Pathogenesis of *Babesia caballi* Infection in Experimental Horses

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(Received 21 April 1998/Accepted 24 June 1998)

ABSTRACT. The present study was designed to investigate the role of cytokines in the pathogenesis of *Babesia caballi* in experimentally infected horses. The expression of cytokine mRNA was determined by using reverse transcription-polymerase chain reaction in two *B. caballi*-infected horses for 2 weeks after the infection. In one horse, there was up-regulation of interferon-gamma, tumor necrosis factor-alpha (TNF- α) and interleukin-2 mRNAs, while in the second horse, expression of only TNF- α mRNA was up-regulated. No change was observed in interleukin-4 mRNA in both of the horses. To know the relation between nitric oxide (NO) production and pathogenesis, NO production was assayed in three dexamethasone treated-*B. caballi*-infected horses. Production of NO in all 3 horses increased significantly before death, although the parasitemia level remained very low. Treatment with NO inhibitor resulted in the suppression of NO production and increased parasitemia level in a horse, which died of the infection. The pathological examination showed that the main cause of the death was dyspnoea and pulmonary edema. Histopathologically, diffuse global mesangial proliferative glomerulonephritis was also observed. These results suggested that NO may be a critical effector molecule of immune defense against parasite. TNF- α and NO might be contributing to the pathogenesis in *B. caballi* infection. — **KEY WORDS:** *Babesia caballi*, cytokine, nitric oxide, pathogenesis.

—J. Vet. Med. Sci. 60(10): 1127–1132, 1998

Babesia caballi is an intra-erythrocytic protozoa which causes piroplasmosis in horses. *B. caballi* infection is characterized by high fever, dyspnoea, and edema [6]. The disease usually runs an acute course although the parasitemia remains very low, even less than 1.0%. The underlying mechanism by which such a low parasitemia causes an acute disease is still not well understood and there is scarcity of reports in the literature on this aspect.

There are many reports concerning the pathogenesis of malaria, in which, production of cytokines and free radicals have been associated with the pathogenesis of the disease [12, 26]. Cell-mediated immunity and cytokines have been identified to play a crucial role in the pathogenesis of malaria [5]. T-helper 2-dominant responses reportedly down-regulate the susceptibility to murine cerebral malaria [27]. The depletion of CD4⁺ T cells, or the administration of antioxidants or antibodies against certain cytokines, resulted in protection of the mice against fatal cerebral malaria complications [15]. Resistance to cerebral malaria in tumor necrosis factor alpha/beta (TNF- α / β) deficient mice is associated with the reduction in intercellular adhesion molecule-1 expression and up-regulation of nitric oxide (NO) synthesis [21].

In bovine and canine babesiosis, the host immune responses, especially excess production of cytokines have been shown to worsen the clinical signs of the disease [12, 26]. However, there are few reports about the host immunity and immunopathology in equine babesiosis thus underlying

the importance of identifying the role and production of cytokines. The present study was, therefore, designed to investigate the pathogenesis by analyzing cytokine production in horses experimentally infected with *B. caballi*.

MATERIALS AND METHODS

Animals: Six adult race horses were divided into 3 groups. Two horses in group A were inoculated with *B. caballi* and kept as untreated control. Three horses in group B were administered 16.7 mg dexamethasone (Corson : Dainippon Pharmaceutical Co., Ltd., Japan) intravenously (i.v.) twice a day from 7 days before infection till the end of the examination period. One horse of group C was administered 16.7 mg of dexamethasone i.v. twice a day from 7 days before infection till the end of the examination period and 10 g of aminoguanidine (Tokyo Kasei Kogyo Co., Ltd., Japan) [9] from 6 days before infection till the end of the examination period.

All these 6 horses were inoculated with 200 ml of the *B. caballi*-infected blood (parasitemia level was less than 0.1%) and monitored for clinical symptoms and appearance of parasitemia. Giemsa-stained blood smears were examined to determine the level of parasitemia. All experiments were conducted under the p3 level of complete isolater in Equine Research Institute, Japan Racing Association (JRA).

Parasite: *B. caballi* stock was obtained from Dr. R. D. Angusk (United States Department of Agriculture, National Veterinary Service). The parasite was stored in liquid nitrogen after propagating twice in a splenectomised horses by serial passage.

Detection of cytokine mRNA by reverse transcription

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polymerase chain reaction (RT-PCR): Peripheral blood mononuclear cells (PBMC) were isolated from 2 horses of group A for two weeks after the infection. RNA extracted from PBMC was used for the detection of cytokine mRNA. To synthesize cDNA, 1 μ g of each RNA sample was incubated with Rous-associated virus 2 reverse transcriptase (RAV2, Takara Shuzo Co., Ltd. Japan) and 2.5 mM C(dT)₁₅. Then, 0.1 μ g each of cDNA was subjected to PCR in a volume of 20 μ l, containing 0.2 μ M deoxynucleoside triphosphate mixture, 0.2 μ M each of primers, 1.5 μ M MgCl₂, PCR buffer and 0.5 U of *Taq* polymerase (Gibco BRL, Life Technologies). To determine expression of interferon-gamma (IFN- γ), interleukin-2 (IL-2), TNF- α and hypoxanthine phosphoribosyltransferase (HPRT) mRNA, RT-PCR was performed as described previously [10]. The first cycle consisted of 94°C for 2 min, followed by 35 cycles of 94°C for 45 sec, 54°C for 45 sec, 72°C for 80 sec, extension was allowed to complete at 60°C for 7 min and samples were then chilled at 4°C. For detection of IL-4 mRNA by RT-PCR, the following primers were used; sense; 5'-GGTCTCACCTCCCAACTGCT-3' and antisense; 5'-CTTCTTGGCTTCATTACAG-3'. The expected size of the product is 332 bp. The first cycle consisted of 94°C for 2 min, followed by 35 cycles of 94°C for 1 min, 52°C for 2 min and 72°C for 2 min. Extension was allowed to complete at 72°C for 4 min and the samples were then chilled at 4°C. After amplification, 10 μ l each of sample was loaded onto 2% agarose, and electrophoresed. The products were visualized by ethidium bromide staining. The housekeeping gene of mRNA HPRT was used as control for RNA quality and concentration. Amplified bands were assessed for their relative intensities between the bands by using densitometer. The cytokine levels were expressed as the ratio to the intensities before the infection.

Measurement of nitric oxide (NO) production: To assay for reactive nitrogen intermediates in serum, we used the procedure as reported previously [8].

Histopathology: Experimentally infected horses were euthanized by injecting suxamethonium chloride solution (Succin, Yamanouchi Pharmaceutical Co., Ltd., Japan) and thiopental sodium (Ravonal, Tanabe Pharmaceutical Co., Ltd., Japan) i.v. On necropsy, liver, spleen, kidney, lung, adrenal gland, pancreas, heart and brain were collected and fixed in 10% buffered formalin. After fixation, tissues were embedded in paraffin, sectioned at 3 to 4 μ m, and stained with hematoxylin and eosin, Masson trichrome, or periodic acid-methenamine-silver stain for histological examination.

RESULTS

Clinical symptoms and parasitemia level: Both horses of group A survived infection with *B. caballi*. In contrast, all four infected horses of groups B and C showed dyspnoea, and died at day 6, 7 and 8 post infection due to pulmonary edema. Clinically, all the 6 horses showed symptoms of reduced appetite, fever, weight loss, and coughing. Both horses in group A showed a transient fever (Fig. 1-a).

However, persistent high body temperature over 40°C was observed in horses of group B and C (Fig 1-b). Two of 3 horses from group B suffered from persistent fever up to 3 days post infection while the third horse suffered up to 4 days post infection.

Amongst the six experimental horses, one in group C developed the highest parasitemia (0.74%, Fig 1-b), another in group B developed relatively higher parasitemia (0.23%) while all others developed very low parasitemia (less than 0.2% , Fig. 1-a).

Histopathology: Grossly, bilaterally discolored kidneys and wet, heavy, edematous lungs associated with mild to moderate splenomegaly were observed. Histologically, diffuse global mesangial proliferative glomerulonephritis characterized by mild to severe mesangial cell proliferation and mild matrix increase was observed in all cases (Fig. 2-a). In such cases, however, capillary wall thickening and sclerotic glomeruli were rare. Bowman's spaces and tubules were completely filled with proteinaceous fluid and hemoglobin (Fig. 2-b). Diffuse edema was observed in the lungs where alveoli were filled with acidophilic, homogenous material (Fig. 2-c).

Cytokine mRNA expression: The cytokine mRNA expression in PBMC samples from group A horses was examined by RT-PCR for 2 weeks post inoculation and the results are presented in Table 1. In the horse No. 1, an increase in the expression of IFN- γ , TNF- α , IL-2 mRNA was observed. The peak cytokine mRNA expression was associated with the increase in body temperature. In the other horse No.2, only increased expression of TNF- α mRNA was observed while mRNA expression of other cytokines remained unchanged. The expression levels of IL-4 mRNA of both horses did not change after the infection.

Nitric oxide (NO) production: NO production in 3 groups of horses was determined and the results are shown in Fig. 3. There was no significant change in the group A horses during the experimental period. However, higher NO production was observed in the late phase of the infection in the group B horses, just before death. The level of NO production in group C horse, which died 6 days post infection, was low and comparable to that of group A horses.

DISCUSSION

Cytokines are important effector molecules for the protection against pathogens, especially in clearing intracellular pathogens [11, 14, 17, 22]. Although cytokines are the key molecules in host protective immunity, over production of cytokines may damage the host tissues. For example, excess amount of TNF- α , IFN- γ and NO may contribute to the cause and development of glomerulonephritis [1, 20]. Excess amount of TNF- α and IFN- γ are reported to aggravate endotoxic shock in septicemia [2]. In the infections due to intracellular pathogens, like malaria, excess amount of cytokines and free radicals may induce host tissue damage and injury [5].

Fig. 1. The kinetics of parasitemia and body temperature in *B. caballi*-infected horses. a) Group A horses. b) Groups B and C horses. +: Horses died at this time. —: temperature (°C), —: parasitemia level (%).

Oxygen-derived free radicals substantially contribute to the pathogenesis of parasitic diseases [3]. In bovine and canine

babesiosis, production of cytokine and free radicals are reported to be partly involved in pathogenesis [12, 26].

Fig. 2. The histopathological examination of *B. caballi*-infected horse. a) Moderate global mesangial cell proliferation (arrows) and mild thickening of matrix are

observed in renal glomerulus. Periodic acid-methenamine-silver (PAM) stain. bar=40 μ m. b) Many tubules in renal medulla are filled with hemoglobin (arrows). Masson trichrome stain. bar=40 μ m. c) The alveoli of lung are filled severely with edematous fluid. Hematoxylin and eosin stain. bar=800 μ m.

However, there is no report on the correlation between pathogenesis and cytokine production in *B. caballi* infection in horses.

The clinical symptoms observed in these experimentally infected horses were similar to those reported in the fatal cases of *B. caballi* infection in endemic areas [6]. Expression levels of TNF- α , IFN- γ , IL-2 and IL-4 mRNAs in *B. caballi*-infected horses (group A) were determined. Up-regulation of TNF- α level in both horses with low parasitemia was observed (Fig. 1, Table 1). Horses treated with dexamethasone before challenge (group B), showed enhanced NO production and subsequently died due to the infection (Fig. 3). In contrast, the horses not treated with dexamethasone before challenge (group A), did not show increased level of NO and survived from the infection. This implied that the increased NO level may be positively correlated with pathogenesis and contribute to parasite-induced tissue damage. Horses in groups A and B had lower parasitemia as compared to the horse in group C which was treated with NO synthetase inhibitor, aminoguanidine. Thus NO (in group B horses) and TNF- α (in group A horses) might have helped in parasite clearance, as reported in malaria [22, 23] and other parasite infections [13, 16].

At necropsy, the most remarkable lesions found were bilateral diffuse discoloration of kidney and lung edema which might have led to dyspnoea. Group B horses which died of the infection showed the higher level of NO production just before death (Fig. 3). Excess amount of NO is well known to cause the endothelial injury [25, 28]. There is a possibility that NO induced endothelial injury might have led to pulmonary edema (shown in Fig. 2).

On the other hand, aminoguanidine and dexamethasone-treated horse (group C) showed no increase in NO level after the *B. caballi* challenge but died of infection. The inhibition of NO synthetase might have induced production of active oxygen which also causes tissue injury [7, 24]. However more work is required to be undertaken as we examined only one case in group C. Nevertheless, the results suggest a possibly important contribution of active oxygen in pathogenesis of *B. caballi* infection. TNF- α , IFN- γ and NO contribute to induce glomerulonephritis and injury of endothelium in malaria cases [1, 4, 18–20]. Similarly, in *B. caballi* infected horses, NO and TNF- α and other cytokines may supplement protective immune responses against the parasite if produced in optimal quantities but excess amount of these molecules may contribute to the pathogenesis.

Table 1. The kinetics of cytokine mRNAs in *Babesia caballi* infected horse (group A).

Horse	Cytokines	Days after infection				
		0	3	6	10	14
Horse 1	IL-2	1.0	3.0	2.1	NT	—
	IFN- γ	1.0	11.8	36.3	NT	24.5
	TNF- α	1.0	1.9	4.4	NT	1.8
Horse 2	IL-2	1.0	1.0	0.1	—	—
	IFN- γ	1.0	0.8	0.7	0.7	0.9
	TNF- α	1.0	0.6	3.3	1.1	3.7

The cytokine levels were expressed as the ratio to the intensities at day 0. NT: not tested. —: under the detectable level.

ACKNOWLEDGMENTS. This study was partly supported by grants from the Ministry of Agriculture, Forestry and Fisheries, and from the Japan Racing Association (JRA). The authors thank Dr. Rakha NK for critical reading of this manuscript.

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Fig. 3. The kinetics of nitric oxide (NO) production in *B. caballi*-infected horses. The NO levels were expressed as the ratio to the levels before the infection. +: Horses died at this time.

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