Pathological and Microbiological Studies of Japanese Hare (Lepus brachyurus angustidens) Naturally Infected with Francisella tularensis subsp. holarctica

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(Received 21 June 2009/Accepted 25 August 2009)

ABSTRACT. An adult male hare (Lepus brachyurus angustidens) was discovered in a moribund condition in the bush in the mountains of Aomori prefecture in Japan. Upon gross inspection, many ticks were found on the neck and the external ear regions, and more than half the ticks contained blood in the intestine. The skin around the tick bite wounds was alopecic and mildly thickened. At necropsy, enlargement of the cervical lymph nodes and spleen were observed. Histologically, acute necrotizing splenitis, lymphadenitis, hepatitis, pneumonia, myelitis, adrenalitis, and encephalitis with bacterial organisms were observed. The cutaneous lesions were chronic and cysts had formed in the areas marked by tick bites. Immunohistochemically, the organisms in the skin, liver, spleen, lymph nodes, lungs, adrenal glands, brain, bone marrow, and ticks were positive for F. tularensis antigen. Microbiological and polymerase chain reaction results were consistent with F. tularensis subsp. holarctica. Because the cutaneous lesions were more chronic than those in the visceral organs and F. tularensis was detected in the ticks, we inferred that F. tularensis was transmitted to the hare via tick bites.

KEY WORDS: histopathology, Japanese hare (Lepus brachyurus angustidens), tularemia (yato-byo), Francisella tularensis, F. tularensis subsp. holarctica, F. tularensis subsp. holarctica


Tularemia (yato-byo) is a zoonotic disease caused by Francisella tularensis (F. tularensis), a Gram-negative non-motile, coccobacillus bacterium [26]. F. tularensis has several subspecies, and type A (F. tularensis subsp. tularensis) is considered the most virulent for humans, with an infectious dose of less than 10 colony-forming units and a mortality of 5% to 6% in untreated cases of cutaneous disease [7, 25]. In contrast, type B (F. tularensis subsp. holarctica) does not cause disease in rabbits, and the mortality rate associated with the cutaneous disease in human is less than 0.5% [6, 25]. Of the species in the genus Francisella, only F. tularensis subsp. holarctica were isolated in Japan [10, 17, 27] and approximately 1400 cases of tularemia in humans have been reported. However, the annual incidence has decreased and since the middle of the 1960s, there have been fewer than 10 cases per year [24]. The majority of cases have been associated with contact with infected wild rabbits [23, 24]. Among rabbit species, two subspecies are considered to be chiefly associated with tularemia: Lepus brachyurus angustidens (L. b. angustidens) and L. b. brachyurus [22]. The former is mainly distributed in the northeastern part of the main island of Japan. Although many human cases have been reported in this area, the detailed pathology of L. b. angustidens naturally infected with F. tularensis is not fully understood because clinical signs were not found during necropsy of animals found dead in the field and experimentally infected hares have recovered from the infection without the typical lesions of tularemia [6, 21]. In this report, we describe in detail the pathological and microbiological findings of L. b. angustidens infected with F. tularensis subsp. holarctica.

MATERIALS AND METHODS

Animal: An adult male hare (L. b. angustidens), weighing 2.6 kg, was discovered in a moribund condition in the bush in the mountains of Aomori prefecture in Japan on May 24, 2008. The hare did not run away when approached. Upon manipulation, only slight falling off was observed. Shortly thereafter, the hare ran into the woods. When the observer returned to the same site, the recumbent hare was found. Although the hare was breathing and had a weak pulse, but soon stopped breathing and died. Upon gross inspection, many ticks were found on the neck and the external ear regions, and more than half the ticks contained ingested blood. A V-like laceration was observed on the left external ear (Fig. 1). The skin around the tick bite wounds was alopecic and mildly thickened.

Pathological examination: All the tissues and ticks were fixed in 10% neutral-buffered formalin solution and embedded in paraffin, according to conventional methods. Paraffin sections (3 μm thick) were stained with hematoxylin and eosin (HE). Special stains, including Gram, reticulin silver impregnation, and Giemsa stains, were used to evaluate the presence of infectious agents. To isolate the bacteria, a part of the spleen sample was stored at 4°C until use.

Immunohistochemistry: For detection of the F. tularensis antigens in the tissues, serial sections were deparaffinized
Figs. 1–3. Skin (Fig. 1); Many ticks (arrows) and V-like laceration of the left external ear are observed (Fig. 1). Spleen (Figs. 2, 3); Marked splenomegaly (Fig. 2) and multifocal white spots (Fig. 3) are observed throughout the parenchyma. HE. Bars=2 cm (Fig. 2) and 0.5 cm (Fig. 3).

Figs. 4–7. Skin; Granuloma composed of mononuclear cells and multinucleated giant cells (arrow) are seen in the external ear skin (Fig. 4). Cyst formation in the subcutis and scar formation (*) at the site of a tick bite are observed (Fig. 5). Necrosis and bacterial colonies (arrows) in the keratin layer are observed (Fig. 6). Bacterial colonies are seen within and outside the blood vessels (Fig. 7). HE. Bars=50 μm (Fig. 4), 1 mm (Fig. 5) and 30 μm (Figs. 6, 7).
and the endogenous peroxidase activity were neutralized with 3% hydrogen peroxidase. The sections were incubated overnight at 4°C with monoclonal antibodies (clone M11H7) [16] directed against F. tularensis antigens at dilutions of 1:3,200. The Histofine® Simple Stain MAX-PO (M) (Nichirei Biosciences Inc., Tokyo, Japan) was used as the secondary antibody. Detected antigens were visualized with 3-3'-diaminobenzidine-tetrahydrochloride reagent or 3-amino-9-ethylcarbazole solution (Nichirei Biosciences Inc.). Mayer’s hematoxylin was used for counterstaining.

Tissues from uninfected domestic rabbit was used as control.

Electron microscopy: For electron microscopy, 10% neutral-buffered formalin-fixed skin & spleen, lymph nodes, and liver were cut into 1 mm³ blocks, fixed in 1% buffered osmium tetroxide, and embedded in epoxy resin. Sections (about 70 nm thick) were stained with uranyl acetate and lead citrate, and examined with a transmission electron microscope (H-7000; Hitachi, Tokyo, Japan).

Bacterial isolation and identification: To isolate the bacteria, homogenized spleen tissues were plated on conventional as well as enriched culture media suitable to support growth of Francisella species [6]. All cultures and experiments were performed in level-3 biosafety laboratories according to Committees on Biosafety and Animal Handling and Ethical Regulation of the National Institute of Infectious Diseases, Japan.

To confirm the F. tularensis sequences, real-time polymerase chain reaction (RT-PCR) targeting the fopA gene [9] and multitarget genes [19, 30] was performed on formalin-fixed hare spleen, tick samples, and bacteria isolated from the spleen. DNA was extracted from the formalin-fixed tissues using the DNeasy Blood & Tissue Kit (Qiagen, Dusseldorf, Germany). The cultured bacteria were suspended in 100 μl of Solution I from the SepaGene kit (Sanko Junyaku, Ibaraki, Japan) and incubated at 94°C for 15 min. After incubation, the solution was centrifuged at 13,000 rpm for 10 min and the supernatant was used for RT-PCR. The reaction mixture and primers for each gene were as previously described [9, 19, 30]. RT-PCR amplification was performed as described previously using a LightCycler 330 instrument and LightCycler FastStart DNA Master Hybridization Probe (Roche Diagnostics, Manheim, Germany) [9, 19, 30]. The final PCR products were separated by electrophoresis on a 2.0% NuSieve 3:1 agarose gel (FMC BioProducts, Rockland, ME, U.S.A.). For more specific genetic diagnosis, conventional PCR targeting the 16S rRNA gene [8], fopA gene [11], tul4 gene [28], ppi-helicase gene [13], and RD1 gene [2] was performed on the spleen samples and cultured bacteria. DNA was extracted from the supernatant used for the RT-PCR with the SepaGene kit. The amplification conditions for the PCR were: initial denaturation at 94°C for 5 min, 30 cycles at 94°C for 30 s, 55°C (ppi), 58°C (16S), 60°C (fopA, tul4, RD1) for 30 s, and 72°C for 30 s, followed by a final extension at 72°C for 7 min. The final PCR products were separated by electrophoresis and DNA sequence analysis was performed as for the RT-PCR.

RESULTS

Necropsy: At necropsy, marked enlargement of the spleen (10 × 2 × 1 cm) (Fig. 2), enlarged cervical lymph nodes (1.5 × 1 × 0.5 cm), and many white spots on the liver, spleen (Fig. 3), lymph nodes, and bone marrow were observed. The lungs were edematous and a foam-like secretion was retained in the bronchi, and one well-demarcated nodular lesion (0.7 × 0.7 × 0.5 cm) was present in the right anterior lobe. The pulmonary lymph nodes were mildly swollen.

Histopathology: Histologically, there were tick bite wounds in the primary lesion, accompanied by chronic necrotizing granuloma with bacterial infection. In the skin of the cerebral and external ear, heterophils, lymphocytes, plasma cells, and multinucleated giant cells (Fig. 4) had infiltrated the dermis to the subcutis, and had sometimes formed cystic lesions (Fig. 5) surrounded by connective tissue. The centers of the cysts were filled with red blood cells, plasma material, and cell debris. Bacterial colonies were occasionally observed within the keratin layer of the skin (Fig. 6). In the stroma, collagenolysis, edema, and hemorrhage were observed. Multiple bacterial colonies were found within and outside the small vessels (Fig. 7). There were no histological changes in the ticks, but bacterial colonies were observed not only in the ingested blood but also in the cavity of the intestine without blood. The blood was hemolytic. The lesions of the visceral organs (liver, spleen, lymph nodes, lungs, and adrenal glands), brain, and bone marrow were characterized by necrosis with acute inflammation. Liver changes presented as multifocal acute necrosis with an irregular outline, especially near the portal vein (Fig. 8). The lesions contained amorphous cell debris, necrotic hepatocytes, and mild infiltrations of lymphocytes and heterophils. Multiple bacterial colonies were observed in the sinusoid, necrotic foci (Fig. 9), and in the cytoplasm of hepatocytes and Kupffer cells. Hepatocytes that contained bacteria swollen to 2–3 times the size of uninfected hepatocytes. The spleen and cervical and pulmonary lymph nodes showed massive necrosis (Fig. 10). Many bacteria similar to those in the liver were observed as free cells or colonies in the necrotic foci (Fig. 11) and in the cytoplasm of heterophils and macrophages. Bacterial thrombi were occasionally observed in the lymph nodes. Diffuse pulmonary edema and localized necrotizing lesions were seen in the lungs. Multifocal necrosis with bacteria were found in the cortex of the adrenal glands, but no inflammatory reaction was observed. In the brain, multifocal necrosis, with hemorrhage and bacterial colonies, was observed in the cerebral cortex and midbrain. Multifocal necrosis with bacterial colonies was observed in the bone marrow. There were no histopathological changes in the other organs, including the spinal cord.

The bacteria were clearly stained by reticulin silver impregnation and Giemsa stains, but were not stained by Gram stain.

Immunohistochemistry: Most of the lesional bacteria
found in systemic organs were intensely positive for *F. tularensis* antigen (Figs. 12–15). The bacteria were seen as rods or granules in the cytoplasm of heterophils, monocytes, macrophages, and hepatocytes, and sometimes formed antigen aggregates. Antigen-positive granules were also seen in the cavities of the small vessels and in the cytoplasm of vascular endothelial cells, free or as aggregates. In the ticks, scattered and aggregated antigen-positive cells were also observed in the pool of ingested blood and in the cavity of the intestines (Fig. 16). No immunostaining was seen in the cytoplasm of the intestinal epithelial cells, salivary glands, or genital organs of the ticks.

**Electron microscopy:** Bacteria were found in the cytoplasm of monocytes (Fig. 17), macrophages, heterophils, and hepatocytes. They were round to rod or almond-like in shape, and measured 200–700 nm in length. The bacteria had well-defined borders along the center and their margins. Most bacteria were enclosed by a phagosomal membrane and the others were located in the cytoplasm without a membrane. The centers of the bacteria showed high electron
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density and were surrounded by electron-lucent zones. The electron microscopic characteristics were similar to those previously reported for *F. tularensis* [3, 12].

**Microbiology:** After incubation for two days, colonies were seen only on the cystine glucose HI agar. The bacteria were Gram-negative and had thick capsules. Because according to these results the isolated bacteria were likely to belong to the genus *Francisella*, they were smeared onto cystine glucose HI agar and Eugon chocolate agar, upon which *Francisella* grows well. After incubation for two days, the bacteria had grown on both agars, and were oxidase-negative and catalase-positive. In acid production in cystine trypticase agar base, it was glucose, maltose positive and glycerol, sucrose negative (Table 1). Thus, the characteristic biological properties of the bacteria were similar to those of *F. tularensis* subsp. *holarctica* [6].

The sizes of the visualized PCR bands corresponding to the 16S rRNA (expected size of 1142 bp), *fopA* (expected size of 708 bp), and *tul4* (expected size of 407 bp) genes were identical to those of the *F. tularensis*-positive control (Fig. 18), and the size of the PCR band corresponding to RD 1 (expected size of 1135 bp) and *ppi*-helicase (expected size of 220 bp) were identical to that of *F. tularensis* subsp. *holarctica* (Fig. 19). Based on these results, the organism was finally identified as *F. tularensis* subsp. *holarctica*.

**DISCUSSION**

Although tularemia is mainly a disease of wild lagomorphs and rodents [20], it occurs sporadically among domestic animals [1, 4, 15]. The lesions previously described in domestic animals were necrosis of the lymph nodes, spleen, lung, liver, and bone marrow. In the hare, the pathological findings of tularemia vary. The pathology of tularemia in European brown hares (*Lepus europaeus*) has been described as chronic granulomas with necrosis, particularly in the lungs and kidneys [18, 21, 29]. The granulomas contain heterophils, mononuclear leucocytes, and multinucleated giant cells, as well as calcified areas. In contrast, the postmortem findings in hares (*Lepus timidus*) dying of tularemia in the autumn in Sweden included focal coagulative necrosis in the liver, spleen, lymph nodes, and bone marrow, with high numbers of *F. tularensis* [21]. The lesions in the hare reported in the present study were similar to those of *Lepus timidus*, with the exception of cutaneous, lung, brain, and adrenal gland lesions. Gross and microscopic necrotizing lesions in the spleen, cervical lymph nodes, and liver were the most apparent lesions in the hare in our study. The apparent differences in the pathology observed in various hares could result from differences in the pathogenicity of the different strains of bacteria, their modes of infection, the immune status of the hosts, and the varying susceptibility of the hares.

The immunohistochemical identification of *F. tularensis* in formalin-fixed tissue is valuable for establishing a rapid etiological diagnosis under circumstances where fresh tissues may not be available for the isolation and identification of the organism. In our study, the intensity and localization of positive staining were similar to those previously reported for other animals [4, 14, 31], and most commonly included cellular debris within the necrotic lesions and intra-cellularly in monocytes, macrophages, heterophils and...
hepatocytes. This is the first use of an immunohistochemical assay to localize *F. tularensis* antigen in ticks. Positive staining was localized in the cavity of the intestines of the ticks, but no positive staining was observed in the salivary gland, genital system, or intestinal epithelial cells. These findings suggest that *F. tularensis* is transmitted among the ticks and to hares by tick bites.

The infection is often transmitted by arthropods, including ticks, biting flies, and possibly mosquitoes [5, 26], but it can also be acquired orally, via the respiratory route, by the bites of infected vertebrates, or from direct contact with infected tissue [6, 22]. In the present study, the route of transmission of *F. tularensis* to the hare was not identified, but the cutaneous lesions caused by tick bites were more chronic than those in the visceral organs, and bacterial antigens were detected in both the blood-injected and the noninjected ticks. The cervical lymph nodes were markedly more swollen than the other lymph nodes at necropsy. It is common that the lymph nodes draining the infection site become swollen [28]. Therefore, we assumed that the primary lesions were formed on the skin by tick bites, and that the bacteria in the intestines of the ticks were transmitted to the skin of the hare, and then rapidly spread, either hematogenously or lymphogenously, to the cervical lymph nodes and multiple organs, and infected hare died by acute septicemia.

ACKNOWLEDGMENT. This work was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of science (JSPS: No. 6604–19380171–0044) and by a grant (H20-Shinkou-Ippan-013) of Ministry of Health, Labor and Welfare of Japan.

REFERENCES


