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Juvenile toxicity of cyclosporin in the rat

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ABSTRACT

The pups from 32 litters of SD rats were given 0, 1, 3 or 10 mg/kg-d of cyclosporin by oral gavage from 4 to 28 days of age. 10 mg/kg-d resulted in a persistent impairment of the primary antibody response at 10 weeks of age. Indications of systemic toxicity, including the death of 10/64 pups and severely depressed weight gain, were also observed at this dose level. Arteriopathy of the heart and tubular basophilia and edema in the cortico-medullary region of the kidney were observed at 3 and 10 mg/kg-d. In conclusion, while pharmacological effects were seen at all dose levels, the adverse effects of cyclosporin on the development of the immune system in the rat only occurred at a dose level that also induced systemic toxicity.

1. Introduction

Cyclosporin is used as an immunosuppressant to prevent organ transplant rejection in children [1]. The presence of the drug has been demonstrated in the breast milk following treatment of the mother and the drug concentration may attain high levels in the suckling infant [2]. Treatment of rodents with cyclosporin during in utero and post-natal development has been reported to result in morphological and functional abnormalities of the thymus and spleen [3]. Cyclosporin also affects *ex vivo* thymocyte development [4] and transient effects on T-cell maturation have been reported following lactational exposure of rat pups [5]. A previous experiment in our laboratory [6] demonstrated a persistent depression of the primary antibody response in adult rats that had previously been treated with cyclosporin for 4 weeks starting at 4 days of age, but not in rats born to mothers treated during gestation. It has been suggested that the pediatric use of cyclosporin may contribute to autoimmune disease later in life [7]. Fortunately, only discrete transient influences on immune function have been detected in children exposed to cyclosporin [8].

A pediatric evaluation or waiver is now a mandatory component of all new drug applications in North America and Europe [9]; toxicity studies in juvenile animals are an integral part of this assessment [10,11]. The developing immune system of children has been identified as a likely target for the adverse effects of chemicals [12,13] and medicines [14,15]. At present, however, there is no regulatory guidance on how to monitor the development of the immune system during the course of juvenile toxicity assessments. Methods are available for the detection of immune modulation or developmental toxicity resulting in immune depression [16], but more research is needed in order to develop and validate methods for the identification of hazards resulting in an increased susceptibility to allergy or autoimmune disease [17].

The first aim of the present experiment was to identify a no effect level for the adverse effects (NOAEL) of cyclosporin on the development of the immune system and to investigate whether such effects occur at dose levels that are otherwise non-toxic for the juvenile rat. An additional aim was to identify and refine methods suitable for the evaluation of the immune system within the context of regulatory juvenile toxicity studies. The findings of our previous experiment [6] were not particularly relevant to the risk assessment of cyclosporin in children, since only a single dose level was tested, which caused mortality and growth retardation in addition to the immune effects. In the present experiment, multiple dose levels were assessed with the aim of identifying threshold doses for the various manifestations of toxicity in the juvenile rat. The immune effects would become a serious concern only if they occur at dose levels that are otherwise non-toxic in the juvenile. The experiment was designed to focus on the immune endpoints that had already proven to be sensitive to the effects of cyclosporin, in order to limit the number of animals used. In particular, a single functional test of immune function was employed (i.e. the T-dependant antibody response to sheep red blood cells—SRBC) at the expense of other available methods, such as the humoral response to Keyhole Limpet Hemocyanin (KLH) or the delayed-type hypersensitivity response. Additional endpoints were evaluated in order to better characterize the effects seen in the previous study conducted in our laboratory, such as the relative proportion of CD4+/CD8+ double positive T cells. These cells are prevalent in the developing rat but represent only a minor subpopulation of T-lymphocytes in the peripheral circulation

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of the adult [18]. Finally, histopathological examinations were performed in order to detect any changes induced following juvenile exposure to cyclosporin. The dose level of cyclosporin from the previous experiment (10 mg/kg-d) was included as the high dose level in the present experiment in order to evaluate additional parameters (e.g. immature lymphocyte subset types) and perform a more complete toxicological evaluation (e.g. histopathology of the non-immune organs) for comparison with the effects at the lower dose levels (3 and 1 mg/kg-d).

2. Materials and methods

2.1. Test substance

Cyclosporin, Sandimmun®, was obtained from Novartis Pharma (Basel, Switzerland). The drug was dissolved in olive oil (Cooper, Melun, France) at concentrations of 0.2, 0.6 and 2 mg/mL for oral administration to the juvenile rats.

2.2. Test system

Thirty-two lactating Sprague–Dawley female rats (Crl: OFA (SD)—Charles River Laboratories, France), each with a litter of four male and four female pups, arrived on the day after giving birth. The animals were housed in a barrier-protected unit in an AAALAC-accredited facility (22 °C, 12-h light cycle, at least 15 air changes per hour, at least 40% relative humidity). The dams were housed in plastic cages measuring 365 mm × 225 mm × 180 mm with autoclaved sawdust as bedding. After weaning at 21 days of age, the young rats were housed in groups of four of the same sex and dose group in stainless steel mesh cages measuring 555 mm × 350 mm × 200 mm.

2.3. Study design

The pups from 32 litters were given 0, 1, 3 or 10 mg/kg-d of cyclosporin by gavage from 4 to 28 days of age. Each litter comprised one male and one female pup from each treatment group. All of the pups were individually identified by foot tattoo before weaning then by ear tattoo after weaning. Half of the pups in each of the treated and control groups were terminated and necropsied at 4 weeks of age, 1 day after the last dose. The other half of the pups were retained without further treatment for a 6-week maturation period and then necropsied at 10 weeks of age.

The following evaluations were performed: determination of lymphocyte subsets in peripheral blood, spleen and thymus, serum immunoglobulin titres, primary antibody response to SRBC following sensitization, spleen and thymus weight, cellularity and histopathology.

Physical development and functional tests generally used in regulatory pre- and post-natal development studies were performed in this experiment before and after weaning. Blood samples were collected from all pups before termination at weeks 4 and 10 for clinical laboratory determinations.

2.4. Physical and functional development

The pups were examined daily. They were weighed daily from post-natal day (PND) 4 to 10, then on PND 12, 14, 16, 18 and 21 and twice weekly after weaning. Food consumption was recorded twice weekly for each cage of rats from weaning up to termination. Tibia length was measured for each pup on PND 4, 7, 14 and 21. The days of pinna unfolding, incisor eruption and eye opening were recorded for each pup. The surface righting reflex was assessed for each pup on PND 8, the gripping response was assessed on PND 17, and the pupillary and auditory reflexes were assessed on PND 21. Sexual maturation was assessed from the day of vaginal opening for females or balano preputial skinfold cleavage for males.

2.5. Clinical laboratory determinations

Clinical laboratory determinations were performed for each pup shortly before termination (i.e. half of the pups at 4 weeks of age, half at 10 weeks). Blood was withdrawn from the retro-orbital sinus under isoflurane anesthesia just before termination. A standard panel of hematology and serum chemistry parameters was analyzed using an Advia 120 hematology instrument (Bayer Corporation, Tarrytown, NY) and AU640 (Olympus, France).

2.6. Lymphocyte subset determinations in the spleen, thymus and peripheral blood

The blood samples collected for clinical pathology (see above) were also used for lymphocyte subset determinations.

At necropsy of the pups, at either 4 or 10 weeks of age, the spleen and thymus were removed aseptically and representative fragments of each organ were homogenized. A sample of each tissue was then used to prepare a cell suspension of approximately 10^7 cells/mL in RPMI 1640 suspension medium supplemented with 10% fetal bovine serum containing 100 IU/mL penicillin and 100 μg/mL of streptomycin (Bio Whittaker Europe, Verviers, Belgium).

Lymphocyte phenotype analysis was performed by flow cytometry (FACScanto, Becton-Dickinson, Franklin Lakes, NJ) using antibodies purchased from BD Biosciences Pharmingen (San Diego, CA). The following lymphocyte subsets were counted: CD45RA ref. 554884 (B lymphocytes), CD3 ref. 557030 (T-lymphocytes), CD4 ref. 554837 (helper T-lymphocytes), CD8 ref. 558824 (cytotoxic T-lymphocytes), CD3/CD4/CD8 (non-mature lymphocytes) and CD3/CD4/CD25 (activated lymphocytes) with CD25 ref. 554866.

2.7. Immunoglobulin determinations

An indirect ELISA method was used to determine the total serum IgG or IgM concentrations. The diluted serum samples were placed in 96-well plates coated with a goat anti-rat IgG or IgM antibody (ref. 112.005.008, Jackson Immunoresearch laboratories, West Grove, PA and Marm-4, Marm-4, AbCys, Paris, France; respectively). The fixed immunoglubulins were in turn bound to a peroxidase-linked goat anti-rat IgG or anti-rat IgM polyclonal antibody (ref. 3030.05, Southern Biotechnology Associates Inc., Birmingham, Alabama and UPB 91250, Uptima-Interchim, Montluçon, France; respectively). The rat IgG or IgM content of each well was then quantified by colorimetry, following reaction with a peroxidase substrate, using a plate reader (Thermomax, Molecular Devices, Saint-Gregoire, France). The optical density at 450 nm of the samples was calibrated against a standard curve produced by the same method using a standard IgG or IgM solution (ref. Cappel 55951, ICN Pharmaceuticals, Aurora, Ohio and ref. IR473, AbCys, Paris, France; respectively).

2.8. Primary antibody response to SRBC (plaque-forming cell assay)

Four days before termination (weeks 4 and 10), each rat was given an intravenous injection of 4 mL/kg of a suspension of 10^9 mL^{-1} of sheep red blood cells (SRBC, ref. 72141, BioMérieux, Craponne, France). At necropsy, the spleen was weighed and a fragment was used to prepare a cell suspension as described above. The cell suspension was diluted to a concentration of 10^6 cells/mL and 600 μL of this suspension was mixed with 75 μL of a 2×10^9 SRBC suspension. 75 μL of reconstituted guinea pig complement (ref. S-1639, Sigma–Aldrich, St Quentin Fallavier, France) was added. The suspension was then incubated for 1 h at 37 °C in a Cunningham chamber, prior to counting of the plaques formed by each antibody-producing cell.

2.9. Histopathology and immune organ cellularity

The spleen, thymus, mandibular and mesenteric lymph nodes, Peyer's patches, sternum, heart, liver, kidneys, ureters and adrenal glands were sampled from all animals sacrificed at either 4 or 10 weeks of age corresponding to the end of the treatment period and the end of treatment-free period, respectively. The heart, liver, kidneys, spleen and thymus were weighed. The sampled tissues were fixed in 10% neutral-buffered formalin and embedded in paraffin blocks. Decalcification of the sternum was performed in Kristenson solution for 48 h. Standard histopathological evaluation was performed on 4-μm-thick, Haematoxylin & Eosin or Martius Scarlet Blu stained sections. Weighed fragments from the spleen and thymus were homogenized in RPMI 1640 suspension medium prior to counting the cells in an Advia 120 hematology instrument. The total organ cellularity was estimated by multiplying the determined cell density of the homogenized fragment by the total organ weight.

2.10. Statistical analyses

A predefined decision tree was applied to the majority of datasets. The Shapiro–Wilk test and Levene's test were used to test for normality and homogeneity of variances, respectively. Kolmogorov's and Bartlett's tests were used for organ weights. When these tests were non-significant ($p < 0.05$), analysis of variance followed by Dunnett's were used to compare each treated group with the control group. Otherwise, the Kruskal–Wallis test followed by Wilcoxon's test were used for the intergroup comparisons (or the non-parametric Dunn's test for organ weights). Mean cage food consumption was analyzed using only the Dunnett's test. Physical development data were analyzed using the Fisher's exact test, with Bonferroni correction.

3. Results

3.1. Mortality and clinical signs

Increased mortality was observed at the high dose level of 10 mg/kg-d. A total of 10 out of 64 pups (six males and four females) given this dose level died during the first 2 weeks of treatment. Four control pups also died, as did one pup given 1 mg/kg-d and three pups given 3 mg/kg-d.

The majority of pups given 10 mg/kg-d had swelling of the head, hindlimbs and/or lips starting during the second week of treatment. These clinical signs remained evident for three males and four females until 3 weeks after the end of the treatment period.