The Journal of Veterinary Medical Science

NOTE

Pathology

Immunohistochemical phenotyping of macrophages and T lymphocytes infiltrating in peripheral nerve lesions of dourine-affected horses

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J. Vet. Med. Sci.
82(10): 1502–1505, 2020
doi: 10.1292/jvms.20-0172

Received: 25 March 2020
Accepted: 31 July 2020
Advanced Epub:
12 August 2020

ABSTRACT. Dourine is a deadly protozoan disease in equids caused by infection with *Trypanosoma equiperdum*. Neurological signs in the later stage of infection may be caused by peripheral polyneuritis and related axonal degeneration. This neuritis involves T lymphocytes, B lymphocytes, and macrophages, and is observed in cases without obvious neurological signs. However, the pathogenesis of neuritis remains unclear. We identified M2 macrophages and CD8 T cells as the predominant phenotypes in neuritis of dourine-affected horses with or without neurological signs. In contrast, the populations of M1 macrophages and CD4 T cells were small. This result indicates that inflammation was chronic and suggests that dourine-associated neuritis occurs at the early stage of infection.

KEY WORDS: dourine, immunohistochemistry, peripheral polyneuritis, *Trypanosoma equiperdum*

Dourine is a sexually transmitted protozoan disease in equids caused by infection with *Trypanosoma equiperdum* [14, 16]. Affected horses manifest various clinical signs depending on the clinical stage. The main clinical sign in clinical stage 1 is edema of the genital organs. Cutaneous lesions are observed in clinical stage 2. Eventually, infected horses exhibit neurological signs in clinical stage 3 [1, 5]. The neurological signs of dourine often include lameness and paralysis of the hind limbs as well as facial paralysis [1, 8, 20]. Therefore, these neurological signs are one of the factors causing serious damage to the horse industry. A histological examination of dourine-affected horses in Italy suggested that the neurological signs of dourine were attributed to lesions in the peripheral nerves, such as edema and neuritis, rather than those in the central nervous system [15]. We also previously revealed that peripheral neuritis, which was characterized by the infiltration of T cells, B cells, and macrophages, already existed before the manifestation of neurological signs; neurological signs were probably due to axonal degeneration related to neuritis [10]. However, the pathogenesis of peripheral neuritis remains unclear.

In recent years, a number of researchers have classified macrophages into M1 and M2 phenotypes according to their functions. During acute inflammation, macrophages are polarized toward M1 phenotype macrophages, which promote inflammation through the production of inflammatory cytokines, chemokines, and reactive oxygen species. In contrast, M2 phenotype macrophages suppress inflammation by producing anti-inflammatory cytokines, and regenerate injured tissues at the later stage of inflammation [4, 17].

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Table 1. Immunohistochemical positive rates of macrophage and T cell markers

	Case 1		Case 2		Case 3			Case 4
	Facial nerve	Ischiadic nerve	Facial nerve	Median nerve	Facial nerve	Trigeminal nerve	Ischiadic nerve	Facial nerve
Associaion with neurological signs[a)]	+	+	-	-	+	+	-	+
Immunohistochemical positive rates in each marker[b)]								
iNOS	-	-	-	-	-	-	±	±
Arginase	+	+	++	++	++	++	+	++
CD204	±	±	±	±	+	±	+	+
CD4	-	-	-	±	±	-	+	±
CD8	-	-	+	+	+	++	++	++
Granzyme	-	-	±	-	-	-	±	±

a) +: this nerve is associated with neurological sign, -: not associated with neurological sign. b) ++: positive cells are more than 20%, +: 5–20%, ±: less than 5%, -: no positive cells.

T cells have also been classified into CD4 T cells and CD8 T cells according to differences in the coreceptors expressed at the plasma membrane. CD4 T cells, which are named helper T cells, activate various inflammatory cells, such as macrophages, CD8 T cells, and B cells, to control infections *via* cytokine messages. CD8 T cells, which are named cytotoxic T cells, directly attack neoplastic cells and host cells infected with intracellular pathogens [2]. In addition, a subset of T cells expressing the NK1 marker has been identified. These T cells are called natural killer (NK) T cells and attack virus-infected cells and neoplastic cells [7].

In the present study, we identified the phenotypes of macrophages and T cells infiltrating in the peripheral nerves lesions of dourine-affected horses by immunohistochemical staining in order to clarify the pathogenesis of peripheral polyneuritis.

Four horses infected with dourine in Mongolia were examined in the present study. The diagnosis of dourine was made by the detection of protozoa using a polymerase chain reaction and a microscopic examination of blood and/or a genital swab, the detection of an anti-Trypanosoma antibody using an enzyme-linked immunosorbent assay and immunochromatographic test, and the presentation of characteristic clinical signs, such as edematous swelling of the genital mucosa and facial paresis. The detailed clinical information and gross and histological lesions of these animals were previously reported by Mungun-Ochir *et al* [10]. In the present study, peripheral nerves associated with neurological signs (Case 1: fascial and ischiadic nerves, case 3: fascial and trigeminal nerves, case 4: facial nerve) and those not associated with neurological signs (Case 2: facial and median nerves, Case 3: ischiadic nerve) were examined (Table 1).

Immunohistochemistry using anti-iNOS mouse monoclonal antibody (1:200, R&D Systems, Minneapolis, MN, USA), rabbit anti-human arginase 1 antibody (1:400, LSBio, Seattle, WA, USA), and mouse anti-human macrophage scavenger receptor A (MSR-A: CD204) (1:40, Trans Genic Inc., Kumamoto, Japan) was performed for the identification of M1 and M2 macrophages. To identify CD4 T cells, CD8 T cells, and NK T cells, anti-equine CD4 monoclonal antibody (1:50, Kingfisher Biotech, Saint Paul, MN, USA), anti-CD8α monoclonal antibody (1:100, Washington State University, Pullman, WA, USA), and rabbit anti-granzyme B polyclonal antibody (1:400, Spring Biosciences, Pleasanton, CA, USA), respectively, were utilized. Sections were immersed in citrate buffer for antigen retrieval (15 min, 97°C). Endogenous peroxidase was then blocked with 0.3% H_2O_2 (5 min, room temperature). After incubation with the primary antibody (4°C, overnight), the sections were incubated with MAX-PO polymer reagent (Nichirei Bioscience, Tokyo, Japan) as a secondary antibody (30 min, room temperature). Immunolabeling was detected using 3,3′-diaminobenzidine. Hematoxylin was utilized as a counter stain. The positivity rate of markers was evaluated in the most severe foci of inflammation. In each slide, five microscopic fields at ×200 magnification were selected. At least 100 inflammatory cells were counted in each field, and the percentage of positive cells for each antibody was evaluated as 1 of 4 stages: ++ more than 20%, + 5–20%, ± less than 5%, and − no positive cells. In Case 1, the percentage of positive cells for each antibody was calculated using the total number from 5 fields because of the small number of inflammatory cells.

The results of the immunohistochemical examination are summarized in Table 1. While iNOS-positive cells were not found or uncommon if observed (Fig. 1a), the majority of macrophages were positively stained with anti-arginase (Fig. 1b) and CD204 antibody in all nerves of all four cases. The population of CD8-positive cells (Fig. 1c) was larger than those of CD4-positive cells (Fig. 1d) and granzyme-positive cells. Case 1 had no CD4, CD8, or granzyme-positive cells in the evaluated fields. Arginase and CD204-positive cells and CD8-positive cells were the predominant phenotypes in both nerves associated with and without neurological signs. Therefore, regardless of the association with neurological signs, M2 macrophages and CD8 T cells were more predominant phenotypes than M1 macrophages and CD4 and NK T cells in dourine-associated peripheral neuritis.

M1 and M2 macrophages both play important roles in host defenses against parasitic infections and other contagious diseases such as viral and bacterial infections [9]. M1 macrophages produce inflammatory cytokines and induce inflammation at the early stage of infection; as inflammation progresses, the dominancy of macrophages switches towards the M2 subtypes [17]. M2 macrophages suppress inflammation by producing anti-inflammatory cytokines [17]. However, M2 macrophage may also induce inflammation. According to an experimental mouse model of chronic helminth infection, chronic exposure to anti-inflammatory signals activated some aggressive abilities of M2 macrophages such as proinflammatory cytokine production [13]. Although M2 macrophages promote or suppress inflammation by changing cytokine signals, they are the predominant phenotype in chronic

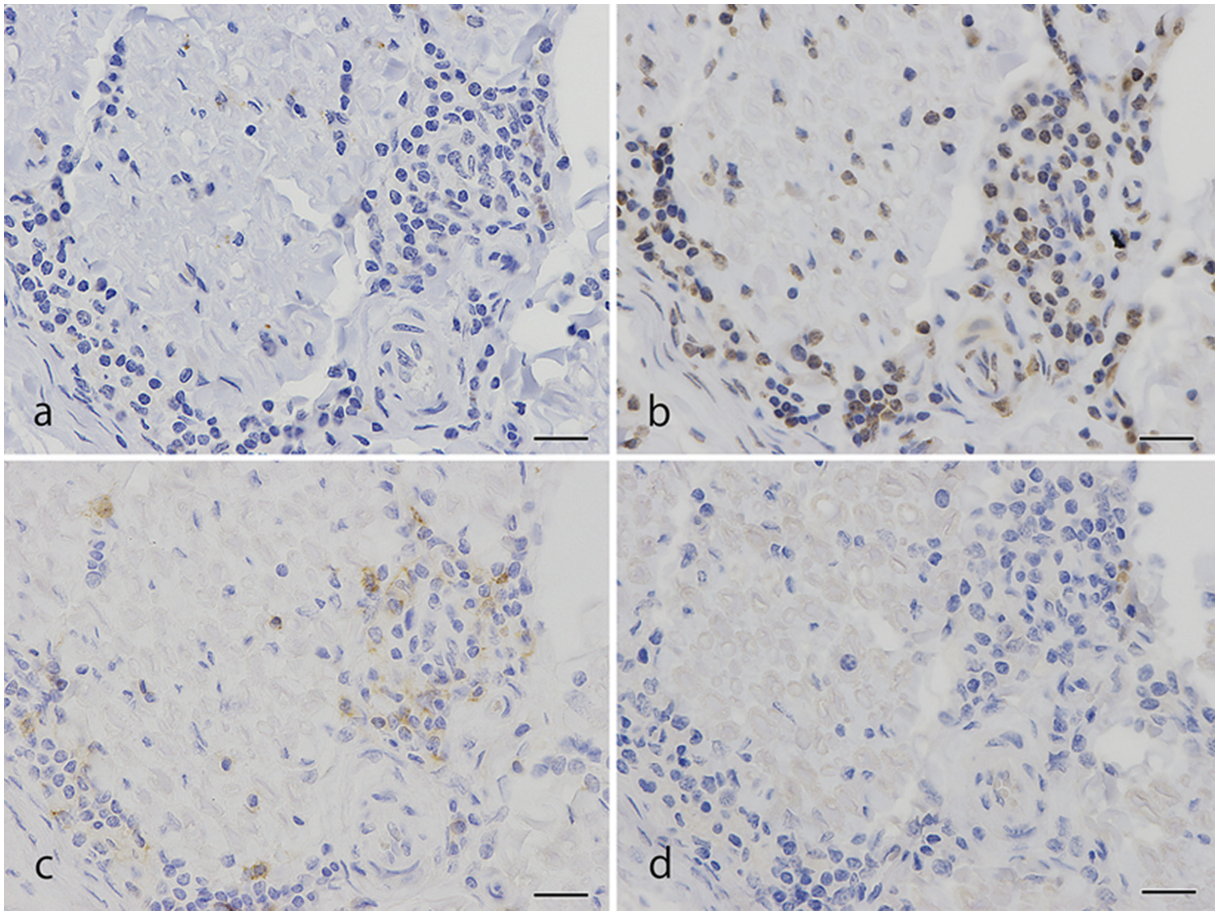

Fig. 1. Immunohistochemistry of the facial nerve in Case 4, iNOS (a), arginase (b), CD8 (c), and CD4 (d). Macrophage markers revealed that iNOS positive macrophages were rare (a); in contrast, the majority of macrophages were stained with arginase (b). T lymphocytes immunolabeled with CD8 were frequently observed (c), while only a few T lymphocytes expressed CD4 (d). Immunohistochemical staining with hematoxylin counter stain. Bars=50 μm.

inflammation regardless of their functions [3, 13, 17, 21]. In the present study, the abundant infiltration of M2 macrophages was observed in all nerves. No significant differences were observed in the degree of infiltration of M2 macrophages between peripheral nerves associated with or without neurological signs. The function of M2 macrophages remains unclear in dourine-associated neuritis. Peripheral neuritis in dourine-affected horses are considered to be chronic.

CD4 T cells and CD8 T cells both play important roles in responses to protozoan infections [2]. In the present study, CD8 T cells were more frequently observed than CD4 T cells. In general, CD8 T cells are effective at attacking cells that are parasitized with intracellular protozoa such as *Trypanosoma cruzi* [19]. However, *T. equiperdum* does not have the ability to invade host cells. An *in vitro* culture of *T. equiperdum* also did not require the intracellular stage for growth and replication [18]. Previous studies on sheep infected with *Trypanosoma congolense* (an obligate extracellular parasite) revealed that the numbers of CD4 T cells, CD8 T cells, and B cells increased during the early stage of the inflammatory reaction; CD8 T cells then became the predominant phenotype of inflammatory cells [6, 11, 12]. The presence of CD8 T cells in dourine-associated neuritis may also be due to chronic inflammation against *T. equiperdum*, and the present result showing the predominance of M2 macrophages supports this finding.

In Case 1, although only a few M2 macrophages were observed, there were no CD4, CD8, or granzyme-positive cells in the evaluated fields. We previously revealed that the lesion on the peripheral nerves in Case 1 was mainly fibrosis [10]. Fibrosis is a common histological lesion produced during chronic inflammation. Therefore, the peripheral nerve lesion of Case 1 may have been at the terminal stage of inflammation.

In conclusion, the phenotypes of macrophages and T cells infiltrating in peripheral nerves of dourine-affected horses were identified as the M2 and CD8 subtypes, respectively. These phenotypes were predominant even in the case lacking neurological signs. This result suggests that peripheral neuritis occurs at the early stage of infection. The present study provides important information not only on the pathogenesis of dourine, but also for the establishment of treatments for the neurological signs of this disease.

ACKNOWLEDGMENT. The present study was supported by the Mongolian Science and Technology Research Partnership for Sustainable Development (JICA/AMED SATREPS) project (17jm0110006h0005).

REFERENCES

1. Ahmed, Y., Hagos, A., Merga, B., Soom, A. B., Duchateau, B., Goddeeris, B. M. and Govaere, J. 2018. *Trypanosoma equiperdum* in the horse −a neglected threat? *Vlaams Diergen. Tijds.* **87**: 66–75.
2. Allen, J. E. and Liu, L. X. 2004. Immunity to parasitic and fungal infections. pp. 469–496. *In*: Immunology, Infection, and Immunity (Pier, G. B., Lyczak, J. B. and Wetzler, L. M. eds.), ASM Press, Washington, D.C.
3. Arnold, L., Henry, A., Poron, F., Baba-Amer, Y., van Rooijen, N., Plonquet, A., Gherardi, R. K. and Chazaud, B. 2007. Inflammatory monocytes recruited after skeletal muscle injury switch into antiinflammatory macrophages to support myogenesis. *J. Exp. Med.* **204**: 1057–1069. [Medline] [CrossRef]
4. Atri, C., Guerfali, F. Z. and Laouini, D. 2018. Role of human macrophage polarization in inflammation during infectious diseases. *Int. J. Mol. Sci.* **19**: e1801. [Medline] [CrossRef]
5. Claes, F., Büscher, P., Touratier, L. and Goddeeris, B. M. 2005. *Trypanosoma equiperdum*: master of disguise or historical mistake? *Trends Parasitol.* **21**: 316–321. [Medline] [CrossRef]
6. Coustou, V., Guegan, F., Plazolles, N. and Baltz, T. 2010. Complete in vitro life cycle of *Trypanosoma congolense*: development of genetic tools. *PLoS Negl. Trop. Dis.* **4**: e618. [Medline] [CrossRef]
7. Green, W. R. 2004. Cell-mediated immunity. pp. 373–398. *In*: Immunology, Infection, and Immunity (Pier, G. B., Lyczak, J. B. and Wetzler, L. M. eds.), ASM Press, Washington, D.C.
8. Hagos, A., Abebe, G., Büscher, P., Goddeeris, B. M. and Claes, F. 2010. Serological and parasitological survey of dourine in the Arsi-Bale highlands of Ethiopia. *Trop. Anim. Health Prod.* **42**: 769–776. [Medline] [CrossRef]
9. Liu, Y. C., Zou, X. B., Chai, Y. F. and Yao, Y. M. 2014. Macrophage polarization in inflammatory diseases. *Int. J. Biol. Sci.* **10**: 520–529. [Medline] [CrossRef]
10. Mungun-Ochir, B., Horiuchi, N., Altanchimeg, A., Koyama, K., Suganuma, K., Nyamdolgor, U., Watanabe, K. I., Baatarjargal, P., Mizushima, D., Battur, B., Yokoyama, N., Battsetseg, B., Inoue, N. and Kobayashi, Y. 2019. Polyradiculoneuropathy in dourine-affected horses. *Neuromuscul. Disord.* **29**: 437–443. [Medline] [CrossRef]
11. Mwangi, D. M., Munyua, W. K. and Nyaga, P. N. 1990. Immunosuppression in caprine trypanosomiasis: effects of acute *Trypanosoma congolense* infection on antibody response to anthrax spore vaccine. *Trop. Anim. Health Prod.* **22**: 95–100. [Medline] [CrossRef]
12. Mwangi, D. M., Hopkins, J. and Luckins, A. G. 1996. *Trypanosoma congolense* infection in sheep: cellular phenotypes in lymph and lymph nodes associated with skin reactions. *J. Comp. Pathol.* **114**: 51–61. [Medline] [CrossRef]
13. Mylonas, K. J., Nair, M. G., Prieto-Lafuente, L., Paape, D. and Allen, J. E. 2009. Alternatively activated macrophages elicited by helminth infection can be reprogrammed to enable microbial killing. *J. Immunol.* **182**: 3084–3094. [Medline] [CrossRef]
14. OIE 2013. Terrestrial Manual. Dourine, Office International des Epizooties (OIE), Paris.
15. Pascucci, I., Di Provvido, A., Cammà, C., Di Francesco, G., Calistri, P., Tittarelli, M., Ferri, N., Scacchia, M. and Caporale, V. 2013. Diagnosis of dourine in outbreaks in Italy. *Vet. Parasitol.* **193**: 30–38. [Medline] [CrossRef]
16. Schlafer, D. H. and Foster, H. S. 2016. Female genital system. pp. 358–464. *In*: Pathology of Domestic Animals, 6th ed. (Macie, M. G. ed.), Elsevier, St. Louis.
17. Sica, A. and Mantovani, A. 2012. Macrophage plasticity and polarization: *in vivo* veritas. *J. Clin. Invest.* **122**: 787–795. [Medline] [CrossRef]
18. Suganuma, K., Narantsatsral, S., Battur, B., Yamasaki, S., Otgonsuren, D., Musinguzi, S. P., Davaasuren, B., Battsetseg, B. and Inoue, N. 2016. Isolation, cultivation and molecular characterization of a new *Trypanosoma equiperdum* strain in Mongolia. *Parasit. Vectors* **9**: 481. [Medline] [CrossRef]
19. Tarleton, R. L. 2015. CD8+ T cells in *Trypanosoma cruzi* infection. *Semin. Immunopathol.* **37**: 233–238. [Medline] [CrossRef]
20. Vulpiani, M. P., Carvelli, A., Giansante, D., Iannino, F., Paganico, D. and Ferri, N. 2012. Reemergence of dourine in Italy: clinical cases in some positive horses. *J. Equine Vet. Sci.* **33**: 468–474. [CrossRef]
21. Xu, W., Zhao, X., Daha, M. R. and van Kooten, C. 2013. Reversible differentiation of pro- and anti-inflammatory macrophages. *Mol. Immunol.* **53**: 179–186. [Medline] [CrossRef]