Changes of splenic interleukin-12p70 concentration in mice infected with Babesia microti and Babesia rodhaini

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Changes of splenic interleukin-12p70 concentration in mice infected with *Babesia microti* and *Babesia rodhaini*

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ABSTRACT

Interleukin-12 (IL-12) is one of the most important factors for the host's protective immune response against protozoan infection. To evaluate the effect of bioactive form of IL-12(IL-12p70), expression of IFN-γmRNA and IL-4 mRNA in splenic CD4 positive T cells, anti-parasite delayed type hypersensitivity (DTH), and serum and splenic IL-12p70 concentration were examined in *Babesia microti* and *B. rodhaini* infected mice. The expression of IFN-γmRNA as a marker of Th1 cell differentiation was detected in splenic CD4 positive T cells in *B. microti* infected mice. Activation of DTH response as a marker of cell-mediated immune response was also observed from 2 days after the infection with *B. microti*, while no response was detected in *B. rodhaini* infected mice. Serum IL-12p70 was not detected in both *B. microti* and *B. rodhaini* infected mice, however, splenic IL-12p70 concentration increased significantly in *B. microti* infected mice with peaks at 30 hrs and 4 days after the infection. From these results, splenic IL-12p70 increased at 30 hrs after the *B. microti* infection was considered to closely relate with Th1 cell differentiation followed by cell-mediated immune response in mice.

INTRODUCTION

It has been widely accepted that *Babesia microti* infection was non-lethal with a transient increase of parasitemia, whereas *B. rodhaini* infection was lethal with a remarkable increase of parasitemia in BALB/c mice. Our previous reports demonstrated that the helper T cell (Th cell) differentiation into helper T cell type 1 (Th1 cell), which was induced by Interleulin-12 (IL-12) at early phase of infection, was an important factor on the different course of infection between *B. microti* and *B. rodhaini* (Hashiguchi-Kato et al., 2004; Shimada et al., 1991; Shimada et al., 1992). IL-12 secreted from antigen presenting cells, included macrophage and dendritic cell, had a heterodimeric structure consisted of 35 kilo Dalton (kD) and 40 kD subunits (IL-12p35 and IL-12p40), which combined together formed biological active form IL-12 (IL-12p70) (Gubler et al., 1991; Ling et al., 1995; Schoenhaut et al., 1992). Until an available kit was prepared for IL-12p70, only IL-12p40 could be measured as a parameter of IL-12. The monomer and

homodimer of IL-12p40 showed an inhibitory effect on biological activity of IL-12p70 (Germann and Rude, 1995; Gillessen et al., 1995; Ling et al., 1995). Therefore, measurement of IL-12p70 was necessary for understanding of the effect of IL-12 on Th1 cell differentiation. This study deals with serum and splenic IL-12p70 concentration, expression of IFN-γ mRNA and IL-4 mRNA expression in splenic CD4 positive T cells, and delayed type hypersensitivity (DTH) response in *B. microti* and *B. rodhaini* infected BALB/c mice at early phase of infection.

MATERIALS AND METHODS

Mice and Protozoa

Eight-week-old male BALB/c mice were purchased from SLC Inc. (Shizuoka, Japan). *Babesia micorti* (Munich strain) and *B. rodhaini* (Australian strain) have been maintained in our laboratory by serial passages of parasitized blood to mice.

Experimental infection

Parasitized blood with *B. microti* or *B. rodhaini* was collected by cardiac puncture and injected peritoneally with $1x10^4$ parasitized red blood cell per head in 0.2 ml of sterile saline.

Babesia lysate antigen (BLA)

The blood of approximately 60% of parasitemia was collected by cardiac puncture in heparinized syringe and washed 3 times with physiological saline. The cell pellet was freezed and thawed with liquid nitrogen for 3 times and centrifuged at 14,000 g for 30 min at 4℃. The supernatant was used as a BLA for anti-parasite DTH response.

Anti-parasite DTH response

Anti-parasite DTH response was measured as a marker of the activation of cell-mediated immune response. Briefly, mice were injected subcutaneously with 50μl of BLA in the right footpad after the measuring of thickness. Equal volume of physiological saline was injected in the left footpad as a control. At 24 hrs after the treatment, thickness of both footpads was measured again. Swelling rate was calculated by the following formula: Footpad swelling rate (%) = (thickness of right footpad at 24 hrs after the injection – thickness of right footpad before the injection) ×100 / thickness of right footpad before the injection.

Splenic CD4 positive T cells

Spleen was removed from the mice infected with *B. microti* or *B. rodhaini*, minced, and suspended in phoshate buffered saline (PBS) with 2 % fetal bovine serum (FBS). The cell suspension was filtered through stainless mesh and hemolyzed by NH_4Cl_2-Tris (0.84 %, pH 7.65). The residued cells were eluted on cell enrichment immunocolumn for obtaining CD4 positive T cells as helper T cells (CELLECT-PULS MOUSE CD4 KIT; Cytovax, Biotech. Inc, Canada). The cells obtained were used for the expression of IFN-γmRNA and IL-4 mRNA by reverse transcriptase polymerase chain reaction (RT-PCR).

Expression of IFN-γmRNA and IL-4 mRNA

Expression of IFN-γmRNA and IL-4 mRNA was examined as a marker of Th1 cell and Th2 cell differentiation, respectively. Briefly, total RNA extraction was obtained from splenic CD4 positive T cells by the RNeasy total RNA kits (QUIAGN, GmbH, Germany). First-strand cDNA was synthesized from 1μg of extracted RNA for each sample by the Super Script Preamplification System (first strand cDNA synthesis kit: GIBCO, BRL, Japan). PCR amplification was performed in 25μl, containing 1μg of cDNA, 0.5μM of each

primer (IFN-γ and IL-4: Cont. Lab. Prod., CA, USA, G3PDH: Becton and Dickinson Co., CA, USA), 0.2 mM dNTP (Appl. Biosys., CA, USA), 0.5 U of Taq Gold polymerase (Appl. Biosys., CA, USA), and PCR buffer containing $MgCl_2$ (Appl. Biosys., CA, USA). PCR amplification for IFN-γmRNA was 1 cycle for 10 min at 94℃, 40 cycles for 1 min at 94℃, for 1 min at 60℃, and for 2 min at 72℃, and 1 cycle for 10 min at 72℃. For IL-4 mRNA, the amplification was 1 cycle for 10 min at 94℃, 40 cycles for 1 min at 94℃, for 1 min at 60℃, and for 2 min at 72℃, and 1 cycle for 10 min at 72℃. For G3PDH mRNA as a control, the amplification was 1 cycle for 10 min at 94℃, 30 cycles for 45 sec at 94℃, for 45 sec at 60℃, and for 2 min at 72℃, and 1 cycle for 7 min at 72℃. The PCR products were analyzed by 2 % agarose gel electrophoresis and visualized by ethidium bromide staining.

Serum and splenic IL-12p70 concentration

The serum was obtained from *B. microti* or *B. rodhaini* infected mice. Concentration of serum IL-12p70 was measured by the solid phase sandwich enzyme linked immuno sorbent assay (ELISA) KIT (Biosource Cytoscreen, Mouse IL-12p70, CA, USA). Splenic IL-12p70 concentration was examined according to the method with a minor modification (Fernandez-Lago et al., 1999). Briefly, spleen was removed from *B. microti* or *B. rodhaini* infected mice and homogenized in 1% (wt/vol) 3- [3-cholamidopropyl] –dimethyl ammnonio]-1- propanseulfonate at a concentration of 100 mg/ml. After the homogenization, the solution was kept for 24 hrs at 4℃. Then, the supernatant was collected by the centrifugation at 14,000 g for 30 min at 4℃ and stored at -80℃ until use. Concentration of splenic IL-12p70 was measured by the ELISA KIT mentioned above.

RESULTS

Parasitemia

The parasitemia in *Babesia microti* infected mice increased from 9 days after the infection, showing a peak level of approximately 60 % at 19 days, and then decreased. In *B. rodhaini* infected mice, it increased from 6 days after the infection, inducing the death of the host (Fig. 1).

Anti-parasite DTH response

Babesia microti infected mice showed a significant increase of the anti-parasite DTH response from 2 days after the infection, whereas *B. rodhaini* infected mice showed no response (Fig.2).

Expression of IFN-γmRNA and IL-4 mRNA in splenic CD4 positive T cells

Expression of IFN-γmRNA as a marker of Th1 cell in splenic CD4 positive T cells was detected at 2, 4 and 6 days after the infection in *B. microti* infected mice, whereas at only 6 days after the infection in *B. rodhaini* infected mice. No expression of IL-4 mRNA in splenic CD4 positive T cells was detected in both infected mice(Fig.3).

Serum and splenic IL-12p70 concentration

Serum IL-12p70 level was below the detection limit in both *B. microti* and *B. rodhaini* infected mice during the experimental period. However, splenic IL-12p70 level increased significantly at 30 hrs after the infection in *B. microti* infected mice, showing 2 peaks of 30 hrs and 4 days after the infection, whereas no remarkable change was observed in *B. rodhaini* infected mice (Fig.4).

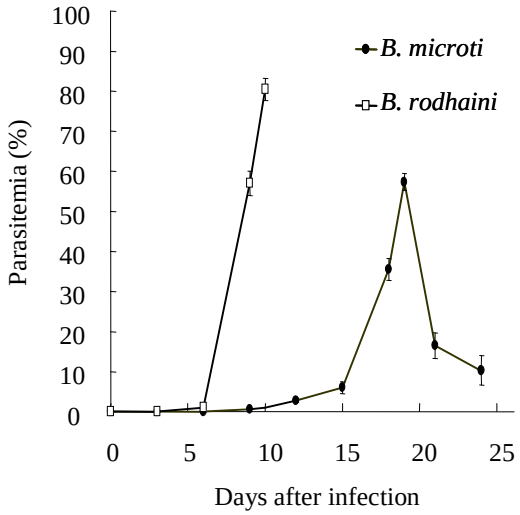

Fig.1. Changes of parasitemia in *B. microti* and *B. rodhaini* infected mice.

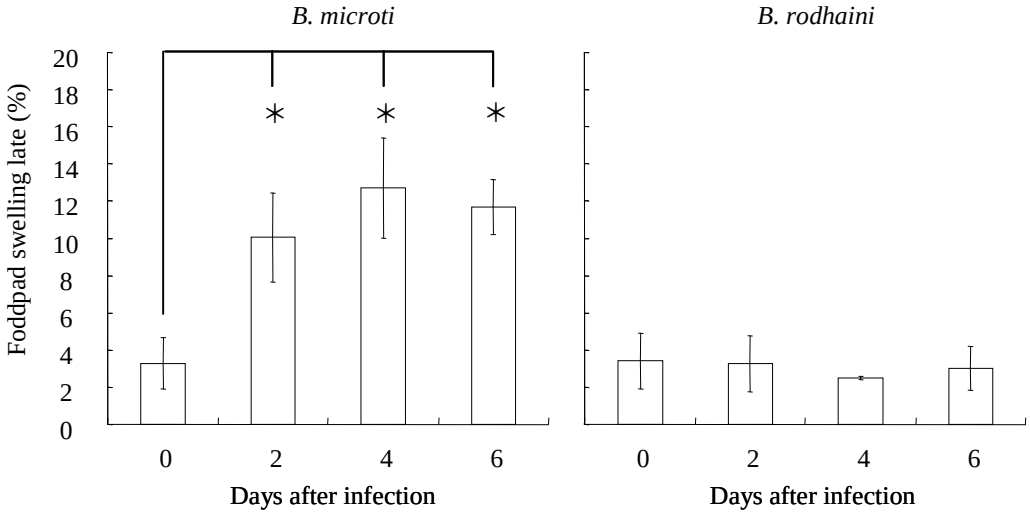

Fig.2. Delayed-type hypersensitivity responses in *B. microri* and *B. rodhaini* infected mice.
＊: Significant increase ($p < 0.05$) compared to the initial level.

DISCUSSION

From the results of this study, *B. microti* infected mice showed a non-lethal infection with a transient increase of parasitemia and an enhancement of cell-mediated immunity, showing as the anti-parasite DTH response. Splenic helper T cells ware differentiated into Th1 cells due to the expression of IFN-γmRNA in CD4 positive T cells observed from 2 days after the infection. In constant, *B. rodaini* infected mice showed a high mortality without the response of the cell-medicated immunity and Th1 cell differentiation until 6 days after the infection. Each infectious course and immunological response observed in *B. microti* and *B.* rodaini infected mice was the same to that demonstrated in previous reports (Ruebush et al., 1986; Shimada et al., 1991). The Th1 cell differentiation at early phase of infection of *Babesia spp.*, followed by the enhancement of cell-mediated immunity, was reported to be closely related with the course of infection, which was quite difference between *B. microti* and *B.* rodaini infection (Shimada et al., 1992; Shimada et al., 1996; Sam and

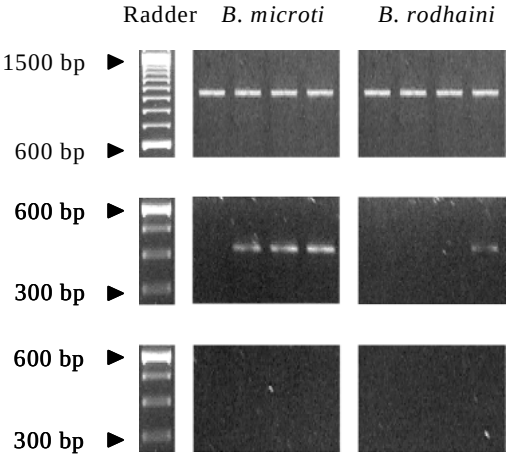

Fig.3. Expressions of IFN-γ mRNA and IL-4 mRNA in splenic CD4 positive T cells from mice infected with *B. microti* and *B. rodhaini*.

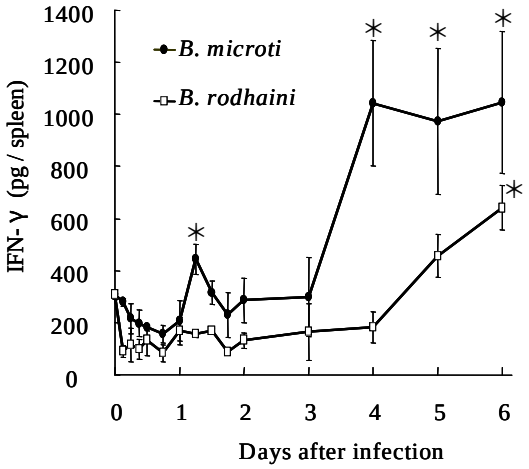

Fig.4. Changes of Serum and splenic IL-12p70 concentration in B. microti and B. rodhaini infected mice.

* : Significant increase ($p<0.05$) compared to the initial level.

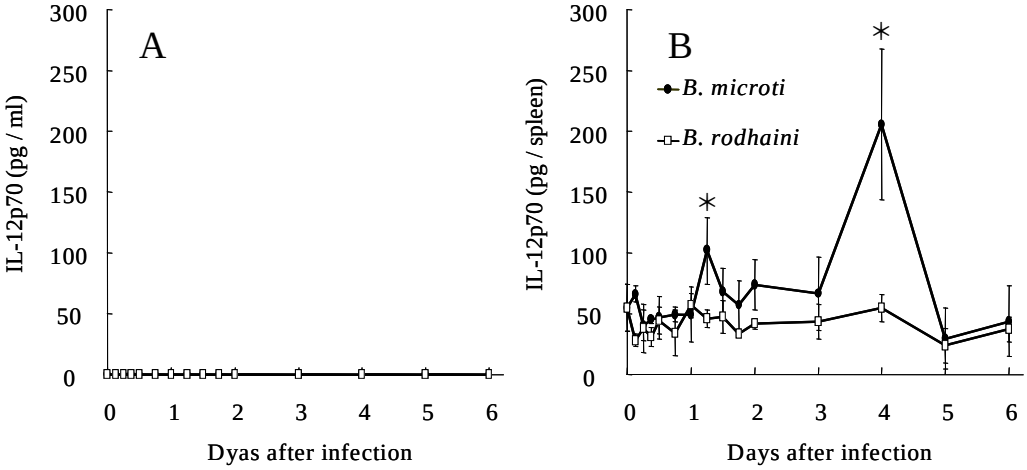

Fig.5 Changes of Serum (A) and splenic (B) IL-12p70 concentrations in *B. microti*- and *B. rodhaini*- infected BALB/ mice.
＊: Significant increase ($p < 0.05$) compared to day 0.

Stevenson, 1999-b).

On the other hand, it is well known that IL-12 is a major cytokine to differentiate Th cell into Th1 cell and to activate natural killer cell (NK cell) (Germann and Rude, 1995; Schoenhaut et al., 1992). Bioactive form of IL-12, however, is IL-12p70 consisted of IL-12p40 and IL-12p35 (Germann and Rude, 1995; Gubler et al., 1991; Schoenhaut et al., 1992). Many researchers measured IL-12p40 level as IL-12, although monomer and homodimer of IL-12p40 showed an imhibitory effect on the biological activity of IL-12p70 (Gillessen et al., 1995. Kato et al., 1996; Ling et al., 1995). Sam and Stevenson (1999-a) indicated that *Plasmodium chabaudi* infected C57BL/6 mice, which is a resistant strain, showed an increase of both IL-12p40 and IL-12p70 level in serum, however infected A/J mice, which is a susceptible strain, showed an increase level of only IL-12p40. Similar results were also reported in *Leishmania donovani* (Quinones et al, 2000) and *Toxoplasma gondii* infection (Robben et al., 2004). Therefore, measurement of IL-12p70

concentration is important for understanding the effect of IL-12 on immnological response of the host, especially Th1 cell differentiation.

In this study, IL-12p70 concentration was detected in only spleen cell homogenates. Our previous reports demonstrated that spleen cells revealed the most important immnological response, especially Th1 cell differentiation and were closely related to the course of infection in *B. microti* and *B. rodhani* infeceted mice (Shimada et al., 1991; Shimada et al., 1992; Shimada et al., 1996). Since anti-parasite DTH response and mRNA expression were observed at 2 days after the infection with *B. microti*, the first increase of splenic IL-12p70 at 30 hrs after the infection was considered to induce Th1 cell differentiation. From these results, splenic IL-12p70 increased with 2 peaks after the infection with *B. microti*, in earlier of which at 30 hrs probably induced Th1 cell differentiation followed by the enhancement of cell-mediated immunity, closely related to the different course of infection between *B. microti* and *B. rodhani* infected mice.

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