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Male-specific pulmonary hemorrhage and cytokine gene expression in golden hamster in early-phase *Leptospira interrogans* serovar Hebdomadis infection

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ABSTRACT

Leptospirosis causes severe clinical signs more frequently in men than in women, but the mechanism underlying the gender differences in leptospirosis remains unclear. In this study, petechial hemorrhage was observed in male but not in female hamster lung tissues infected with *Leptospira interrogans* serovar Hebdomadis at 120 h pi, demonstrating that male hamsters were more susceptible to the development of a severe disease upon *Leptospira* infection. No leptospiral DNA was detected in the lung tissues at 120 h pi when pulmonary hemorrhage was observed, indicating that pulmonary hemorrhage is attributable to the immune reactions of the host rather than from the direct effect of leptospires. The upregulation of nitric oxide synthase genes in the hamsters without pulmonary hemorrhage, *inos* and *enos* in female hamsters at 96 h pi and *enos* in male animals without hemorrhage at 120 h pi, may suggest that nitric oxide has a suppressive effect on leptospirosis-associated pulmonary hemorrhage.

1. Introduction

Leptospirosis is a zoonotic disease caused by infection with the pathogenic bacteria *Leptospira* spp. The pathogenic bacteria of *Leptospira* spp. colonize the kidneys of various mammalian maintenance hosts and are excreted in the urine. Humans become infected with leptospirosis mainly by contact with contaminated water or soil through their skin or mucous membrane [1,2].

Leptospirosis is an acute febrile illness, which has a diverse clinical spectrum ranging from a mild influenza-like illness to a severe disease characterized by jaundice, hemorrhage, acute renal failure, and eventually death [1,3]. Leptospirosis-associated severe pulmonary hemorrhagic syndrome, a severe form of the disease characterized by pulmonary hemorrhage, has recently emerged as an important cause of mortality [1,4]. Although most leptospirosis patients are asymptomatic or exhibit mild illness, 5–10% of them can potentially develop a severe form of the disease [5–7].

The inoculum dose of leptospires at the time of infection and the pre-existing immunity due to a previous infection have been considered possible determinants of disease severity [8]. The clinical severity of the disease has also been considered to be associated with specific leptospiral serovars/serogroups. In a previous study, the infections with different *Leptospira* serovar strains resulted in various outcomes in a hamster model of leptospirosis. For example, *L. interrogans* serovar Manilae caused a lethal infection in hamsters with higher induction of inflammatory cytokine gene expression than serovar Hebdomadis [9]. On the other hand, only some of the human patients infected with the same *L. interrogans* strain (clone) developed severe disease [8,10,11], indicating the involvement of genetic determinants of disease severity in patients pertaining to innate or acquired immunity [8].

Several epidemiological studies have shown that leptospirosis is significantly more frequent in male patients than in females

Abbreviations: COX, cyclooxygenases; Ct, cycle threshold; *gapdh*, *glyceraldehyde 3-phosphate dehydrogenase*; LOX, lipoxygenases; NO, nitric oxide; pi, post-inoculation; TE, Tris-EDTA; XO, xanthine oxidase.

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[12–18]. Serosurveys on inhabitants in particular areas also indicated males had a significantly higher risk of infection [19–21]. The higher proportion of men engaged in professions and daily activities with much more opportunity to become infected with leptospires has been considered the reason for this gender difference [7]. In addition, the male dominance in seroepidemiological studies may suggest that males are more susceptible to *Leptospira* infection. Nevertheless, certain seroepidemiological studies have demonstrated equal prevalence of anti-*Leptospira* antibody in men and women in some regions [5,22,23]. These facts suggest that men are considered more susceptible for developing a clinical disease than women. Furthermore, some studies have indicated that men more commonly develop severe leptospirosis [13–15]. Grave manifestations, such as jaundice, renal failure, and hemorrhage in the lungs, stomach, or subconjunctiva as well as on the skin or mucous membranes, were more common in male patients [13]. On the other hand, there is a report on higher risk of women acquiring severe pulmonary hemorrhagic syndrome (SPHS) in Brazil [24]. However, the causes of these gender differences in leptospirosis remain unclear.

In order to gain insights into the mechanism underlying the gender difference in the severity of *Leptospira* infection, we compared the histopathology, leptospiral burden, and expression of cytokine genes in male and female hamsters infected with *L. interrogans* serovar Hebdomadis [9].

2. Materials and methods

2.1. Leptospira strain and culture conditions

In the present study, *L. interrogans* serovar Hebdomadis strain OP84 [9,25] was cultured at 30 °C in liquid-modified Korthof's medium supplemented with 10% rabbit serum [2]. The strain was stored at −80 °C in Korthof's medium containing 10% glycerol and passaged *in vitro* less than three times for use in challenge experiments.

2.2. Experimental infection

Six-week-old male and female specific-pathogen-free golden hamsters (*Mesocricetus auratus*) were used in the present study. The hamsters were subjected to intraperitoneal inoculation with 1×10^6 cells of *L. interrogans* serovar Hebdomadis strain OP84 in the log phase suspended in 500 μl of Korthof's medium. The control group consisted of hamsters inoculated with the same volume of the medium alone. The number of leptospiral cells was determined using a 0.010-mm-deep counting chamber (Nitirin, Tokyo, Japan). For the preliminary experiment, three male and female hamsters inoculated with the aforementioned strain of serovar Hebdomadis were euthanized by isoflurane inhalation at 120 h post-inoculation (pi). Then, six male and female hamsters were inoculated with the strain of serovar Hebdomadis or medium, and three animals of each group were euthanized by isoflurane inhalation at 72 and 96 h pi for the time course experiment. To confirm histopathological changes observed at 120 h pi in the preliminary experiment, eight male and female hamsters inoculated with the Hebdomadis strain and three male and female hamsters inoculated with the medium alone were euthanized at 120 h pi. At the time of dissection, one kidney of a female hamster (animal ID: F4) was abnormally atrophied, which was excluded from this study. The procedures for collection of blood and tissue samples and their storage for histopathological examinations and DNA and RNA extraction were performed as previously described [9]. The paraformaldehyde-fixed tissues were dehydrated and embedded in paraffin, followed by sectioning and staining with hematoxylin and eosin. The slides were scanned in a microscope using 100× magnification, and the presence or absence of petechial hemorrhage in lung tissues and the deposition of urinary casts in renal tubules were recorded. All animal experiments were approved by the Animal Research Committee of the National Institute of Infectious Diseases (Tokyo, Japan).

2.3. DNA extraction

DNA extraction from blood, kidney, liver, and lung tissues was conducted as described previously [9]. The concentration of extracted DNA samples was determined using a NanoDrop Lite Spectrophotometer (Thermo-Scientific, Rockford, IL, USA), and the samples were subsequently diluted to a concentration of 100 ng/μl using Tris-EDTA (TE) buffer (pH 8.0).

2.4. Total RNA extraction and cDNA synthesis

Total RNA extraction and cDNA synthesis from blood, kidney, liver, and lung tissues were conducted as reported earlier [9]. The concentration of cDNA was adjusted to 100 ng/μl with RNase-free water for relative quantification ($2^{-\Delta\Delta Ct}$ method) or with TE buffer for absolute quantification.

2.5. Quantification of leptospiral DNA in hamster tissues

Real-time PCR was conducted for absolute quantification of *flaB* encoding leptospiral flagellin for detection of leptospiral DNA in hamster tissues as previously described [9]. A standard curve for quantification was generated using serially diluted genomic DNA of known concentrations, extracted from the serovar Hebdomadis strain OP84. The hamster housekeeping gene *glyceraldehyde 3-phosphate dehydrogenase* (*gapdh*) was simultaneously quantified for verifying the quality and quantity of the extracted DNA. The results were expressed as genome equivalents per microliter of whole blood or per 200 ng of DNA from other tissues. The experiments were performed in duplicate using two independently extracted DNA samples from the blood and lung tissue of infected hamsters. The number of genome equivalents in the reaction mixture was calculated as reported elsewhere [9]. Statistical comparison of the copy numbers of the leptospiral genome observed in infected male and female hamster tissues was conducted using *F*-test and *t*-test.

2.6. Comparison of cytokine gene expression in hamster tissues

Quantification of the expression of the following 13 hamster cytokine genes was conducted: *cox2, ifngamma, il1beta, il2, il4, il6, il10, inos, ip10, mip1alpha, tgfbeta,* and *tnfalpha* [9] and *enos* (forward primer: 5′-CAGCAGCACTGATGGAGATGTC-3′ and reverse primer: 5′-CCAAGAGGATACCAGTGGATCTG-3′, designed in this study from the sequence of AJ863053). For this purpose, the comparative cycle threshold (Ct) method ($2^{-\Delta\Delta Ct}$ method) was applied using *rpl18* as a calibrator gene, or absolute quantification was performed through a standard curve generated by amplifying serial dilutions of known concentrations of plasmids containing each target sequence [9]. The relative gene expression was calculated as the ratio of the level of expression in infected to control hamsters. The experiments were performed in duplicate using two independently extracted RNA samples from the blood and the lung tissue of the hamsters. The cytokine gene expressions in the tissues of male and female hamsters were statistically compared using *F*-test and *t*-test.