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Characterization of *Leptospira* infection in suckling and weaning rat pups

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ABSTRACT

Rats are known to be the most important reservoirs of *Leptospira* spp. However, the leptospiral dose and age at which rats become resistant to *Leptospira* infection are not yet well elucidated. Aimed to characterize leptospirosis in rat pups, we found that suckling pups (4-, 7-, and 14-day old) are susceptible to leptospires and resistance starts from the weaning age (23-day old). Susceptibility of rat pups was also affected by the infecting dose of the organisms. Jaundice, decrease in body weight, and neurological symptoms prior to moribundity was evident in infected suckling pups. However, 23-day-old infected pups did not manifest any pathological changes and were able to survive the infection similar to adult rats. Based on these results, we propose the suckling rat pup as a novel animal model of human leptospirosis to investigate pathogenesis, development of host resistance, and the mechanisms involved in rats becoming maintenance hosts for leptospires.

1. Introduction

Leptospirosis, caused by pathogenic *Leptospira*, is well known as a worldwide zoonosis, which is endemic in tropical and subtropical areas [1]. Its clinical manifestations make it difficult to differentiate from other tropical infections [1]. In spite of the fact that this disease is spreading from various animals to other animals and to humans, the mechanisms involved in the pathogenesis of *Leptospira* are still largely unknown, making it a major public health problem [1,2].

Leptospira are thin, highly motile and helically coiled bacteria that belong to the family *Leptospiraceae* [1]. Pathogenic leptospires invade the susceptible host's body through abrasions or lesions of the skin or through the mucous membranes. These organisms then spread to all of the organs following circulation through the blood stream [3]. The duration of the bacteremic phase is from 4 to 7 days and would be followed by very mild symptoms or severe illness, which may sometimes lead to death [3,4]. This outcome of infection may be due to direct effects of the pathogen or genetically determined host immune responses [4].

Mild leptospirosis is difficult to distinguish from other infections as it has non-specific symptoms such as fever,

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chills, headache, muscle aches, abdominal pain, and conjunctival suffusion during the acute phase [4]. On the other hand, jaundice, acute renal failure, and bleeding or hemorrhage in the lungs are characteristics of severe illness, known as Weil's disease [1].

Hamsters and guinea pigs have been used as susceptible animal models of leptospirosis [2,5–9]. The use of gerbils [10] and marmoset monkeys [11] in experimental leptospirosis have also been reported. Although mice are considered resistant to *Leptospira* infection, it has been reported that *tlr*4 mutant mice (C3H/HeJ) [12,13] and cyclophosphamide-treated mice [14] are susceptible to this infection but frequency and severity of nephritis is lower in *Inos* knockout mice [15]. However, these mouse models of infection do not mimic the natural susceptibility among rodents. Four commonly used wild-type mouse strains (i.e., A/J, CBA, BALB/c, and C57BL/6) that are known to be resistant to lethal infection with *Leptospira* have developed specific pathologies as outcomes of sub-lethal infections [16].

In 1917, rats were found to be one of the sources of human *Leptospira* infection [17]. Rats are known to harbor the bacteria in their kidneys without showing any symptoms of leptospirosis [2,18,19]. Also, it is a general maintenance host for serovars belonging to serogroups Icterohaemorrhagiae and Ballum [20]. Based on the experiment conducted by Athanazio et al., clearance of leptospires in all tissues of rats, except in kidneys, was found within 9 days after infection [18]. Moreover, the experimentally infected rats were found to excrete high dose of leptospires in the urine (around 10^7 leptospires/ml) [21]. Urine excretion in the environment was thought to be one of the probable causes of *Leptospira* transmission among rats in nature. Although there was a report by Kemenes (1966) showing rat body weight limits in fatal cases of *Leptospira interrogans* infection [22], it is still unclear at what age rats become resistant to *Leptospira* infection and the mechanisms by which rats become maintenance hosts for leptospires. In order to explore these, we aimed to characterize leptospiral infection in suckling and weaning rat pups.

2. Materials and methods

2.1. Rat infection

Pregnant Wistar rats at 14th day of gestation (SLC, Hamamatsu, Japan) were provided with food and drink ad libitum. The time immediately after the birth of pups was day 0. 4-, 7-, 14-, and 23-day old pups were then subcutaneously infected with 10^8, 10^6, 10^4 and 10^2 organisms of *L. interrogans* serovar Manilae strain K64 [23,24]. Low passage (<10×) in vitro sub-cultured organisms were suspended in PBS and used for infection [23] (see Section 3.2 for the number of pups per group). Pups injected with PBS only were used as controls (uninfected). The pups were monitored daily for their body weight and clinical signs of illness such as loss of appetite, decreased or loss of activity, etc. Pups showing continuous weight loss, jaundice, and loss of activity were considered to be moribund, and were euthanized by inhalation of sevoflurane (Sevofrane, Maruishi Pharmaceutical Co., Ltd, Osaka, Japan). Infected and uninfected rat pups were observed until 30 days post bacterial inoculation.

2.2. Total bilirubin

To measure the amount of total bilirubin in sera, the blood was aseptically collected by cardiac puncture of moribund 7-day old pups injected with 10^2 and 10^8 leptospires (lowest and highest doses) as well as uninfected pups. Total bilirubin in serum was measured by using Bilirubin Kit-K (Alfresa Pharma Corporation, Osaka, Japan). This was done by mixing 20 μl of each serum sample with Dyphylline, Diazo and Fehling reagents (200 μl per reagent) and color reaction was measured using spectrophotometer (600 nm).

2.3. Organ culture

Kidneys, spleens, livers, lungs, and brains were collected from 4- and 7-day-old infected pups that became moribund after infection. These organs were aseptically removed, placed in disposable 2.5 ml syringe, and crushed into 4 ml sterile Korthof's medium with 5-fluorouracil (5-FU; 100 μg/ml), and incubated at 30 °C [23,24]. The next day, 500 μl of the culture supernatant was sub-cultured in fresh Korthof's medium and further incubated. The presence or absence of *Leptospira* in the cultures was observed until 60 days.

2.4. Urine

Urine was aseptically collected by massaging the dorsal-caudal area of the pups until urine was excreted. Ten microliter of urine was observed directly under dark-field microscope, and ≤50 μl of urine were cultured in Korthof's medium and observed for 60 days. *Leptospira*-positive urine was determined by the presence of leptospires in direct microscopy and/or in urine culture.

2.5. Quantitative microscopic agglutination test (MAT)

Whole blood was collected from the retro-orbital plexus of surviving 14- and 23-day old infected pups during the 2nd and 4th week post infection. Serum was obtained by centrifuging the whole blood at 3000 rpm for 30 min. The serum samples were then heat inactivated for 30 min at 56 °C, cooled down, and stored at −20 °C until MAT was performed.

MAT was done using pathogenic *L. interrogans* serovar Manila strains K64 and saprophytic *L. biflexa* strain Patoc I as antigens to detect the presence of anti-*Leptospira* antibodies and their titers in the sera of rat pups. MAT was carried out using the quantitative method specified in the World Health Organization (WHO)-International Leptospirosis Society (ILS) Guidance on Human Leptospirosis [25]. Titers greater than or equal to 1:20 were considered significant (MAT-positive) [23].