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Murine neonatal infection provides an efficient model for congenital ocular toxoplasmosis

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ABSTRACT

Congenital infection is one of the most serious settings of infection with the apicomplexan parasite *Toxoplasma gondii*. Ocular diseases, such as retinochoroiditis, are the most common sequels of such infection *in utero*. However, while numerous studies have investigated the physiopathology of acquired toxoplasmosis, congenital infection has been largely neglected so far. Here, we establish a mouse model of congenital ocular toxoplasmosis. Parasite load and ocular pathology have been followed for the first 4 weeks of life. Ocular infection developed slowly compared to cerebral infection. Even after 4 weeks, not all eyes were infected and ocular parasite load was low. Therefore, we evaluated a scheme of neonatal infection to overcome problems associated with congenital infection. Development of infection and physiopathology was similar, but at a higher, more reliable rate. In summary, we have established a valuable model of neonatal ocular toxoplasmosis, which facilitates the research of the underlying physiopathological mechanisms and new diagnostic approaches of this pathology.

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1. Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite occurring worldwide in humans and animals. Although usually asymptomatic in immunocompetent individuals, toxoplasmosis may cause severe disorders in newborns of mothers infected with *T. gondii* during pregnancy (Jones et al., 2003). *T. gondii* infection is an important cause of ocular toxoplasmosis (OT) and may be a potential complication of both acquired (Bosch-Driessen et al., 2002; Silveira et al., 2001) or reactivated congenital toxoplasmosis (CT) (Bosch-Driessen et al., 2002). Retinochoroiditis is the most frequent manifestation of CT (Vutova et al., 2002; Wallon et al., 2004). The major symptoms associated with OT are loss of visual acuity, strabismus, microphthalmia, cataract, retinal detachment, iridocyclitis, nystagmus, glaucoma and choroidal neovascularization (Kodjikian et al., 2006; Mets et al., 1996). The incidence of ocular involvement in congenitally infected and untreated children rises from 20% at birth to more than 82% at adolescence without treatment (Roberts and McLeod, 1999). Antimicrobial treatment *in utero* may reduce OT (Kodjikian et al., 2006), but this is still controversial (Gras et al., 2001).

Several models of experimental acquired OT have been described in mice (Lu et al., 2005), hamsters (Gormley et al., 1999) and rabbits (Garweg and Boehnke, 2006). Despite of their shortcomings, murine models are probably most promising, due to a completed genome project (Jones et al., 2006), numerous knock-out strains and the reproduction of certain features of human OT, and may be suitable for large-scale controlled studies of the pathogenesis of acquired OT (Atmaca et al., 2004).

Few studies have so far been dedicated to congenital OT in murine models (Hay et al., 1984, 1981; Lee et al., 1983). In these studies, congenitally infected mice developed, in adult age, vasculitis, choroiditis, vitritis, retinitis and necrosis in affected eyes. Congenital OT has been also studied in C57BL/6 embryos (Tedesco et al., 2007), where histological analysis of fetal eyes showed alteration of the retina accompanied with an intense inflammatory infiltrate.

We also investigated the pathology following neonatal infection. Two reasons justify this approach as an approximate model of congenital toxoplasmosis. First, the differentiation of the mouse retina occurs during the first 3 weeks after birth in a process very similar to human third trimester retinal development (Connolly et al., 1988; Dorrell et al., 2004). The second, in the usual infection scheme,

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at mid-pregnancy, most fetuses are infected only shortly before birth (Pfaff et al., 2007). Consequently, following neonatal infection, we expect the same kinetics of infection and physiopathology of the immature retina, but with a much higher efficiency.

The aims of our study were to follow congenital OT during the first weeks of life in Swiss-Webster mice, infected with the avirulent type II Prugniaud (PRU) strain of *T. gondii* and to compare this to a neonatal model of infection.

2. Materials and methods

2.1. Mice and parasites

Male and female outbred Swiss-Webster mice, 8–12 weeks of age, were purchased from Centre d'Elevage R. Janvier (Le Genest-Saint-Isle, France). Animals were bred under specific-pathogen-free conditions, according to national and local regulations. Animal studies in our laboratory were approved by national committees (Accreditation No. 67-482-11).

A cyst suspension of the avirulent PRU strain of *T. gondii* was used to infect pregnant females or newborn mice. The cysts were prepared from the brains of Swiss-Webster female mice perorally infected 2 months before. The brain was homogenized in one mL of PBS and cysts numbers determined microscopically in 20 μL of suspension (Brinkmann et al., 1986).

2.2. Congenital infection model (CI)

Female mice were placed in the male's bedding for 48 h to synchronize the oestrus and were then caged by two with one male for one night. The following day was designated as day zero. Conception was ascertained by the observation of the vaginal plug at day zero and an increase of at least 10 g in the body weight of the female mice at day 12 (Freyre et al., 2006). The pregnant females were gavaged with 10 cysts in 100 μL of PBS on day 12 of pregnancy and were then individually placed in a cage. Blood samples from pregnant and infected mice were collected on day 18 of pregnancy and the blood parasitic load was quantified by quantitative real-time PCR, in order to determine the correlation between maternal parasitemia and newborn survival. The newborn mice were not separated from their mother. Death of pups was recorded daily. Pups were sacrificed on day 0, and after 2 and 4 weeks after birth. Eyes and brains were harvested and analyzed by qualitative and quantitative PCR.

To test lactogenic transmission, mice born from infected mothers were replaced by newborn mice from a non-infected mother. Four weeks later, eyes and brain were harvested and analyzed by PCR for *T. gondii* detection.

2.3. Neonatal infection model (NI)

Newborn mice were infected subcutaneously with five cysts in 100 μL of PBS on day 7 post-natal. Pups were sacrificed 4 weeks after infection. Eyes and brains were harvested and analyzed by quantitative PCR.

2.4. Detection of T. gondii in eyes and brain of the pups

DNA was extracted from the whole eye and from 25 mg of brain tissue using the QIAmp DNA tissue Mini Kit isolation kit (Qiagen, Courtaboeuf, France) and stored at −20 °C. As positive control, *T. gondii* tachyzoites (RH strain) were obtained from mouse ascites.

The presence of *T. gondii* in brains and eyes of pups was detected by PCR using the nucleotide sequence of the 18S rRNA gene (Villard et al., 2003). Primers were 5′-GGC ATT CCT CGT TGA AGA TT-3′ (sense) and 5′-CCT TGG CCG ATA GGT CTA GG-3′ (antisense), which amplify a 88 bp fragment. PCR reaction was performed in 50 μL reaction mixture containing 10 μL DNA template, 2 μM of each primer, 200 μM dNTP (Invitrogen), 2.5 mM $MgCl_2$, and 1.25 U Hot Start Taq DNA Polymerase (Qiagen). Amplification was performed on a Perkin Elmer thermo cycler (GeneAmpPCR system™ 2400) by 15 min incubation at 94 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C, 30 s at 72 °C and a final 5 min at 72 °C. PCR products were analyzed by electrophoresis on a 2% agarose gel and visualized on a UV screen using ethidium bromide staining.

Real-time PCR was performed on a LightCycler (Roche Diagnostics) using the DNA Master Hybridization Probes kit (Roche Diagnostics). Primers for amplification were 5′-AGGAGAGATTACA GGACTGTAG-3′ (sense) and 5′-GCGTCGTCTCGTCTAGATCG-3′ (antisense). The two hybridization probes were 5′-CCGGCTTGGCTGC TTTTCCTG-3′, which was labeled at the 5′ end with LC-red 640 and phosphorylated at the 3′ end, and 5′-GAGTCGGAGAGGGAG AAGATGT-3′, which was fluorescein labeled at the 3′ end. PCR was performed in a final volume of 20 μL, with 5 μL template DNA. Samples were first incubated for 10 min at 95 °C, then amplification was performed for 50 cycles as follows: 10 s at 95 °C, 20 s at 58 °C and 20 s at 72 °C.

A dilution series of *T. gondii* DNA from RH strain mixed with solubilized eye tissues was prepared and included in each amplification run. Results were quantified by interpolating the fluorescence signal of each positive sample against the standard curve.

2.5. Histopathology

Pups from infected and non-infected females were sacrificed on week 0, 2, and 4 after birth. For the neonatal infection model, pups were sacrificed on week 4. Eyes were enucleated and immediately preserved in 4% buffered formaldehyde (Labonord, Templemars, France) for a maximum of 6 h. Fixed eyes were embedded in paraffin using an automate (TECK VIP 300 Sakura Finetek France SAS, Villeneuve d'Ascq, France). Each piece was serially cut in 10 sections of 4 μm, hematoxylin–eosin stained with a staining machine (DRS 2000, Sakura Finetek France SAS). Ten sections per eye were microscopically analyzed at a first round. In case of pathological findings, all the neighboring sections were examined.

2.6. Statistical analysis

Fisher's and Student's tests were used for statistical analyses and p values below 0.05 were considered to denote significant differences.

3. Results

3.1. Offspring viability

In the congenital infection model, only 223 out of 565 (39.5%) newborn mice survived. The others were either stillborn (24%) or died in the first week after birth (36%). *T. gondii* was detected by PCR in all dead mice. At birth, maternal blood parasitemia was compared to the percentage of surviving newborns (Table 1). Percentages of surviving mice were not related to maternal parasitemia. In the neonatal infection model, all infected pups survived.

3.2. Eye infection and parasitic load

3.2.1. Congenital infection model

After birth, parasite detection in the eye increased with the age of pups (X^2 test, $p < 0.001$). A pup was deemed to be affected with OT when at least one eye was tested positive by PCR. Groups of 20 pups were studied for each age (cumulative data from three differ-

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