor immunotoxiciteit in de reproductiestudie deze immunotoxiciteit zou aantonen. Deze immuunparameters bestonden uit routine hematologie, gewicht en microscopie van lymfoïde organen, beenmerg cellulariteit, totale serum immunoglobuline concentraties, relatief vóórkomen van lymfocyten populaties in de milt, "natural killer cell" activiteit van lymfocyten in de milt, mitogene stimulatie van lymfocyten uit de milt, en de primaire en secondaire antilichaamrespons tegen schapen rode bloedcellen. Bovendien werd de weerstand tegen *Trichinella spiralis* parasieten onderzocht.

Er werden enige effecten op het immuunsysteem waargenomen. Deze effecten werden niet gezien bij niet-functionele testen van het immuunsysteem, terwijl met functionele testen enige effecten werden waargenomen (een (statistisch niet-significante) reductie in natural killer cell activiteit en een toegenomen IgE respons na *Trichinella spiralis* infectie). De resultaten geven aan dat diazepam niet een ernstige immunosuppressie induceren in de nakomelingen die via hun moeders tijdens de dracht zijn blootgesteld. Achteraf zou voor de studie naar de wenselijkheid van opname van immunotoxicologische parameters derhalve beter voor een meer actieve verbinding gekozen zijn. Niettemin rechtvaardigen de resultaten verder onderzoek om de bruikbaarheid van een uitbreiding van het OECD 414 protocol met immuunparameters te testen.

Summary

A prenatal developmental toxicity study was conducted in rats which received the pharmaceutical diazepam during days 14 to 20 of gestation. Literature reports claim that diazepam impaired immune function in the offspring of rats, which had received treatment during the third trimester of gestation. Diazepam is currently used in the clinic for treatment of pregnant women. The aim of the study was to study whether inclusion of parameters of immunotoxicity would identify immunotoxic activity of this compound in this test. These parameters included routine hematology, weights and microscopy of lymphoid organs, bone marrow cellularity, total serum immunoglobulin levels, counts of splenic lymphocyte subsets, splenic natural killer cell activity, splenic lymphocyte responsiveness to mitogens, and primary and secondary antibody responses to sheep red blood cells. In addition, the host resistance to *Trichinella spiralis* parasites was studied.

Non-functional immune parameters were not affected in this test. Some effects were observed with functional immune assays, i.e. a (statistically insignificant) reduction in NK-cell activity and an increased IgE response after infection with *Trichinella spiralis*. These results indicate that diazepam is not a strong immunosuppressant under the conditions in this experiment. In retrospect, it would have been more advantageous if we would have selected a more potent immunosuppressant to perform these studies. Nonetheless, the results warrant further research to test the usefulness of extending the current OECD 414 protocol by introducing immune parameters.

1. Introduction

A number of animal toxicity test protocols is available to routinely assess reproductive toxicity. One of these is the so-called Segment II developmental toxicity study, in which the test substance is administered to gravid rats during days 6-15 of gestation (i.e. GD 6-15). This time frame extends from implantation to the end of organogenesis (i.e. closure of the hard palate). The assay is aimed at the detection of potential adverse effects on the dam, and on the development of the embryo and fetus. An important endpoint is the occurrence of malformations in the offspring. The protocol is described in the Organisation for Economic Cooperation and Development (OECD) 414 guideline. This guideline that is not specifically directed at drugs is currently debated for two reasons.

First, the OECD is in a process of extending the period of exposure from GD 6-15 to GD 6-20 (OECD, 1996). In comparison with the current standard protocol, this would add the developmental stage starting at the closure of the hard palate to the end of pregnancy and would allow further assessment of fetal development and growth. However, such an extension of treatment duration might alternatively result in decreased sensitivity due to reduced survival of malformed fetuses and hence a failure to detect adverse effects at parturition due to early resorption.

Second, recent developments suggest the potential usefulness of incorporating parameters of immunotoxicity into the OECD 414 test protocol. So far, immunotoxicity has mainly been studied in adult animals employing multiple immunological assays. Tiered test strategies have been developed to assess immunotoxicity in rodents by introducing immune parameters in the routine protocol of a repeated oral dose toxicity study. These tiered approaches primarily focus on immunosuppression (Vos and Van Loveren, 1994). In our Institute, a tiered battery of immunotoxicity parameters has been developed in the framework of a 28-day repeated oral dose toxicity test in rats (Vos, 1982, Van Loveren and Vos, 1989; Vos and Van Loveren, 1994). Amongst others based on this work, the existing OECD 407 guideline on 28-day repeated oral dose toxicity testing in rats was modified some years ago by incorporating a number of immune parameters (Koëter, 1994; Basketter et al., 1994, OECD 1995). In reproductive toxicity assays, immune parameters are currently not routinely studied. Rat data, however, indicate that the late prenatal period of exposure (i.e. during ontogeny of the immune system) may be particularly sensitive in terms of reduced host resistance to infections later on in adulthood. This was shown in experimental animal studies with various chemicals, including for drugs such as aciclovir (a virusstatic agent; Stahlmann et al., 1992) and diazepam (a benzodiazepine anxiolytic agent; Schlumpf et al., 1994a) at therapeutically relevant dose levels. These animal observations suggest that changes in the functional capacity of the immune system may represent an important hazard of prenatal exposure to chemicals, including pharmaceuticals.

To explore the usefulness of an extended OECD 414 protocol (i.e. the prolonged duration of treatment and the introduction of immune parameters), we conducted an exploratory Segment II assay in rats (De Waal et al., 1998). The period of treatment was either GD 6-15 or GD 6-20 of gestation. In both instances, the protocol was enriched by immune parameters derived from the updated OECD 407 protocol and additional immune parameters. The benzodiazepine diazepam was chosen as a model compound. The parameters of the immune system that were selected are similar to those used in 28-day oral toxicity testing, as performed according to OECD guideline 407 version 1995 (i.e. weight of the lymphoid

organs, in addition to histopathology of lymphoid organs and tissues). In addition, analyses of subpopulations of spleen lymphocytes, total serum immunoglobulin levels, spleen lymphocyte responsiveness to mitogens, and natural killer (NK-)cell activity of spleen cells were performed.

In this pilot study (De Waal et al., 1998), the teratology screen showed no treatment-related abnormalities in the offspring. The extension of treatment from GD 6-15 to GD 6-20 did not influence the outcome of the developmental parameters. Some effects of exposure were observed in the immunotoxicology screen. However, a causal relationship to treatment was questionable because of a lack of dose-response. It therefore was not possible to unequivocally detect effects of *in utero* exposure to diazepam on the developing immune system in neonatal rats.

Using alternative parameters (i.e. cytokine profiles of spleen cells and host resistance to *Trichinella spiralis*), the group of Schlumpf consistently reported delayed immunotoxicity in the offspring of rats that subcutaneously received diazepam at dose levels as low as 1.25-5 mg/kg/day from GD 14 to 20 (Schlumpf et al., 1989, 1994a,b). Perhaps the parameters that were subject of study in our laboratory were less sensitive than those applied by Schlumpf and coworkers. In addition, there were a number of differences between the study design that was used by the group of Schlumpf and ours, that may have influenced the results (De Waal et al., 1998). One important difference was that in the study by De Waal et al. (1998) only non functional parameters of the immune system were evaluated, whereas in the study by Schlumpf et al., (1989, 1994 a,b) assays of immune function were applied.

In the context of the general aim to investigate the validity of including immune parameters in reproduction toxicity protocols, we have conducted a developmental immunotoxicity study in rats. We hoped to clarify whether differences in study design could explain the apparent discrepancies between the findings of the group of Schlumpf (Schlumpf et al., 1994a,b) and ours (De Waal et al., 1998). The study design of our pilot experiment was modified as follows:

- Diazepam was administered during GD 14-20 (as was done by Schlumpf and coworkers in their experiments);
- Oral dosing (as in our pilot experiment) was compared with subcutaneous injection (as employed in the experiments of Schlumpf and colleagues);
- The dose level shown by the group of Schlumpf to exert developmental immunotoxicity in rats (i.e. 1.25 mg/kg/day subcutaneously) was compared with the 30 mg/kg/day dose level (orally administered) which appeared to be devoid of clear-cut developmental toxicity in our pilot experiment.
- The *Trichinella (T.) spiralis* host resistance model was introduced as a read-out of developmental immunotoxicity (as studied by Schlumpf and colleagues in their experiments).

2. Experimental procedures

2.1 Animals

Specific pathogen-free male (n = 5) and female (n = 80) Wistar rats (WU) were obtained from the breeding stock of our Institute. The animals were less than 20 weeks of age. They were housed on macrolon cages. Drinking water and a conventional diet (RMH-GS, Hope Farm B.V., Woerden, The Netherlands) were provided ad libitum. The animals were rested for one week prior to mating. In the animal room, a light-dark regimen of 12 hours was maintained (light on from 12.00 hour a.m. until 12.00 hour p.m.). Temperature and relative humidity were maintained at 20-24 ^{o}C and 50-75%, respectively.

2.2 Test material

Diazepam was supplied by BUFA B.V., Oegstgeest, The Netherlands. The compound was dissolved in maize oil as vehicle (Sigma, The Netherlands).

2.3 Dose selection

Diazepam is a long-acting benzodiazepine, which is used to treat humans as an anxiolytic, anti-epileptic, or hypnotic agent. In rats, the pharmacodynamically active oral dose of diazepam ranges from 30 mg/kg (i.e. the ED_{50} based on the induction of sedation) to 88 mg/kg (i.e. the ED_{50} based on the induction of muscle relaxation) (Jansen Van 't Land and Van Der Laan, 1989). Developmental immunotoxicity, however, has been reported to occur at far lower dose levels. In experiments demonstrating immune suppression as a consequence of prenatal treatment, rats were subcutaneously injected with only 1.25 mg/kg/day from day 14 to 20 of gestation (Schlumpf et al., 1994a,b). Treatment of rats with 1.25-2.5 mg/kg/day subcutaneously from day 14 to 20 of gestation produced transient behavioral deficits in rat offspring (Schlumpf et al., 1989).

In our previously performed pilot experiment, pregnant rats were treated with diazepam by daily gastric intubation at dose levels of 0, 3, 30 and 300 mg/kg/day. The period of treatment lasted either from GD 6 to 15, or from GD 6 to 20. During the experiment, it was decided to lower all dose levels because of overt toxicity at the 30 and 300 mg/kg/day dose levels. Dose levels of 0, 0.3, 3 and 30 mg/kg/day were orally administered during the remainder of the study. In this experiment, it was not possible to unequivocally detect effects of maternal exposure to diazepam on the developing immune system in neonatal rats with the set of immune parameters examined (De Waal et al., 1998).

The aim of the present study was to compare the data obtained by Schlumpf and coworkers (1994a,b) with ours (De Waal et al., 1998). Therefore, two dose levels were chosen: 1.25 mg/kg/day (i.e. the dose employed by the group of Schlumpf) and 30 mg/kg/day (i.e. the dose previously employed in our pilot experiment).

2.4 Experimental design

Female rats were mated between 9.00 and 10.00 hour a.m., numbered and randomly distributed in five groups of 12 animals each. The animals were treated with diazepam by once daily administration from GD 14 to 20 as follows:

- Group 1: 0 mg/kg/day by subcutaneous injection (i.e. the subcutaneous control group);
- Group 2: 1.25 mg/kg/day by daily subcutaneous injection;
- Group 3: 0 mg/kg/day by oral administration (gastric intubation) (i.e. the oral control group);
- Group 4: 1.25 mg/kg/day by oral administration (gastric intubation);
- Group 5: 30 mg/kg/day by oral administration (gastric intubation).

The dosing volume was 1 ml per 200 g body weight for orally administered diazepam, and 0.05 ml per 100 g body weight when the compound was injected subcutaneously.

Maternal body weights and food consumption were examined on day 0, 6, 12, 18 and 21 of gestation. The dams were observed daily for clinical signs.

The pups were randomised at birth and their number was culled to four per litter. The remaining pups and their dams were sacrificed subsequently. At the age of six weeks (i.e. at weaning), the four rats born in each litter were used as follows:

- One weanling rat was subjected to an immunotoxicology screen;
- One weanling rat was subjected to T. spiralis to examine host resistance;
- Two weanling rats were subjected to test T-cell dependent humoral immune responsiveness against sheep red blood cells (SRBC).

2.5 Immunotoxicology screen

At six weeks after birth, one weanling rat per litter was sacrificed by exsanguination from the abdominal aorta during ether anesthesia. The numbers of animals investigated were: 0 mg/kg subcutaneously, n = 9 males; 1.25 mg/kg subcutaneously, n = 10 males; 0 mg/kg orally, n = 9 males; 1.25 mg/kg orally, n = 8 males and 1 female; 30 mg/kg orally, n = 9 males and 1 female. The immunotoxicology screen included the following parameters:

2.5.1 Hematology

For hematology, blood was collected in EDTA-coated tubes. White blood cell counts and differentiation was performed in a Multispecies Hematology Analyzer (Bayer BV, Division Diagnostics, Mijdrecht, The Netherlands). The following parameters were determined: red blood cell (RBC) count, platelet (PLT) count, hemoglobin (Hb), hematocrit (Ht) and white blood cell (WBC) count and differentiation.

2.5.2 Total serum antibody levels

The total serum IgM, IgG, IgE and IgA immunoglobulin levels were measured as described previously (De Waal et al., 1998).

2.5.3 Organ weights

Heart, lungs, liver, spleen, thymus, kidneys, adrenals, testes, brain, mesenteric lymph nodes, popliteal lymph nodes and mandibular lymph nodes were weighed.

2.5.4 Histopathology

Heart, lungs, liver, kidneys, adrenals, testes, stomach, jejunum, colon, thyroid, esophagus, brain, and pituitary gland were fixed in neutral, aqueous phosphate-buffered 4% formaldehyde. Thymus, mesenteric lymph nodes, mandibular lymph nodes and popliteal lymph nodes were in part fixed in 4% formaldehyde; from these organs, the remaining tissue was quickly deep frozen by liquid nitrogen for immunohistochemistry. The spleen was divided into three parts. One part was fixed in 4% formaldehyde, the second part was deeply frozen and the third part was used to prepare cell suspensions. The intestines were sampled according to the Swiss role technique (Moolenbeek and Ruitenberg, 1981) and fixed in 4% formaldehyde. Bone marrow from one femur was fixed in 4% formaldehyde. Bone marrow from the other femur was collected by flushing 2 ml Impuls Cytometer Fluid through the femur using a 21-gauge needle. The concentration of nucleated cells was determined in a Coulter counter. Additionally, cytospin preparations were prepared and stained with May-Grunwald Giemsa for differential count of the cells. The formaldehyde-fixed tissues were embedded in paraffin. Five-micrometer-thick haematoxylin-eosin stained tissue sections were prepared for microscopic examination. When indicated by the histopathological results, additional recuts were prepared. Histopathology data were documented using the PATHOS data-entry and reporting system (Pathology Operating Systems Ltd, Harrogate, England) or manually.

2.5.5 Lymphocyte subpopulations in the spleen

Immunofluorescence analysis of lymphocyte subpopulations was performed using a single-laser FACScan (Becton and Dickinson Immunocytometry Systems, Mountain View, CA). Monoclonal antibodies MARK-1, ER-2, OX-8 and OX-19, all conjugated with FITC, were used to identify B-cells, $CD4^+$-, $CD8^+$-, and $CD3^+$-cells, respectively.

2.5.6 Natural killer (NK) activity of spleen cells

Spleen cells were incubated with ^{51}Cr-labelled LAC cells with different target : effector ratio's (200 : 1; 100 : 1; 50 : 1; 25 : 1). Also, ^{51}Cr-labelled YAC cells were incubated with medium (spontaneous release) or 1% Triton solution (maximum release). After a 4-hr incubation period at 37°C, the cells were harvested and the ^{51}Cr-release from these labelled YAC cells was counted using a gamma-counter. Specific release was calculated according to standard procedures.

2.5.7 Mitogen stimulation of spleen cells

The proliferative response of spleen cells to mitogens was determined using the lymphocyte stimulation test. The following mitogens, which specifically stimulate the proliferation of T- and/or B-cells were used: phytohaemagglutinin (PHA), concavalin A (ConA), and lipopolysaccharide (LPS). Spleen cells were incubated in the absence (control) or presence of mitogen for 48 hr at 37°C (5% CO_2). Then, ^3H-thymidine was added and again the cells were incubated for 20-22 hr at 37°C (5% CO_2).

2.6 SRBC antibody response

Two weaning male rats per litter were immunised intraperitoneally (i.p.) with 0.5 ml of a 20% SRBC suspension (containing 2x 10^9 cells) at the day of sectioning of their littermates at the age of six weeks. A booster injection (0.5 ml 20% SRBC) was given 15 days later. The primary IgM and secondary IgG antibody responses were determined by ELISA on day 7 (n = 1 animal per litter) and 20 (n = 1 animal per litter) after primary immunisation, respectively, after bleeding the animals (Van Loveren et al., 1991).

2.7 Host resistance to T. spiralis

The resistance to T. spiralis was assessed as previously described (Van Loveren et al., 1995). At the age of six weeks, one weanling rat per litter was infected with 1000 T. spiralis muscle larvae by gastric intubation. Six weeks after the infection, the animals were sacrificed by decapitation under $C0_2/O_2$ anesthesia, the serum was sampled, and tongue muscle samples were fixed in neutral, aqueous phosphate-buffered 4% formalin for counting of the larvae. The serum was stored at –20°C until it was analysed for T.spiralis-specific IgM, IgG, IgE and IgA antibody levels. These tongue tissue samples were routinely processed for histology and stained with haematoxylin-eosin and Giemsa. Morphometric analysis of tongue sections was done using the IBAS 2000 image analysis system (Kontron, Munich, FRG). In addition, the yield of muscle larvae was determined in eviscerated carcasses by the digestion method (Van Loveren et al., 1995).

2.8 Statistical analysis

Data are expressed as mean ± SD. They were analysed by applying a one-way ANOVA. When indicated by ANOVA, differences between individual groups were tested by student's t-test (two sided) based on the mean of square residual from the ANOVA in question.

3. Results

The treatment of dams with diazepam during GD 14 to 20 did not affect the body weight of their offspring at the age of 6 weeks (Table 1). The relative organ weights were not affected either (Table 2).

An extensive histopathology screen did not reveal any treatment-related microscopic changes in the tissues of the weanling rats examined (Table 3). Lymphoid depletion was only observed in four isolated cases. In one animal, the dam of which had received 1.25 mg/kg/day of diazepam by repeated subcutaneous injection, lymphoid depletion was observed in the T-cell areas of the spleen (PALS, periarteriolar lymphoid sheath), and the paracortical area of both the mandibular and popliteal lymph node. In another animal out of the same group, lymphoid depletion was only seen in the spleen. In the group where the dams were orally treated with 1.25 mg/kg/day of diazepam, lymphoid depletion was observed in the mesenteric lymph node of one animal. After oral administration of 30 mg/kg/day lymphoid depletion was once observed in the popliteal lymph node (Table 3).

No changes in hematological parameters were found (Table 4). The differential counting did not reveal any abnormalities in the distribution of subpopulations of nucleated leukocytes in the circulating (Table 5). The number of nucleated leukocytes in the femur bone marrow was not affected either (Table 6).

FACScan analysis did not reveal any effects on the number of B-cells, or CD4-, CD8- and CD3-positive T-cells in the spleen (Table 7). The NK-cell activity was reduced in splenic lymphocyte cultures obtained from the offspring of dams which were orally treated with 30 mg/kg/day of diazepam. This effect was particularly prominent at the 25 : 1 (target : effector) ratio, but did not reach statistical significance (Table 8). In the lymphocyte stimulation test, no treatment-related changes were found (Table 9). Total serum immunoglobulin levels were not affected either (Table 10). The primary immune response to SRBC remained unchanged (Table 11).

Subcutaneous treatment of dams with 1.25 mg/kg/day of diazepam tended to decrease the host resistance of the offspring to infection with *T. spiralis* parasites as judged by a non-significant increase in larval burden in tongue preparations (Table 12) The number of *T. spiralis* larvae in tongue samples was not changed upon oral treatment (Tables 12). No differences were observed in the severity of the inflammatory reactions surrounding the encapsulated muscle larvae in the tongue (Table 13). The number of *T. spiralis* larvae in eviscerated carcasses was non-significantly increased in the offspring of orally treated dams, while no effect was observed upon subcutaneous dosing (Table 14). The anti-*T. spiralis*-specific IgE antibody response was significantly increased in the offspring of dams which received 1.25 or 30 mg/kg/day of diazepam via the oral route. The anti-*T. spiralis*-specific IgM, IgG and IgA antibody responses showed no treatment-related changes (Table 15).

4. Discussion

In this study, the immunotoxic potential of diazepam was evaluated in the offspring of rats which received this pharmaceutical either via the subcutaneous or oral route. A dose of 1.25 mg/kg/day of diazepam was administered via both routes. In addition, a considerably higher dose of 30 mg/kg/day was used employing the oral route of administration only. The latter dose was incorporated in the study design to allow a comparison with a previous pilot experiment, in which we could not unequivocally detect effects of *in utero* exposure to diazepam at maternal dose levels up to 30 mg/kg/day administered orally on the developing immune system of neonatal rats. At the 1.25 mg/kg/day dose level employed subcutaneously, Schlumpf and colleagues demonstrated that in their hands diazepam administered during GD 14 to 20 caused impaired host resistance to *T. spiralis* parasites in the offspring (Schlumpf et al., 1994). In an earlier study, using non-functional parameters of the immune response, we have observed some immunotoxic effects, but could not ascribe these to exposure due to the lack of dose-response relationships (De Waal et al., 1998). In the experiment we presently report on, we performed various immune function tests (including the *T. spiralis* host resistance assay) in addition to an immunotoxicity screen of non-functional parameters.

The non-functional parameters did not reveal any immunotoxic potential. Out of the immune function assays, only the NK-cell activity assay and the *T. spiralis* host resistance assay showed treatment-related effects. The secondary antibody response to SRBC could not be evaluated because the weanling animals studied did not yet develop IgG antibodies.

The NK-cell activity showed some reduction in splenic lymphocyte cultures obtained from the offspring of dams which were treated orally with 30 mg/kg/day of diazepam. This effect, however, did not reach statistical significance. In contrast, the NK-cell activity tended to be increased upon subcutaneous administration of diazepam. Again, the latter effect was not statistically significant.

The anti-*T. spiralis*-specific IgE antibody response was significantly increased in the offspring of dams which received 1.25 or 30 mg/kg/day of diazepam via the oral route. This change may suggest a stimulation of immune responses. Alternatively, the results may suggest an increase in circulating *T. spiralis* antigen and therefore a decrease in host resistance. This could not be confirmed by a change in the larval burden in tongue preparations. The yield of *T. spiralis* larvae in eviscerated carcasses showed some increase upon oral dosing, however, this change was not statistically significant and was not dose-proportional. It is doubtful whether the increase in anti-*T.spiralis* IgE response upon oral dosing is causally related to treatment, because the control IgE levels in the offspring of vehicle-treated dams were remarkably low upon oral administration as compared with those observed after subcutaneous dosing. This also applies to the control anti-*T.spiralis* IgM, IgG and IgA levels. The reason for this apparent difference in control anti-*T.spiralis* antibody levels measured upon oral *versus* subcutaneous administration is unknown.

Besides the changes in NK-cell activity and anti-*T.spiralis* IgE antibody response no other effects were seen in the offspring of dams which were treated by the oral route. Subcutaneous *in utero* exposure to diazepam resulted in a trend suggesting a decrease in host resistance to infection with *T.spiralis* parasites as judged by a non-significant increase in larval burden in tongue preparations. Apparently, either oral or subcutaneous prenatal diazepam treatment caused signs of diminished immunocompetence in *T.spiralis*-infected weanling rats. These

results favour further investigations to demonstrate the usefulness of extending the current OECD 414 protocol by incorporating immune parameters.

Most microscopic lesions observed in this experiment are considered to belong to the background histopathology of the rat strain used. Occasionally, lymphoid depletion was observed in the spleen and/or lymph nodes in the offspring of diazepam-treated dams. The low incidence of lymphoid depletion, however does not warrant the conclusion that this phenomenon was caused by the diazepam treatment.

Our results employing the *T.spiralis* host resistance model are different from those reported by the group of Schlumpf (Schlumpf et al., 1994a). They treated rats with 1.25 mg/kg/day subcutaneously from GD 14 to 20. The offspring was infected with *T.spiralis* larvae at the age of 8 weeks. Six weeks later, the number of muscle larvae was significantly increased as determined in digested carcasses and by morphometric analysis of the tongue, when compared with offspring of vehicle-treated dams. Moreover, the anti-*T. spiralis*-specific IgG antibody titer was significantly decreased and the anti-*T.spiralis*-specific IgA antibody titer was increased in the prenatally diazepam-treated group. In our hands, however, the effects of *in utero* exposure to 1.25 mg/kg/day of subcutaneously administered diazepam on the developing immune system seem to be less prominent than those observed by Schlumpf and coworkers (1994a). This apparent discrepancy with our results may be due to the rat strain used (i.e. Long Evans rats in the experiment by Schlumpf and coworkers *versus* Wistar rats in our experiment) and/or the time point of *T.spiralis* infection (i.e. 8 weeks after birth in the experiment by Schlumpf and coworkers versus 6 weeks in our experiment). It is not readily apparent, however, why these differences in rat strain and timing of infection would have an impact on the outcome of the experiments.

In conclusion, this experiment does show some marginal immunotoxic effect on the offspring of dams exposed to diazepam during pregnancy. The results also indicate that the immunotoxic activity of diazepam is not very severe. In retrospect, it would have been more advantageous if we would have selected a more potent immunosuppressant to study inclusion of parameters for immunotoxicity in reproduction toxicity studies. Notwithstanding this notion, the present data warrant further research to test the usefulness of extending the current OECD 4141 testing protocol by introducing immune parameters.

Table 1. Effects of prenatal diazepam treatment on body weight of weanling rats (mean ± SD[1])

Subcutaneous administration		Oral administration		
Dose level (mg/kg/day)				
0 (n = 9)[1]	1.25 (n = 10)	0 (n = 9)	1.25 (n = 9)	30 (n = 10)
90 ± 8	88 ± 10	90 ± 8	88 ± 8	83 ± 5

[1] Number of animals (in parentheses)

Table 2. Effects of prenatal diazepam treatment on relative organ weights (mg/g body weight) of weanling rats (mean ± SD)

Organ	Subcutaneous administration		Oral administration		
	Dose level (mg/kg/day)				
	0 (n = 9)[1]	1.25 (n = 10)	0 (n = 9)	1.25 (n = 9)	30 (n = 10)
brain	17 ± 2	18 ± 2	17 ± 2	18 ± 2	18 ± 1
liver	45 ± 3	44 ± 3	44 ± 3	43 ± 3	44 ± 4
testes	6.8 ± 0.7	6.7 ± 0.4	6.5 ± 0.7	6.7 ± 0.6	6.5 ± 0.6
lung	7.1 ± 0.6	7.1 ± 0.6	7.0 ± 0.7	7.1 ± 0.4	7.2 ± 0.6
kidneys	10.8 ± 0.5	10.5 ± 0.6	9.6 ± 1.9	10.2 ± 0.3	10.2 ± 0.9
heart	4.9 ± 0.5	4.8 ± 0.4	5.0 ± 0.4	4.8 ± 0.4	4.8 ± 0.5
pituitary gland	0.04 ± 0.02	0.04 ± 0.00	0.04 ± 0.01	0.04 ± 0.01	0.04 ± 0.01
adrenals	0.26 ± 0.04	0.27 ± 0.05	0.26 ± 0.03	0.25 ± 0.02	0.27 ± 0.04
thyroid	0.07 ± 0.02	0.07 ± 0.02	0.06 ± 0.01	0.07 ± 0.02	0.07 ± 0.01
spleen	4.0 ± 0.5	4.4 ± 0.9	3.8 ± 0.5	3.7 ± 0.5	3.6 ± 0.3
thymus	4.0 ± 0.4	4.3 ± 0.5	4.1 ± 0.6	4.0 ± 0.6	3.9 ± 0.5
mandibular lymph node	1.3 ± 0.3	1.4 ± 0.4	1.6 ± 0.4	1.4 ± 0.3	1.5 ± 0.4
mesenteric lymph node	1.4 ± 0.4	1.5 ± 0.4	1.3 ± 0.2	1.4 ± 0.3	1.5 ± 0.3
popliteal lymph node	0.05 ± 0.01	0.05 ± 0.02	0.05 ± 0.01	0.05 ± 0.01	0.04 ± 0.02

[1] Number of animals (in parentheses)

Table 3. Effects of prenatal diazepam treatment on incidence of microscopic lesions in weanling male rats

	Subcutaneous administration		Oral administration		
	Dose level (mg/kg/day)				
Tissue: - observation	0	1.25	0	1.25	30
Adrenal gland: - extramedullary hematopoiesis minimal	(9) 2	(10) 3	(9) 1	(8) 0	(9) 1
Kidney: - cyst minimal - tubular dilatation minimal slight - basophilic tubules minimal slight - mineralisation minimal slight - tubular degeneration minimal	(9) 0 0 1 8 1 1 0 1	(10) 1 2 0 9 0 1 2 0	(9) 1 0 0 9 0 1 0 0	(8) 0 2 0 7 0 1 0 0	(9) 0 3 0 8 1 1 2 0
Liver: - necrosis minimal - inflammatory cell foci minimal - extramedullary hematopoiesis minimal slight	(9) 1 5 8 0	(10) 0 3 6 4	(9) 0 1 9 0	(8) 0 1 8 0	(9) 0 2 9 0
Lung: - crystals minimal - pleural hyperplasia minimal - alveolar haemorrhage minimal - mucocellular plug minimal - osseous metaplasia minimal	(9) 1 1 0 0 0	(10) 0 0 0 0 0	(9) 1 0 1 0 0	(8) 0 0 0 1 0	(9) 0 0 0 0 1

	Subcutaneous administration		Oral administration		
	Dose level (mg/kg/day)				
Tissue: - observation	0	1.25	0	1.25	30
Mandibular lymph node:	(9)	(9)	(7)	(8)	(7)
- sinus histiocytosis					
slight	2	0	1	3	5
moderate	3	8	4	3	1
marked	3	1	2	2	1
- other tissue present					
slight	0	0	0	1	0
moderate	3	1	0	2	1
marked	5	7	6	5	6
- dev. sec. follicles					
minimal	1	2	1	3	1
slight	0	0	0	1	0
moderate	0	0	0	1	0
marked	0	1	0	0	0
- lymphoid depletion					
minimal	0	1	0	0	0
- medullary plasmacytosis					
marked	0	1	0	0	0
Mesenteric lymph node	(9)	(10)	(9)	(8)	(7)
- sinus histiocytosis					
minimal	1	0	0	1	0
slight	3	6	5	4	4
moderate	5	3	4	3	3
marked	0	1	0	0	0
- dev. sec. follicles					
minimal	2	2	1	0	3
slight	4	2	2	1	1
moderate	2	2	4	7	2
marked	0	0	1	0	0
- lymphoid depletion					
minimal	0	0	0	1	0
- medullary plasmacytosis					
minimal	0	2	3	3	2
slight	0	0	2	1	0
Pituitary gland	(6)	(6)	(4)	(7)	(4)
- cyst					
minimal	1	1	0	0	0
slight	0	1	0	0	0

	Subcutaneous administration		Oral administration		
	Dose level (mg/kg/day)				
Tissue: - observation	0	1.25	0	1.25	30
Popliteal lymph node: - sinus histiocytosis	(7)	(9)	(7)	(8)	(8)
minimal	0	0	1	1	0
slight	1	1	3	2	1
moderate	2	6	2	4	2
marked	4	2	0	1	4
- lymphoid depletion					
minimal	0	1	0	0	0
slight	0	0	0	0	0
marked	0	0	0	0	1
Spleen: - extramedullary hematopoiesis	(9)	(10)	(9)	(8)	(9)
slight	0	0	1	5	0
moderate	0	5	7	3	6
marked	9	5	1	0	3
- dev. sec. follicles					
minimal	0	1	1	0	0
slight	0	1	0	0	0
- lymphoid depletion					
slight	0	1	0	0	0
moderate	0	1	0	0	0

Notes:

1. The figures in parentheses represent the number of male pups from which the tissue or organ was examined microscopically.
2. Tissues or organs not examined were either not recovered at autopsy or were lost during histochemical processing. These organs were scored in the PATHOS system as "no sample". In addition, tissues or organs were diagnosed as "sample inadequate" when not all tissue compartments of an organ could be evaluated histologically.
3. No lesions were observed in bone marrow, brain, heart, large intestine, small intestine, oesophagus, Peyer's patches, stomach, testis, thymus and thyroid.
4. The female weanling rats having received 1.25 (n = 1) and 30 (n = 1) mg/kg/day diazepam p.o., respectively, did not show any remarkable microscopic findings.

Table 4. Effects of prenatal diazepam treatment on hematological parameters in weanling rats (mean ± SD)

Parameter	Subcutaneous administration		Oral administration		
	Dose level (mg/kg/day)				
	0 (n = 9)[1]	1.25 (n = 10)	0 (n = 9)	1.25 (n = 9)	30 (n = 8)
WBC (x 10^9/l)	3.1 ± 1.4	3.1 ± 0.3	3.2 ± 1.0	2.5 ± 0.5	3.1 ± 0.8
RBC (x 10^{12}/l)	5.1 ± 0.3	5.5 ± 0.3	5.3 ± 0.1	5.5 ± 0.6	5.4 ± 0.3
Hb (mmol/l)	6.8 ± 0.4	6.8 ± 0.3	6.8 ± 0.2	6.9 ± 0.3	6.8 ± 0.1
Ht (l/l)	0.36 ± 0.02	0.36 ± 0.02	0.36 ± 0.01	0.37 ± 0.02	0.36 ± 0.01
MCV (fl)	70 ± 2	64 ± 3	66 ± 2	66 ± 3	67 ± 4
MCH (fmol)	1.32 ± 0.03	1.23 ± 0.04	1.27 ± 0.04	1.26 ± 0.06	1.27 ± 0.09
MCHC (mmol/l)	19.0 ± 0.4	19.1 ± 0.6	19.2 ± 0.2	19.0 ± 0.3	19.0 ± 0.3
RDW (%)	25 ± 2	26 ± 1	24 ± 2	26 ± 1	25 ± 4
HDW (mmol/l)	1.32 ± 0.05	1.36 ± 0.064	1.38 ± 0.01	1.32 ± 0.06	1.4 ± 0.1
PLT (x 10^9/l)	1081 ± 117	1064 ± 143	1030 ± 99	1063 ± 85	1058 ± 65

[1] Number of animals (in parentheses)

Table 5. Effects of diazepam treatment on distribution of subpopulations of nucleated leukocytes in the circulation of weanling rats (mean ± SD)

Parameter	Subcutaneous administration		Oral administration		
	Dose level (mg/kg/day)				
absolute counts[1]	0 (n = 7)[2]	1.25 (n = 9)	0 (n = 8)	1.25 (n = 9)	30 (n = 8)
Neutrophils	0.30 ± 0.18	0.22 ± 0.07	0.31 ± 0.12	0.17 ± 0.06	0.22 ± 0.08
Lymphocytes	2.7 ± 1.3	2.8 ± 0.8	2.8 ± 0.8	2.3 ± 0.5	2.7 ± 0.7
Monocytes	0.04 ± 0.03	0.05 ± 0.03	0.05 ± 0.03	0.03 ± 0.01	0.05 ± 0.02
Eosinophils	0.010 ± 0.008	0.010 ± 0.010	0.020 ± 0.030	0.010 ± 0.006	0.010 ± 0.007
Basophils	0.010 ± 0.004	0.010 ± 0.003	0.000 ± 0.005	0.000 ± 0.003	0.000 ± 0.004
Large unstained cells	0.020± 0.016	0.020 ± 0.012	0.030 ± 0.023	0.020 ± 0.008	0.03 ± 0.02
relative distribution[3]	0 (n = 9)[2]	1.25 (n = 10)	0 (n = 9)	1.25 (n = 9)	30 (n = 8)
Neutrophils	10 ± 4	7 ± 3	9 ± 2	7 ± 2	7 ± 1
Lymphocytes	88 ± 4	90 ± 3	87 ± 2	91 ± 2	90 ± 2
Monocytes	1.4 ± 0.4	1.5 ± 0.6	1.5 ± 0.5	1.1 ± 0.4	1.5 ± 0.6
Eosinophils	0.5 ± 0.2	0.4 ± 0.3	0.7 ± 0.7	0.3 ± 0.2	0.4 ± 0.2
Basophils	0.2 ± 0.1	0.2 ± 0.1	0.17 ± 0.08	0.13 ± 0.06	0.17 ± 0.08
Large unstained cells	0.8 ± 0.4	0.8 ± 0.4	0.8 ± 0.4	0.9 ± 0.2	1.0 ± 0.5

[1] Results expressed as absolute counts x 10^3/l
[2] Number of animals (in parentheses)
[3] Results expressed as percentage of total WBC

Table 6. Effects of prenatal diazepam treatment on numbers of nucleated leukocytes in femur bone marrow of weanling rats (mean ± SD)

Parameter	Subcutaneous administration		Oral administration		
	Dose level (mg/kg/day)				
	0 (n = 7)[1]	1.25 (n = 9)	0 (n = 8)	1.25 (n = 9)	30 (n = 8)
WBC (x 10^9/l)	11 ± 1	12 ± 4	10 ± 3	12 ± 3	12 ± 3

[1] Number of animals (in parentheses)

Table 7. Effects of prenatal diazepam treatment on lymphocyte subpopulations in the spleen of weanling rats (mean ± SD)

Marker	Cell type	Subcutaneous administration		Oral administration		
		Dose level (mg/kg/day)				
		0 (n = 9)[1]	1.25 (n = 10)	0 (n = 9)	1.25 (n = 9)	30 (n = 10)
MARK-1	B-cell	29 ± 2	30 ± 8	29 ± 4	28 ± 5	29 ± 2
ER-2	$CD4^+$-cell	18 ± 3	16 ± 4	17 ± 2	17 ± 3	18 ± 2
OX-8	$CD8^+$-cell	18 ± 3	17 ± 4	17 ± 3	18 ± 4	17 ± 3
OX-19	$CD3^3$-cell	30 ± 5	32 ± 5	29 ± 5	30 ± 6	32 ± 6

[1] Number of animals (in parentheses)

Table 8. Effects of prenatal diazepam treatment on splenic NK-cell activity in weanling rats (mean ± SD)[1]

Ratio target : effector	Subcutaneous administration		Oral administration		
	Dose level (mg/kg/day)				
	0 (n = 9)[2]	1.25 (n = 10)	0 (n = 9)	1.25 (n = 9)	30 (n = 10)
200 : 1	100 ± 34	111 ± 41	100 ± 27	99 ± 43	74 ± 43
100 : 1	100 ± 38	108 ± 51	100 ± 37	87 ± 50	62 ± 44
50 : 1	100 ± 40	109 ± 40	100 ± 25	82 ± 58	65 ± 51
25 : 1	100 ± 55	134 ± 72	100 ± 39	81 ± 64	43 ± 55

[1] Results are expressed as a percentage of the respective (s.c. or p.o.) controls calculated per whole organ
[2] Number of animals (in parentheses)

Table 9. Effects of prenatal diazepam treatment on mitogen stimulation index of weanling rats splenic lymphocytes (mean ± SD)

Mitogen	Subcutaneous administration		Oral administration		
	Dose level (mg/kg/day)				
	0 (n = 9)[1]	1.25 (n = 10)	0 (n = 9)	1.25 (n = 9)	30 (n = 10)
ConA	54 ± 19	44 ± 22	51 ± 11	57 ± 20	55 ± 10
PHA	44 ± 16	35 ± 17	37 ± 10	37 ± 6	36 ± 8
LPS	2.8 ± 0.6	3.3 ± 1.1	3.4 ± 1.1	3.2 ± 0.9	3.0 ± 0.9

[1] Number of animals (in parentheses)

Table 10. Effects of prenatal diazepam treatment on total serum immunoglobulin levels in weanling rats (mean ± SD)[1]

Isotype	Subcutaneous administration		Oral administration		
	Dose level (mg/kg/day)				
	0 (n = 7-8)[2]	1.25 (n = 9-10)	0 (n = 6)	1.25 (n = 7)	30 (n = 10)
IgM	95 ± 8	115 ± 29	91 ± 18	97 ± 22	91 ± 20
IgG	104 ± 40	80 ± 30	70 ± 43	71 ± 37	105 ± 35
IgA	95 ± 39	78 ± 37	82 ± 22	80 ± 43	72 ± 25
IgE	49 ± 40	83 ± 86	52 ± 76	74 ± 88	48 ± 46

[1] Values are expressed as a percentage of levels of standard serum. [2] Number of animals (in parentheses).

Table 11. Effects of prenatal diazepam treatment on primary and secondary antibody responses to SRBC in weanling rats (^{2}log ELISA titer, mean ± SD)

Isotype	Subcutaneous administration		Oral administration		
	Dose level (mg/kg/day)				
	0 (n = 9)[1]	1.25 (n = 10)	0 (n = 9)	1.25 (n = 9)	30 (n = 10)
IgM, day 7	29 ± 3	24 ± 2	26 ± 3	22 ± 2	25 ± 3

[1] Number of animals (in parentheses)

Table 12. Effects of prenatal diazepam treatment on *T. spiralis* infection in weanling rats: number of *T. spiralis* larvae in tongue samples (mean ± SD)[1].

Subcutaneous administration		Oral administration		
Dose level (mg/kg/day)				
0 (n = 9)[2]	1.25 (n = 10)	0 (n = 9)	1.25 (n = 9)	30 (n = 10)
1.2 ± 0.9	1.8 ± 1.7	1.0 ± 0.3	1.1 ± 0.6	1.2 ± 0.5

[1] Values are expressed per mm^2 of tissue. [2] Number of animals (in parentheses)

Table 13. Effects of prenatal diazepam treatment on *T. spiralis* infection in male weanling rats: relative distribution of inflammatory responses surrounding *T. spiralis* larvae in tongue samples (mean ± SD).

Classification[1] (% of animals)	Subcutaneous administration		Oral administration		
	Dose level (mg/kg/day)				
	0 (n = 9)[2]	1.25 (n = 10)	0 (n = 9)	1.25 (n = 9)	30 (n = 10)
none	64 ± 6	65 ± 11	65 ± 6	60 ± 9	64 ± 5
minimal	26 ± 6	27 ± 9	28 ± 4	33 ± 8	30 ± 4
moderate	6 ± 2	5 ± 3	6 ± 3	4 ± 2	4 ± 2
marked	3 ± 2	2 ± 2	2 ± 1	3 ± 1	2 ± 2
total	100	100	100	100	100

[1] Semiquantitative evaluation of inflammatory responses surrounding muscle larvae:
None = no or a few inflammatory cells present.
Minimal = some inflammatory cells present.
Moderate = muscle larvae surrounded by one or more layers of inflammatory cells.
Marked = muscle larvae almost completely replaced by inflammatory cells.
[2] Number of animals (in parentheses)

Table 14. Effects of prenatal diazepam treatment on *T. spiralis* infection in weanling rats: yield of *T. spiralis* muscle larvae in carcasses (mean ± SD).[1]

Subcutaneous administration		Oral administration		
Dose level (mg/kg/day)				
0 (n = 9)[2]	1.25 (n = 10)	0 (n = 9)	1.25 (n = 9)	30 (n = 10)
88 ± 29	78 ± 52	55 ± 20	80 ± 38	67 ± 20

[1] Results expressed as total larvae count (x 10^3)
[2] Number of animals (in parentheses)

Table 15. Effects of prenatal diazepam treatment on *T. spiralis*-specific antibody responses in weanling rats (2log ELISA titer, mean ± SD)[1]

Isotype	Subcutaneous administration		Oral administration		
	Dose level (mg/kg/day)				
	0 (n = 9)[2]	1.25 (n = 10)	0 (n = 9)	1.25 (n = 9)	30 (n = 10)
IgM	1.9 ± 1.3	1.5 ± 0.5	1.2 ± 0.7	1.4 ± 0.7	0.9 ± 1.0
IgG	9.1 ± 0.6	8.6 ± 2.8	8.1 ± 0.9	9.0 ± 1.2	8.7 ± 1.3
IgA	4.1 ± 2.0	3.9 ± 1.7	2.7 ± 1.0	3.0 ± 1.1	2.3 ± 0.9
IgE	6.2 ± 1.6	5.8 ± 1.9	4.3 ± 1.2	5.7 ± 1.4*	6.6 ± 1.7*

* Statistically significant as compared with 0 mg/kg/day p.o. ($p < 0.05$)
[1] Values are expressed as increases over pooled serum obtained at the day of infection (day 0)
[2] Number of animals (in parentheses)

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References

Basketter DA, Bremmer JN, Kammuler ME, Kawabata T, Kimber I, Loveless SE, Magda S, Pal THM, Stringer DA & Vohr HW (1994). The identification of chemicals with sensitizing or immunosuppressive properties in routine toxicology. *Fd. Chem. Toxicol.*, **32**, 289-296.

De Waal EJ, Piersma AH, Van der Stappen AJ, Verhoef A, De Jong WH & Van Loveren H (1998). Extension of the OECD 414 rat teratogenicity protocol by increasing the duration of treatment and introduction of parameters of immunotoxicity: an exploratory study using diazepam as a model compound. RIVM report 316601003.

Jansen Van 't Land C. & Van der Laan JW (1989). Vergelijking van verschillende benzodiazepinen met betrekking tot de potentie voor sedatie en spierverslapping en werkingsduur in de rat. RIVM rapport 318510.002.

Koëter HBWM (1994). International harmonisation of immunotoxicity testing. *Hum Exp. Toxicol.*, **14**, 151-154.

Moolenbeek C, Ruitenberg E (1981). The Swiss roll: A simple technique for histogical studies of the rodent intestine. *Lab. Animal.*, **15**, 57-59

OECD (1995). Guideline for the testing of chemicals 407. Adopted 27th of July, 1995.

OECD (1996). Proposal for updating guideline 414: prenatal developmental toxicity study. Organisation for Economic Cooperation and Development (OECD), draft document.

Schlumpf M, Ramseier H, Abriel H, Youmbi M, Baumann JB & Lichtensteiger W (1989). Diazepam effects on the fetus. *Neurotoxicology*, **10**, 501-516.

Schlumpf M, Lichtensteiger W & Van Loveren H (1994a). Impaired host resistance to *Trichinella spiralis* as a consequence of prenatal treatment of rats with diazepam. *Toxicology*, **94**, 223-230.

Schlumpf M, Bütikofer EE, Schreiber AA, Parmar R, Ramseier HR & Lichtensteiger W (1994b). Delayed developmental immunotoxicity of prenatal benzodiazepines. *Toxic. in Vitro*, **8**, 1061-1065.

Stahlmann R, Korte M, Van Loveren H, Vos JG, Thiel R & Neubert D (1992). Abnormal thymus development and impaired function of the immune system in rats after prenatal exposure to aciclovir. *Arch. Toxicol.*, **66**, 551-559.

Van Loveren H & Vos JG (1989). Immunotoxicological considerations: a practical approach to immunotoxicity testing in the rat. In *Advances in Applied Toxicology* (eds. Dayan, A.D. & Paine, A.J.), Taylor & Francis Ltd, London, pp. 143-164.

Van Loveren H, Verlaan APJ & Vos JG (1991). An enzyme-linked immunosorbent assay of anti-sheep red blood cell antibodies of the classes M, G and A in the rat. *Int. J. Immunopharmacol.*, **13**, 689-695.

Van Loveren H, Luebke RW & Vos JG (1995). Assessment of immunotoxicity with the parasitic infection model *Trichinella spiralis.* In: Methods in Immunotoxicology, Wiley-Liss, Inc., New York, Volume 2, 243-271.

Vos JG (1982). Screening and unctional tests to detect immune suppression in toxicity studies. In: *Immunologic Considerations in Toxicology, Vol II* (R.P. Sharma, ed). CRC Press, pp 67-78.

Vos JG & Van Loveren H (1994). Developments of immunotoxicological methods in the rat and applications to the study of environmental pollutants. *Toxicol. in Vitro*, **8**, 951-956.

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