FULL PAPER

Pathology

Pathology of female mice experimentally infected with an *in vitro* cultured strain of *Trypanosoma equiperdum*

Yusuke TANAKA[1]), Keisuke SUGANUMA[2,3]), Kenichi WATANABE[1,3]) and Yoshiyasu KOBAYASHI[1,3])*

[1])Department of Veterinary Medicine, Obihiro University of Agriculture and Veterinary Medicine, Nishi 2-11, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
[2])National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Nishi 2-13, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
[3])Research Center for Global Agromedicine, Obihiro University of Agriculture and Veterinary Medicine, Nishi 2-11, Inada-cho, Obihiro, Hokkaido 080-8555, Japan

ABSTRACT. Dourine, caused by infection with *Trypanosoma equiperdum*, is one of the trypanosomiasis in equids. The clinical course of dourine is long-term, ranging from 1–2 months to several years. Since the pathogenesis of dourine has not yet been elucidated, experimental studies using mouse infection models are needed. Although mice are not susceptible to most *T. equiperdum* strains, some strains can infect mice. Even in such strains, infected mice develop rapidly transient parasitemia and die within 2–8 days. Therefore, mice experimentally infected with these *T. equiperdum* strains are not suitable for mouse infection models to analysis the pathogenesis of dourine. A sequential method of isolating parasites from dourine-affected horses and adapting them to *in vitro* cultures using soft agarose media was recently developed. Various *T. equiperdum* strains adapted to *in vitro* conditions have been established using this technique. We used one of these strains, the *T. equiperdum* IVM-t2 strain. In the present study, *T. equiperdum* IVM-t2 strain inoculated mice developed periodic parasitemia during the experimental period of 60 days. Histopathologically, vaginitis and dermatitis were observed. These findings were comparable to those of dourine-affected horses. Therefore, mice infected with *T. equiperdum* IVM-t2 strain may be a valuable tool for pathological, immunological, and parasitological *in vivo* research, and will contribute to investigations on the mechanisms underlying the disease process and the host-parasite relationship.

KEY WORDS: dourine, histopathology, mouse, parasitemia, *Trypanosoma equiperdum*

Trypanosoma brucei rhodesiense, *T. b. gambiense*, *T. b. brucei*, *T. evansi*, and *T. congolense* are causative agents of trypanosomiasis in humans and livestock [2, 6]. Although mice are not natural hosts of these trypanosomes, various multi-faceted infection models of the disease in mice have been established to date. These mouse infection models have been utilized in pathological and immunological studies to elucidate the pathogenesis of trypanosomiasis [1].

Dourine, one of the trypanosomiasis in equids, is caused by infection with *T. equiperdum* [7, 12, 24]. Infected horses manifest various clinical signs, such as edema of the external genitalia, skin plaques, and neurological signs during the long-term clinical course of 1–2 months to several years [12]. Pathological examinations of infected horses revealed that these clinical signs are caused by inflammation and edema of the genital organs, dermatitis, and peripheral polyneuritis, respectively [10, 13, 20, 24]. However, the pathogenesis of dourine remains largely unknown. Similar to other trypanosomiasis, experimental studies using mouse infection models are needed to elucidate the mechanisms underlying the disease process and the host-parasite relationship in dourine. However, mice are not susceptible to almost all *T. equiperdum* strains [7, 13]. If an infection is established, infected mice rapidly develop transient parasitemia and die within 2–8 days of post infection [5, 8, 15]. *T. equiperdum* strain that infects mice and causes long-term clinical course observed in dourine-affected horses has never been established. Also, detailed histopathology of mice infected with *T. equiperdum* has not been reported.

T. equiperdum strains used in previous studies on experimental infections were prepared by subculturing the blood of horses with parasitemia in mice [8, 13–15]. Suganuma *et al.* recently established a method to isolate *T. equiperdum* from the genital mucosa of

dourine-affected horses and adapt them to *in vitro* cultures using soft agarose media [19]. The usage of *T. equiperdum* strains adapted and grown under *in vitro* conditions provides a more stable and quantitative supply of these parasites for experimental studies. Using this technique, Suganuma *et al.* isolated 5 *T. equiperdum* strains from dourine-affected horses in Mongolia (unpublished data). One of the isolated strains from Mongolian horses, named the *T. equiperdum* IVM-t2 strain, was used in the present study.

BALB/c and C57BL/6 mice are the most commonly used mouse strains for experimental studies on trypanosomes [1]. Previous studies on viral and bacterial diseases indicated differences in susceptibility to pathogens between these two mouse strains [3, 9]. Furthermore, survival time and susceptibility markedly differed between BALB/c and C57BL/6 mice in experimental studies on *T. b. rhodesiense*, *T. b. gambiense*, *T. b. brucei*, *T. evansi*, and *T. congolense* [1].

The susceptibility of BALB/c and C57BL/6 mice to *T. equiperdum* IVM-t2 strain remains unknown. Therefore, we inoculated BALB/c and C57BL/6 mice with the above mentioned strain of *T. equiperdum*. We evaluated parasitemia levels, gross lesions, and histological lesions of mice infected with the *in vitro* cultured strain of *T. equiperdum* for 60 days, and discussed about species differences in these lesions between horses and mice.

MATERIALS AND METHODS

Ethics

The present experiments were approved by the Obihiro University of Agriculture and Veterinary Medicine Committee for Experiments Using Animals (approved number 19–23). All methods were carried out in accordance with International Guiding Principles for Biomedical Research Involving Animals issued by the Council for the International Organizations of Medical Sciences.

T. equiperdum strain

The *T. equiperdum* strain used in the present study was the IVM-t2 strain (*T. equiperdum* isolated at the Institute of Veterinary Medicine from a Töv aimag dourine horse no. 2). This strain was isolated from the genital mucosa of a dourine-affected horse in Mongolia and adapted to soft agarose media using the methods established by Suganuma *et al.* [19].

Experimental design

Eleven 6-week-old female BALB/c and C57BL/6 mice obtained from CLEA Japan Inc. (Tokyo, Japan) were used for experimental infection. Six mice of each mouse strain were intraperitoneally injected with 1×10^6 *T. equiperdum* IVM-t2 strain parasites. As the control group, 5 mice of each mouse strain were intraperitoneally injected with sterilized phosphate-buffered saline, which was used as solvent for trypanosomes. The number of parasites in peripheral blood was counted using a cell counting chamber and blood samples taken from a tail vein under anesthesia by 2.0% isoflurane 3 times a week. In each mouse strain, the parasitemia level was calculated as mean value ± standard deviation. At the blood sampling, body weight was also evaluated. The experimental period was 60 days, and mice that survived through the period were euthanized at 60 days of post infection (dpi) by anesthesia with 5.0% isoflurane and blood drawing from the heart. The liver, spleen, kidney, heart, lung, intestine, uterus, vagina, brain, spinal column including the vertebrae and spinal cord, and sciatic nerve were collected and fixed in 10% neutral buffered formalin. Although the dorsal skin was collected from mice that survived for 60 days, it was not collected from those that died in the early stage of experimental infection.

Histopathological examination

Formalin-fixed samples were routinely processed and embedded in paraffin. Paraffin sections were stained with hematoxylin and eosin (HE) and subjected to a histopathological examination. Fixed spinal columns were washed with tap water for 1 hr, and placed in Anna Morse solution (decalcification solution made of sodium citrate and formic acid) for 48 hr. Decalcified spines were washed with tap water for 12 hr, and sectioned at the seventh cervical and third lumber vertebra for histopathological examination. The degree of inflammation in each section of systemic organs and tissues was evaluated using the following criteria: −=no inflammation, +=mild and focal inflammation, ++=moderate inflammation, +++=severe and diffuse inflammation. The degrees of extramedullary hematopoiesis and hyperplasia of white pulp in the spleen were evaluated. Sections of sciatic nerves were stained with Luxol fast blue (LFB)-HE stain to evaluate the presence or absence of axonal degeneration.

Immunohistochemistry

An immunohistochemical examination using anti-*T. equiperdum* rabbit antisera (1:400, K. Suganuma) as the primary antibody was performed to evaluate the distribution of parasites in each organ and tissue. In addition, to identify the phenotype of inflammatory cells, selected sections were immunostained with anti-CD3 rabbit monoclonal antibody (clone SP7, 1:400, Abcam, Cambridge, UK) and anti-Iba1 rabbit polyclonal antibody (1:500, Wako, Osaka, Japan). Sections were deparaffinized by xylene and hydrated in a series of graded ethanol. Non-specific endogenous peroxidase was blocked with 0.3% H_2O_2 at room temperature for 10 min. Sections were incubated with each primary antibody at 4°C overnight. MAX-PO polymer reagent (Nichirei, Bioscience, Tokyo, Japan) was used as the secondary antibody at room temperature for 30 min. Labeling was visualized by 3,3′-diaminobenzidine, and sections were counterstained by Mayer's hematoxylin. Additionally, selected sections were applied for immunofluorescence for *T. equiperdum*. In immunofluorescence, Alexa Fluor 488-conjugated goat anti-rabbit IgG (1:400, Thermo Fisher Scientific, Hanover Park, IL, USA) was used for secondary antibody. Nuclei were stained with 4′,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA, USA).

RESULTS

Survival rate, transition of body weight, and clinical signs

Five out of 6 (83%) BALB/c mice in the infected group (IG) survived throughout the experimental period, 60 dpi. One BALB/c mouse in IG died during anesthesia for blood collection at 31 dpi. All BALB/c mice in IG and control group (CG) did not show weight loss during the experimental period (Supplementary Fig. 1). Three out of 6 (50%) C57BL/6 mice in IG survived throughout the experimental period. Two mice died and 1 mouse was euthanized because of greatly reduced motility and depression at 12 dpi. These 3 C57BL/6 mice in IG showed severe weight loss compared to other IG mice and CG mice (Supplementary Fig. 1). Two out of the 3 surviving C57BL/6 mice in IG developed redness and alopecia on the dorsal skin from 31 and 43 dpi to 60 dpi, respectively (Fig. 1).

Blood examination

The first peak of parasitemia was detected at 10 and 8 dpi in BALB/c and C57BL/6 mice in IG, respectively. Periodic parasitemia was then observed until 60 dpi in IG of both mouse strains (Fig. 2, Supplementary Fig. 2).

Histopathological examination

The degree of inflammation in each organ and tissue collected from IG mice was summarized in Table 1. Various degrees of vaginitis were observed in all IG mice of both mouse strains. Mild (+) or moderate (++) vaginitis was characterized by perivascular inflammation in the lamina propria and tunica muscularis. In severe (+++) vaginitis, inflammatory cells were diffusely observed at the vaginal lamina propria and tunica muscularis (Fig. 3a). The majority of inflammatory cells were positively stained with Iba-1 (Fig. 3b). Few lymphocytes and plasma cells were also observed. Perineuritis of the sciatic nerve was noted in 6/6 BALB/c mice and 5/6 C57BL/6 mice in IG, and severe inflammation was more frequently detected in C57BL/6 mice of IG (3/6) than in BALB/c mice of IG (1/6). Inflammation of the sciatic nerve was confined to the perineurium and surrounding connective tissues, while inflammatory cells were not found within nerve bundles (Fig. 3c). Mild axonal degeneration was also noted in the sciatic nerve of 2/6 BALB/c mice and 1/6 C57Bl/6 mouse in IG. Edema and diffuse infiltration of macrophages and lymphocytes in the dermis and subcutaneous tissue were observed in dorsal skin presenting alopecia and redness in 2 C57BL/6 mice of IG. The hair follicles and adnexal glands in the inflammatory foci were atrophied and decreased in number (Fig. 3e). Although apparent skin lesions were not grossly observed in BALB/c mice of IG, moderate infiltration of macrophages and lymphocytes into the dermis and subcutaneous tissue of the dorsal skin was noted in 2 out of 5 mice. Hepatitis, characterized by the perivascular infiltration of lymphocytes, macrophages, and plasma cells, was detected in 3/6 BALB/c and 5/6 C57BL/6 mice of IG. Although hepatitis in BALB/c mice was mild and focal, that in C57BL/6 mice was more severe. The necrosis of hepatocytes was also observed in severely affected livers (Fig. 3h). Mild or moderate perivascular inflammation was scattered throughout the kidney, heart, and lung. Although splenitis was not detected, hyperplasia of white pulp was frequently observed in mice of IG (BALB/c: 6/6, C57BL/6: 5/6). No significant changes were noted in the intestines, uterus, brain, or spinal cord of IG mice. In CG mice, inflammation was not detected in systemic organs or tissues, and axonal degeneration of the sciatic nerve and white pulp hyperplasia of the spleen were also not observed. Extramedullary hematopoiesis was noted in CG mice (BALB/c: 1/5, C57BL/6: 4/5) and IG mice (BALB/c: 6/6, C57BL/6: 4/6). In both mouse strains, the degrees of extramedullary hematopoiesis of IG mice were more severe than those of CG mice.

At the time of necropsy, parasitemia was observed in 2/6 BALB/c and 5/6 C57BL/6 mice in IG. In IG mice with parasitemia,
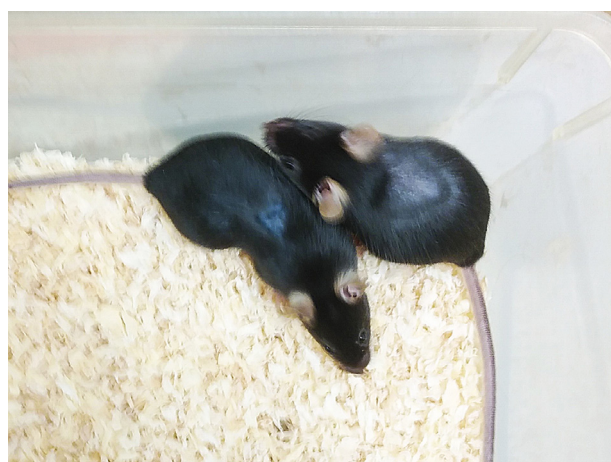

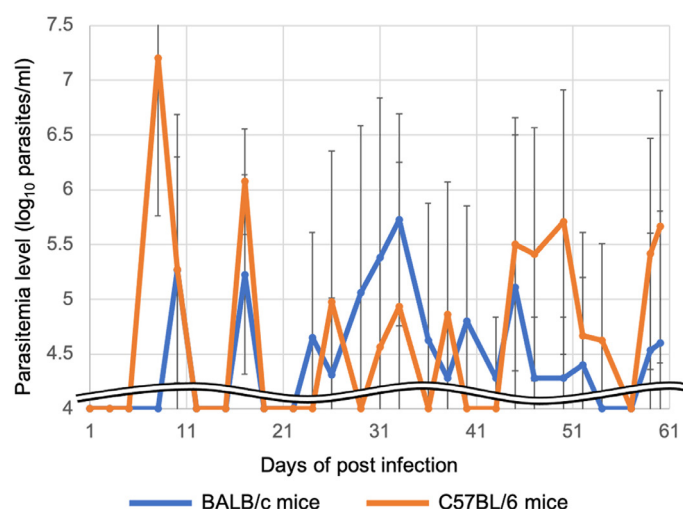**Fig. 1.** C57BL/6 mice in the infected group. Alopecia and redness are observed on the dorsal skin.

Fig. 2. Average parasitemia (mean value ± standard deviation) in the infected group of each mouse strain (log 10 parasites/ml, limit of detection is 1×10^4 parasites/ml). Periodic parasitemia is observed in both mouse strains.

Table 1. Degree of inflammation in BALB/c and C57Bl/6 mice in the infected group

	Infected BALB/c mice				Infected C57BL/6 mice			
	-	+	++	+++	-	+	++	+++
Liver	3/6	3/6	0/6	0/6	1/6	2/6	2/6	1/6
Kidney (interstitium)	2/6	4/6	0/6	0/6	4/6	2/6	0/6	0/6
Kidney (renal pelvis)	3/6	3/6	0/6	0/6	4/6	1/6	1/6	0/6
Heart (epicardium)	2/6	4/6	0/6	0/6	4/6	2/6	0/6	0/6
Heart (endocardium)	0/6	0/6	0/6	0/6	5/6	1/6	0/6	0/6
Lung	2/6	4/6	0/6	0/6	3/6	2/6	1/6	0/6
Sciatic nerve (perineurium and surrounding connective tissue)	0/6	1/6	4/6	1/6	1/6	0/6	2/6	3/6
Vagina	0/6	3/6	2/6	1/6	0/6	2/6	2/6	2/6
Skin (dermis and subcutaneous tissue)	3/5	0/5	2/5	0/5	1/3	0/3	0/3	2/3

-, no inflammation; +, mild and focal inflammation; ++, moderate inflammation; +++, severe and diffuse inflammation.

parasites were detected extracellularly at the inflammatory foci of the heart (BALB/c: 0/2, C57BL/6: 1/5), perineurium and perineural connective tissue of the sciatic nerve (BALB/c: 2/2, C57BL/6: 5/5), vagina (BALB/c: 2/2, C57BL/6: 5/5), and dermis and subcutaneous tissue (BALB/c: 1/1, C57BL/6: 2/2). Even in IG mice without parasitemia, parasites were present in the heart (BALB/c: 2/4, C57BL/6: 0/1), perineurium and perineural connective tissue of the sciatic nerve (BALB/c: 3/4, C57BL/6: 0/1), vagina (BALB/c: 0/4, C57BL/6: 1/1), and dermis and subcutaneous tissue (BALB/c: 1/4, C57BL/6: 1/1). With or without the detection of parasitemia at the time of necropsy, a large number of parasites were present in the perineural connective tissue of BALB/c and C57BL/6 mice in IG (Fig. 3d) and the dermis and subcutaneous tissue of the dorsal skin with alopecia and redness in C57BL/6 mice of IG (Fig. 3f, 3g). The presence of trypanosomes in each organ was also confirmed by immunohistochemistry and immunofluorescence using anti-*T. equiperdum* rabbit antisera (Fig. 3d, 3g). Positive reactions were not observed in any organs or tissues in CG mice.

DISCUSSION

T. equiperdum infects equids and causes dourine. The clinical course of dourine is long-term, ranging from 1–2 months to several years [12, 22]. In experimental infection using mice, the majority of *T. equiperdum* strains did not infect mice [7, 13]. In some studies, *T. equiperdum* infected mice developed rapidly transient parasitemia, and died within 2–8 days from infection [5, 8, 15]. In the present study, 5/6 BALB/c (one mice died in an anesthesia accident) and 3/6 C57BL/6 mice in IG survived throughout the experimental period, and periodic parasitemia was also observed throughout the experimental period in IG of both mouse strains. Therefore, these results indicate that BALB/c and C57BL/6 mice were infected with the *T. equiperdum* IVM-t2 strain for 60 days. Parasites were observed histologically in the heart, perineurium, perineural connective tissue, vagina, dermis, or subcutaneous tissue of *T. equiperdum* IVM-t2 strain infected mice with parasitemia at the time of necropsy. Even in mice with parasitemia levels below the detection limit at the time of necropsy, parasites were also detected in these organs and tissues. Therefore, the heart, perineurium, perineural connective tissue, vagina, dermis, and subcutaneous tissue may have tissue tropism for the *T. equiperdum* IVM-t2 strain. These clinical and pathological features have also been observed in BALB/c and C57BL/6 mice infected with *T. equiperdum* IVM-t2 strain in other experiments (unpublished data).

In the transmission process of *T. equiperdum* among horses, it is considered that trypanosomes invade the genital mucosa and are carried to visceral organs through the blood stream [7, 12]. In the present study, trypanosomes were injected intraperitoneally, and they were subsequently detected in the peripheral blood. Although the route of infection was different from natural infection among horses, mice infected by intraperitoneal injection were thought to reflect the pathological condition of dourine-affected horses with parasitemia.

In the present study, the survival rate of C57BL/6 mice in IG was lower than that of BALB/c mice in IG. In a previous experimental study using mice infected with *T. b. brucei*, *T. congolense*, or *T. evansi*, the death of trypanosomes infected mice is thought to be caused by systemic inflammatory response syndrome (SIRS), renal failure, anemia, or invasion of parasites to central nervous system (CNS) [1]. In the present study, the lesions of kidney were mild, and CNS was free from lesions. On the other hand, severe extramedullary hematopoiesis was detected in the spleen of IG mice. In addition, inflammation in the systemic organs was more severe in C57BL/6 mice than in BALB/c mice. Therefore, in the present study, anemia and SIRS are possibly the cause of death of 3 C57BL/6 mice in IG. In the infection of *T. b. brucei*, IFN-γ produced by Th-1 cells has an important role in immune response to trypanosomes and development of anemia [18]. The predominant helper T cells in the blood and spleen of BALB/c mice are Th-2 cells, while those in C57BL/6 mice are Th-1 cells [4, 11, 16, 23]. The differences of survival rate of BALB/c and C57BL/6 mice in the present study might be due to the differences in dominant helper T cells.

Clinical signs observed in dourine-affected horses include local edema in the genitalia and mammary glands, skin plaques, anemia, and neurological signs, such as paralysis of the hind limbs and facial muscles [12]. Although anemia was reported in a previous experimental infection with *T. equiperdum* strains using mice, no other signs were observed [8, 15]. Since severe extramedullary hematopoiesis was detected in *T. equiperdum* IVM-t2 strain infected BALB/c and C57BL/6 mice, IG mice may
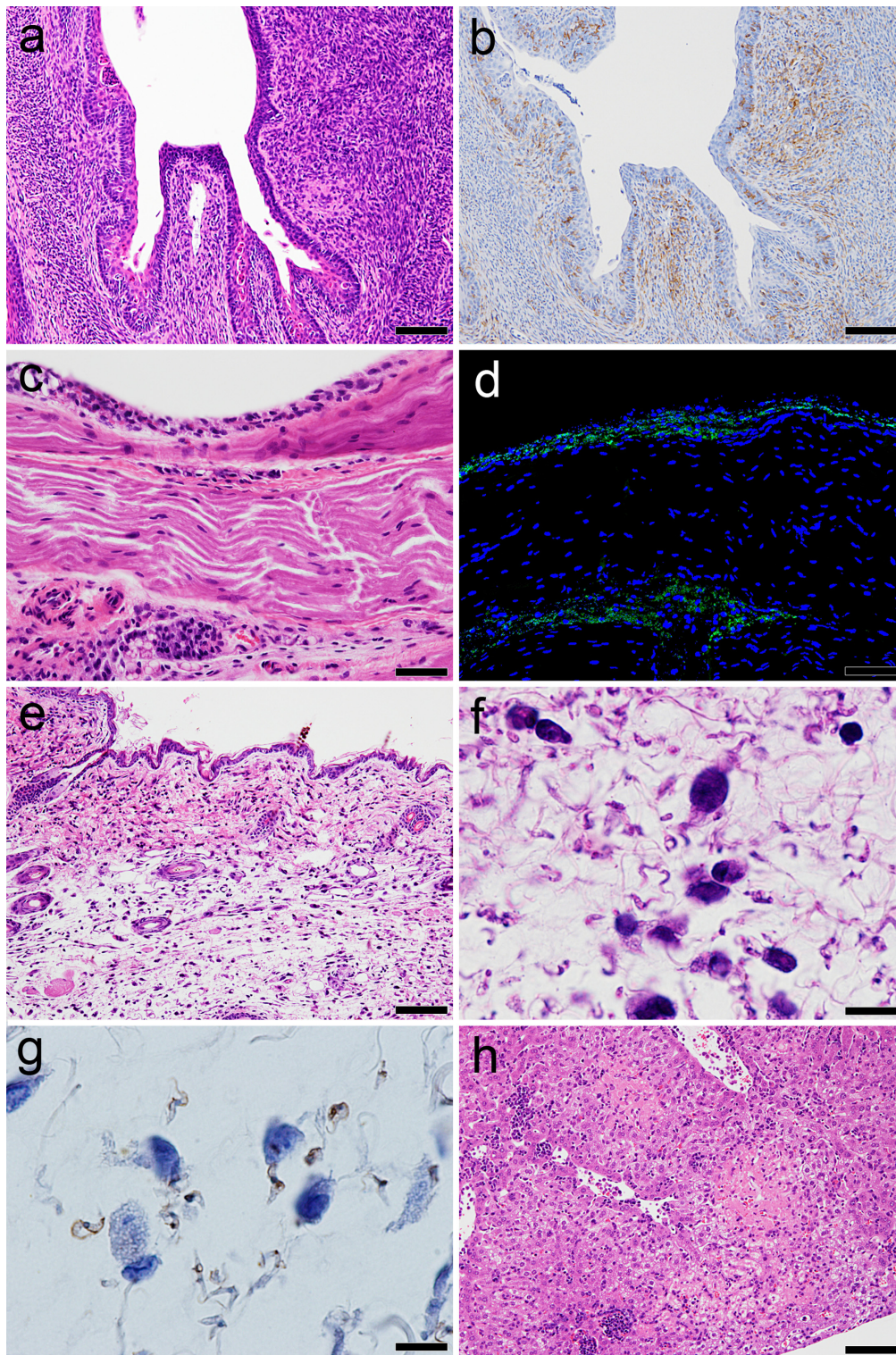
Fig. 3. **a)** Vagina of a C57BL/6 mouse in the infected group (IG). Diffuse inflammation is observed in the lamina propria and tunica muscularis of the vagina. Hematoxylin and eosin (HE) stain. Bar=50 µm. **b)** Vagina of a C57BL/6 mouse in IG. Inflammatory cells in the vagina are positively stained with Iba-1, and determined as macrophages. Anti-Iba1 immunohistochemistry. Bar=50 µm. **c)** Sciatic nerve of a BALB/c mouse in IG. Although inflammatory cells are detected in the perineurium and surrounding connective tissues, they are not observed within the nerve bundles. HE stain. Bar=50 µm. **d)** Sciatic nerve of a C57BL/6 mouse in IG. Numerous trypanosomes (green) are detected in the perineurium and peri-neural connective tissue, but not within nerve bundles. Anti-*Trypanosoma equiperdum* immunofluorescence. Bar=50 µm. **e)** Skin of a C57BL/6 mouse in IG. Lymphocytes and macrophages diffusely infiltrate the dermis and subcutaneous tissue. Edema is also observed. The hair follicles and adnexal glands are atrophied and decreased in number. HE stain. Bar=100 µm. **f)** Skin of a C57BL/6 mouse in IG. Numerous trypanosomes are observed in the subcutaneous tissue. HE stain. Bar=10 µm. **g)** Skin of a C57BL/6 mouse in IG. Trypanosomes in the subcutaneous tissue are positively stained with anti-*T. equiperdum* antibody. Anti-*T. equiperdum* immunohistochemistry. Bar=10 µm. **h)** Liver of a C57BL/6 mouse in IG. Multifocal necrosis of hepatocytes and aggregation of inflammatory cells are observed. HE stain. Bar=100 µm.

have been anemic. The histopathological finding of skin plaques in dourine-affected horses, described as "trypanosomal sand", is characterized by the presence of parasites and severe inflammation in the dermis [17]. The skin plaques of dourine has never been reproduced in experimental animals. In the present study, redness and alopecia of the dorsal skin were observed in C57BL/6 mice, and the presence of parasites and inflammation were observed in the dermis and subcutaneous tissue. The histopathological lesion was similar to "trypanosomal sand".

A previous study on *T. brucei* using a mouse infection model revealed that adipose tissue was a main extravascular parasite niche during its lifecycle [21]. To the best of our knowledge, the relationship between *T. equiperdum* and adipose tissue in dourine-affected horses has not yet been investigated. Parasites were detected in the subcutaneous adipose tissue as well as in the dermis of *T. equiperdum* IVM-t2 strain infected mice by immunohistochemistry, and these results suggested that *T. equiperdum* may also parasitize adipose tissue as a parasite niche.

In the female reproductive organs of dourine-affected horses, inflammation was distributed in the epithelium, lamina propria, and tunica muscularis of the vagina and uterine submucosa [13, 24]. A histopathological examination of the vagina and uterus of BALB/c and C57BL/6 mice infected with the *T. equiperdum* IVM-t2 strain revealed inflammation in the vaginal lamina propria and tunica muscularis. Significant lesions were not observed in the epithelium of the vagina. In the present study, mice were infected with *T. equiperdum* by an intraperitoneal injection, and parasites were distributed to the vagina via the blood stream. On the other hand, *T. equiperdum* is transmitted during coitus in horses and causes vaginitis [12]. Differences in the distribution of inflammation of the vagina between dourine-affected horses and *T. equiperdum* IVM-t2 strain infected mice may be due to the difference in route of infection.

Inflammation and numerous parasites were observed in the perineurium and surrounding connective tissue in BALB/c and C57BL/6 mice infected with the *T. equiperdum* IVM-t2 strain. There were no inflammatory cells within the nerve bundles. In peripheral neuritis of dourine-affected horses, lymphocytes, plasma cells, and macrophages infiltrated the epineurium, perineurium, and nerve bundles [10, 24]. In a previous study, parasites were not histologically detected in the peripheral nerve tissues of dourine-affected horses [10]. Therefore, the nature and distribution of inflammation in peripheral nerves differed between horses and mice. Peripheral neuritis in dourine-affected horses at terminal stage involved axonal swelling and fragmentation, and axonal degeneration was the cause of neurological signs [10]. Although mild axonal degeneration was also observed in 2/6 BALB/c and 1/6 C57BL/6 mice of IG, they did not exhibit neurological signs. Therefore, mice infected with the *T. equiperdum* IVM-t2 strain may not reflect the pathophysiology of dourine-associated peripheral neuritis.

In conclusion, we confirmed that *T. equiperdum* IVM-t2 strain, which adapted to *in vitro* condition, infected mice as long as 60 days. The infected mice exhibited periodic parasitemia, similar to dourine-affected horses. Histopathologically, dermatitis and vaginitis, characteristic lesions of dourine, were also observed. Therefore, mice infected with *T. equiperdum* IVM-t2 strain are useful tool for pathological, immunological, and parasitological *in vivo* research to elucidate the mechanisms underlying the disease process and the host-parasite relationship in dourine.

CONFLICT OF INTEREST. The authors declare no conflicts of interest with respect to the publication of this manuscript.

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