Exploratory Study on Pathogenesis of Far-Eastern Spotted Fever

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Abstract. Far-eastern spotted fever is an emerging disease caused by Rickettsia heilongjiangensis, a tick-borne obligate intracellular bacterium. In this study, R. heilongjiangensis was used to infect BALB/c mice by inoculation of retro-orbital venous plexus to imitate a blood infection caused by tick biting. We found that R. heilongjiangensis rapidly entered the circulation for systemic dissemination and the pathogen existed in liver, spleen, lungs, and brain of the mice at least 9 days post-infection (p.i.). Severe pathological lesions were observed in liver, lungs, and brain at Day 6 p.i. In addition, the elevated levels of inflammatory cytokines, including interferon-γ, tumor necrosis factor, and CC chemokine, were detected in the infected organs at Day 3 p.i. Our results reveal that R. heilongjiangensis may cause an infection in BALB/c mice and the pathological lesions in the infected mice are associated with host inflammatory response induced by R. heilongjiangensis.

INTRODUCTION

Rickettsiae are obligate intracellular bacteria requiring the environment of the host cells for replication. The genus Rickettsia has been classified into three groups: one that includes Rickettsia bellii; the typhus group, which includes Rickettsia prowazekii, the agent of the louse-borne epidemic typhus, and Rickettsia typhi, the agent of the flea-borne murine typhus; the spotted fever group (SFG), which includes more than 20 recognized species that are associated mainly with ticks, but also with fleas and mites.¹

Before 1980, North Asian tick fever caused by Rickettsia sibirica was recognized as the only spotted fever rickettsiosis in Russia and China. In 1992, a rickettsial strain isolated from the blood of a patient with febrile exanthematous illness in Japan was identified as a novel species, Rickettsia japonica, and the rickettsiosis was named as Japanese spotted fever.² Rickettsia heilongjiangensis was first isolated from Dermacentor sylvarum ticks in the northeastern area of China in 1983 and was classified within the R. japonica subgroup of SFG rickettsiae.³ Rickettsia heilongjiangensis was demonstrated to be pathogenic for humans based on serologic findings and pathogen identification.⁴⁻⁵ The disease caused by Rickettsia heilongjiangensis has been named as far-eastern spotted fever (FESF), which includes more recognized species, that are associated mainly with ticks, but also with fleas and mites.¹

Most of the FESF cases have been reported in the summer in the disease-endemic areas,⁴⁻⁶ which coincides with the peak activity of ticks.

To reveal its pathogenic characteristics and pathogenesis of FESF, R. heilongjiangensis was used to infect BALB/c mice, and then the rickettsial burden and pathologic lesions as well as immune and inflammatory responses in the mice were assessed in this study.

MATERIALS AND METHODS

Growth and purification of R. heilongjiangensis. Rickettsia heilongjiangensis (054 type strain), isolated from D. sylvarum in Heilongjiang province of China, was grown in Vero cells in Dulbecco’s minimal essential medium supplemented with 4% heat-inactivated fetal bovine serum and 2 mM L-glutamine for 7–9 days at 33°C in a 5% CO₂.¹⁰ The infected cells were harvested and disrupted by vortexing with glass beads, and the host cell debris was removed by centrifugation at 500 g for 10 min.¹¹ Supernatants containing rickettsiae were centrifuged at 10,400 × g for 30 min.¹² Rickettsial pellet was suspended in K36 buffer,¹³ and then layered on 20–45% linear Renografin gradient for centrifugation at 141,000 × g for 90 min with rotor SW28 (Beckman, Fullerton, CA).¹¹ The light band of the rickettsial layers was collected and subjected to a second round of Renografin gradient centrifugation. The rickettsiae were collected and washed with phosphate buffered saline 3 times and then suspended in culture media. The rickettsiae were determined by a quantitative polymerase chain reaction (qPCR) (see below). The viable organisms were ~95% of the collected organisms that were measured using a bacterial viability kit (LIVE/DEAD BacLightBacterial Viability Kits, Invitrogen, Carlsbad, CA). The absence of other bacteria and fungi was verified by spreading diverse agar plates. The absence of mycoplasma was checked using a mycoplasma test kit (EZ-PCR Mycoplasma Test Kit, Biological Industries, Kibbutz Beit-Haemek, Israel). One part of the purified organisms was treated at 60°C for 30 min and the heat-inactivated organisms were used as controls of the viable organisms.

R. heilongjiangensis infection of mice. Female BALB/c mice (5 weeks of age) were purchased from the Laboratory Animal Center of Beijing in China. The animal usage was approved by the Beijing Administrative Committee for
Laboratory Animals and the animal care met the standard of the committee. Mice were divided into three groups (25 mice for group 1 and 2, 5 mice for group 3). Mice in group 1 were inoculated with 10^5 viable *R. heilongjiangensis* organisms and mice in group 2 were inoculated with an equal number of heat-inactivated organisms by injection of retro-orbital venous plexus (intravenous route). Mice inoculated with phosphate buffered saline were used as a negative control group (group 3). At Days 1, 3, 6, and 9 post-infection (p.i.), 5 mice in group 1 and group 2 were killed and their blood was collected in EDTA tubes and their liver, spleen, lung, and brain tissues were aseptically excised, respectively. Mice in group 3 were killed and their blood and tissue samples were collected at Day 9 p.i. Each tissue sample was divided into two parts, one was stored at -80°C for qPCR analysis of rickettsial load and the other was fixed in 10% neutral formalin for histopathological analysis. Five mice in group 1 and group 2 were bled by tail-cutting at Days 7, 14, and 28 p.i., respectively. Five blood samples/day were mixed and the separated serum sample was used to evaluate the antibody levels against *R. heilongjiangensis*.

**Histopathological analysis.** The formalin-fixed tissue samples were embedded with paraffin and the sections of paraffin-embedded tissues were stained with hematoxylin-eosin to assess the pathological changes in the infected mouse tissues under light microscopy.

**Detection of *R. heilongjiangensis* in blood and tissues.** DNA was extracted from each mouse blood (100 μL) or organ tissue (10 mg) using a DNeasy Blood &Tissue kit (Qiagen, GmbH, Germany). The total DNA of each sample was eluted from the column with 100 μL of elution buffer. The rickettsial DNA copies were measured using qPCR targeting a 98 bp fragment of ompB gene with primers RompB3944 (5′-ATCTGAAGCGGGAGCAATACC-3′) and RompB4041 (5′-CATCAGTATAGAAGGTTTTGCCCATA-3′) and a TaqMan-MGB probe (FAM-TACATTATCAACAGCCTCGTCAP). Reactions were performed in an ABI7300 system (Applied Biosystems, Carlsbad, CA). Each qPCR run included a standard curve of 10-fold serial dilutions of a known concentration of *R. heilongjiangensis* ompB-recombined plasmid that was previously developed in our laboratory (unpublished data).

**Determination of cytokines in tissues after expression of cytokines.** For RNA extraction of tissues, 25 mg of each tissue sample was treated with RNaseasy Midi kit (Qiagen) and the extracted RNA was treated with RNase-free DNase (Qiagen) to remove DNA. Ten nanogram (ng) of each DNA-free RNA sample was used to produce complementary DNA (cDNA) with the oligo (dT) primer and M-MLV reverse transcriptase (TaKaRa, Dalian, China) in qPCR analysis. Briefly, amplification was conducted in a 20 μL volume containing 10 μL of Syber Green PCR Master mixture, 5 μL of cDNA template, 1 μL (10 pmol) of each primer, and 3 μL of H2O. The primers used to amplify genes encoding interferon-gamma (IFN-γ), tumor necrosis factor (TNF), CC chemokine (regulated on activation, normal T cell expressed and secreted, RANTES), and β-actin gene (control) were synthesized according to the sequences described previously. The fold change (FC) in expression of the target gene relative to the β-actin gene was calculated as follows: FC = 2^{ΔΔCt} where ΔΔCt = (Ct_target − Ct_β-actin)sample − (Ct_target − Ct_β-actin)reference. The Ct values were defined as the cycle numbers at which the fluorescence signals were detected, and sample values represented by tissues from viable organism-infected mice and the reference values as tissues from mice inoculated with heat-inactivated organisms.

**Antibody determination.** The Renografin gradient purified *R. heilongjiangensis* was applied by pen point to 12-well microscope slides and the slides were fixed with methanol after drying. The antigen-coated slides were used to detect the antibodies to *R. heilongjiangensis* in sera by indirect immunofluorescence assay (IFA). The serum samples from mice inoculated with viable organisms or heat-inactivated organisms of *R. heilongjiangensis* were diluted from 1:25 to 3,200 for analysis of specific IgG titers by IFA according to our previous description.

**Statistical analysis.** The analysis for each group was performed on at least five samples. Results were expressed as median with 25% and 75% distribution, and minimum and maximum values for qPCR. Cytokine and antibody levels were expressed as mean ± SD. Quantitative data were compared with the Mann-Whitney U test. Differences are considered significant when *P* < 0.05. Statistical analyses were performed using GraphPad Prism version 5.00 for Windows.

**RESULTS**

*R. heilongjiangensis* infection of BALB/c mice. BALB/c mice infected with 10^5 viable *R. heilongjiangensis* organisms by the intravenous route showed decreased activity and ruffled fur from Days 2 to 5 p.i. and they returned to normal physical appearance and activity after 6 days p.i. However, BALB/c mice inoculated with 10^5 heat-inactivated organisms survived without any signs of illness during the course of the experiment. Rickettsemias of BALB/c mice infected with *R. heilongjiangensis* were determined by qPCR. The rickettsial DNA copies were detectable in whole blood from mice infected with viable organisms at Day 1, significantly (*P < 0.05) increased at Day 3 p.i.; the highest levels were determined at Day 9 p.i. (Figure 1A). The rickettsial load in tissues from mice infected with viable organisms was also analyzed by qPCR. The rickettsial DNA copies were detectable in liver (Figure 1B), spleen (Figure 1C), lungs (Figure 1D), and brain (Figure 1E) from mice infected with *R. heilongjiangensis* at Day 1 p.i., respectively, and the highest levels were determined at Day 6 p.i.; the DNA copies in these tissues were significantly (*P < 0.05) increased between Days 1 and 3 p.i. and significantly (*P < 0.05) decreased between Days 6 and 9 p.i.

**Lesions caused by *R. heilongjiangensis*.** Pathological lesions were observed in liver, lungs, and brain of the *R. heilongjiangensis*-infected mice at Day 6 p.i. and the lesions in these organs were much less severe at Day 9 p.i. In liver tissues, inflammatory infiltrates consisting of mononuclear cells and polymorphonuclear leukocytes were focused on the portal area of the liver and slight swelling of liver cells was observed (Figure 2A and B). For lung lesions, interstitial pneumonia was characterized by numerous inflammatory infiltrates of mononuclear cells and polymorphonuclear leukocytes and alveolar interstitial thickening (Figure 2C and D). Microhemorrhages were found in brain parenchyma (Figure 2E and F). In contrast to live *R. heilongiangensis*, heat-inactivated organisms did not induce any hepatic, pulmonary, and cerebral lesions in mice.

**Immune response to *R. heilongjiangensis*.** The humoral immunity was assessed in *R. heilongjiangensis*-infected mice.
Antibodies (IgGs) to *R. heilongjiangensis* were measured in mouse sera by IFA. The specific antibodies were detected in sera from mice infected with viable organisms at Day 7 and progressively increased until Day 21 p.i. (Figure 3). The specific antibodies were also detected in sera from mice inoculated with heat-inactivated organisms, but the antibody levels were significantly ($P < 0.05$) lower than that of viable organism-infected mice.

IFN-γ, TNF, and RANTES transcripts in organs of *R. heilongjiangensis*-infected mice were assessed by qPCR. Their transcripts were significantly increased in liver, spleen, lungs, and brain from viable organism-infected mice at Day 3 p.i. and decreased at Day 6 (Figure 4A, C, E, and G). Though cytokine transcripts were also observed in these organs of mice inoculated with heat-inactivated organisms, the levels were significantly ($P < 0.05$) lower than that of viable organism-infected mice at Day 3, respectively (Figure 4B, D, F, and H).

**DISCUSSION**

Mediannikov and colleagues investigated FESF in the Far East area of Russia and described the characteristics of the disease. Most of the patients naturally infected by *R. heilongjiangensis* had fever, chills, headache, dizziness, myalgias, arthralgias, and anorexia after an incubation period of 4–7 days, but no specific symptoms appeared during the first several days. Almost half of the patients had hepatomegaly accompanied with an increased alanine aminotransferase and/or aspartate aminotransferase activity, indicating that these patients had a liver lesion caused by *R. heilongjiangensis* infection.

Tick-borne SFG rickettsiae are transmitted through the bite of an infected tick. After rickettsial infection in humans, rickettsemia develops with disseminated infection of the vascular endothelium, culminating in meningoencephalitis and interstitial pneumonitis that lead to increased vascular permeability, noncardiogenic pulmonary edema, and even hypotensive shock. In this study, BALB/c mice were infected with *R. heilongjiangensis* by injection of retro-orbital venous plexus. In this murine model, rickettsiae passed the cutaneous immune system by the intravenous inoculation and rapidly disseminated by a systemic endothelial vascular infection. Our results show that rickettsiae were detectable in blood at Day 1 p.i. and the amount of rickettsiae in blood was significantly increased at Day 3 p.i., suggesting a severe rickettsemia had developed in the mice caused by rickettsial survival and growth in vascular endothelial cells. In addition, rickettsiae were loaded in the liver, spleen, lungs, and brain from Days 1 to 9 after *R. heilongjiangensis* infection. The highest level of rickettsial load was observed in these organs at Day 6 p.i. This result
demonstrated that the rickettsiae were rapidly disseminated to multiple organs of the mice, including lungs and brain, the most important organs relating to severe pathologic injury and death of human rickettsiosis. The SFG rickettsiae were identified in vascular endothelial cells in multiple foci in the brain and lung tissues of the patient in a fatal human SFG rickettsiosis.

In *Rickettsia australis*-infected BALB/c murine model, rickettsiosis was developed with progressively severe vasculitis, interstitial pneumonia, and multifocal hepatic necrosis. In this study, *R. heilongjiangensis*-infected BALB/c mice also exhibited most of the features of human spotted fever, including hematogenous dissemination, multifocal inflammatory lesions, and inflammatory infiltrates. Noticeably, the lungs of BALB/c mice were a primary site of the infection and interstitial pneumonitis and cerebral hemorrhages were observed in the infected mice, suggesting that *R. heilongjiangensis* may cause severe rickettsiosis.

In natural SFG rickettsial infection, rickettsiae are initially inoculated by arthropod saliva into blood lakes in the skin generated by the vector during feeding, and the fate and severity of the infection may depend on the path of infection at the primary phase of infection. The first path, from the dermis into capillaries, may lead to rapid systemic spread by infection of the vascular endothelium, whereas the second path, from the dermis into lymphatics of the subcutis, with initial infection of the lymphatic endothelium, may lead to infection of dendritic cells, efficient antigen presentation in lymph nodes and earlier containment of the infection by the adaptive immune response. Under both scenarios, there is a component of systemic endothelial vascular infection that induces the innate immune response. In this study, we also found that *R. heilongjiangensis* infection of BALB/c mice was accompanied by the development of a humoral response. The specific antibodies were detected in sera from mice infected with viable organisms at Day 7 and progressively increased until Day 21 p.i. The specific antibodies appeared concomitantly with suppression of rickettsial infection.

To explore the roles of cytokine in *R. heilongjiangensis* infection, the messenger RNA (mRNA) expression of IFN-γ and TNF was assessed in this study. As a result, IFN-γ and TNF mRNA were highly expressed in the *R. heilongjiangensis*-infected organs at Day 3 p.i. Although proinflammatory factors IFN-γ and TNF are known to protect hosts from intracellular microorganisms, inflammation is by far the most common cause of tissue injury. The severe tissue lesions of the infected organs observed at Day 6 might be induced by the high-expressed IFN-γ and TNF at Day 3 p.i., although their expression declined at Day 6. Additionally, the high-level RANTES transcript was found in liver and spleen at Day 3, particularly in the spleen at Day 6 p.i. (final observed point). The presence of RANTES transcript in the organs suggests that RANTES is associated with the elimination of *R. heilongjiangensis* in bodies because RANTES is involved in granuloma formation and protective Th1 responses.

Cytokines were hypothesized to play roles both in the pathogenesis of vasculopathic rickettsioses and in the immunity against rickettsiae. The significant decline in the mRNA expression of IFN-γ and TNF appeared concomitantly with a marked increase in rickettsial load in the infected organs between Days 3 and 6 p.i., which suggests that the host defenses were suppressed so that rickettsiae grew rapidly during this infection course. Although the expression of IFN-γ and TNF was declined in the *Rickettsia*-infected organs except for the spleen at Day 6 p.i., the rickettsial load in the infected organs was significantly decreased between Days 6 and 9, indicating that the specific antibodies and other immune factors...
that were not measured in this study play important roles in the rickettsial elimination. The hypothetical roles of the various cytokines in infection caused by *R. heilongjiangensis* will be defined by future experiments.

**CONCLUSIONS**

In this study, by intravenous inoculation, *R. heilongjiangensis* established disseminated intracellular infection in BALB/c mice and caused pathological lesions and inflammatory cytokine expression in major organs similar to what is observed in human spotted fever. Our results reveal that *R. heilongjiangensis* may cause an infection in BALB/c mice and the pathological lesions in the infected organs are associated with host inflammatory response induced by *R. heilongjiangensis*.

Received December 23, 2010. Accepted for publication June 6, 2011.

Financial support: This research was supported by a grant (2010CB530200/2010CB530205) from the National Basic Research Program of China and a grant (2008ZX10004-002) from National Science and Technology Major Project (Infectious Disease Control and Prevention) of China.

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