Babesia microti ならびに *Babesia rodhaini* 感染マウスにおける脾臓細胞の免疫学的研究

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Babesia microti ならびに *Babesia rodhaini* 感染マウスにおける脾臓細胞の免疫学的研究

(Immunological studies on splenic cell subpopulations in mice infected with *Babesia microti* and *Babesia rodhaini*)

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Chapter 1

General introduction

Intra-erythrocytic protozoa *Babesia* species, *Babesia microti* (BM) and *Babesia rodhaini* (BR) are causative agents of murine babesiosis [2, 10, 21, 55]. It is well documented that these 2 species cause different course of infection in mice. Briefly, BM infection is chronic and non-lethal, whereas BR infection is acute and lethal [2, 10, 21, 55]. Clark et al. [8, 9] reported that mice pretreated with *Bacillus Calmette-Guerin* (BCG), which induced an enhancement of non-specific immune response, showed resistance against BM and BR infection. The mice pretreated with *Toxoplasma* lysate antigen also revealed some protective states following BR infection [18, 19]. Furthermore, many investigators suggested that the difference in the course of infection observed between BM and BR infected mice was mainly dependent on their immunological responses [10, 21, 55]. It was widely accepted that the enlargement of spleen has always been observed in BM and BR infected mice [10]. Ruebush et al. [39] demonstrated that mice transferred with immunized splenic cells revealed significantly lower parasitemia than mice transferred with immunized lymph node cells after BM infection. Zivkovic et al. [54] also reported that adoptive transfer of immunized splenic cells induced the resistance against BR infection in mice. Since splenectomized mice were found to be more susceptible to both BM and BR infection compared to intact mice [10, 17, 32, 35, 38, 42, 50, 52], splenic cells may play important roles in protective immunity against BM and BR infection.

In addition, Ruebush et al. [39] suggested that splenic cells, especially T cells, were closely related to the protective mechanism against BM infection, from the observations that anti-T cell serum abrogated the protective activity of the immunized splenic cells against BM infection, while anti-immunoglobulin serum showed no

effect on the infection. Cavacini et al. [4] also reported that B cell deficient mice showed the similar resistance against BM infection as immunologically intact mice. Zivkovic et al. [56] demonstrated that the resistance of immunized mice against challenge infection with BR was abolished by the administration of anti-T cell serum and suggested that T cells played an important role in the protection against BR infection, like in that against BM infection. However, the effect of adoptive transfer with immunized splenic cell subpopulation has not been examined in BR infected mice.

On the other hand, Meeusen et al. [27] demonstrated that the treatment with the immune serum against BM induced significantly lower parasitemia in BM infected mice as compared to that in non-treated mice. Inchley et al. [20] also demonstrated that the enlargement of spleen after BM infection resulted from B cell proliferation. Igarashi et al. [18] , however, reported that no difference between splenic T and B cell numbers was observed in BR infected mice. It is uncertain whether the dominant splenic cell subpopulation providing the protection against BM and BR infection, especially BR infection, is T or B cell.

Furthermore, Zivkovic et al. [54] reported that 8 mice out of 14 pretreated with cyclophosphamide at a dose that completely suppressed immunoglobulin production survived against BR infection and speculated that the protective effect of cyclophosphamide was caused by elimination of suppressor T cells. Habicht et al. [15] also reported that the incidence of *Babesia* spp. infection was remarkably enhanced in an immunocompromised host. However, there are few informations on protective mechanism by splenic T cells and also the properties of their subpopulation in BM and BR infection. Therefore, in order to understand the cause of

difference in the course of infection between BM and BR infected mice, it was necessary to elucidate the mechanism of protective immunity induced by splenic cells of distinct subpopulations.

In this study, the effects of adoptive transfer of immunized splenic cells on lethal BR infection, changes of splenic subpopulations following BM and BR infection, effects of Lyt-2 positive (Lyt-2^+) and L3T4 positive (L3T4^+) T cell depletion on immunological responses after BM and BR infection, and the expressions of γ-interferon and interleukin-4 mRNA in splenic L3T4^+ T cells were examined in mice.

Chapter 2

Effects of adoptive transfer with immunized splenic cells on *Babesia rodhaini* infection

Many researchers suggested that splenic T cells played an important role in protective mechanism against BM infection as described above [39]. Ruebush et al. [41] also reported that the development of a strong anti-parasite delayed-type hypersensitivity (DTH) response occurred in accordance with the resistance to BM infection. However, there are few reports on the effects of splenic cell transfer on protective immunological response in BR infected mice. Furthermore, It is uncertain whether the dominant splenic cell subpopulation providing protection against *Babesia* spp., especially BR infection, is T or B cell.

Therefore, mortality, anti-parasite DTH response, and anti-parasite antibody titers after BR infection were examined in mice transferred with anti-serum treated or un-treated splenic cells from immunized mice.

Materials and Methods

i) Mice

Female BALB/c mice were bred in Department of Research Center for Protozoan Molecular immunology, Obihiro University (Hokkaido) from breeding pairs obtained from CLEA Laboratory (Tokyo), and 4 and 8 weeks aged mice were used in this experiment.

ii) Parasite

Babesia rodhaini (Australian strain), kindly provided by Kyushu Branch, National Institute of Animal Health, Ministry of Agriculture, Forestry and Fishery, was maintained by blood passage in BALB/c mice.

iii) Immunization

Four weeks aged mice were inoculated intraperitoneally (i.p.) with $1x10^5$ parasitized erythrocytes (PE). When the percentage of parasitemia calculated by Giemza stained blood smear exceeded 1% in peripheral blood, 4-4' diazoamino-dibezamidine diaceturate (Ganaseg, E, R, Squibb & Sons Inc., Manila, Philippines) was administrated intramuscularly (i.m.) at a dose of 0.75 mg/head successively for 7 days. These mice were challenged with $1x10^4$ PE and those which showed no parasitemia for approximately 4 weeks were used as immunized mice. Eight weeks aged mice were used as recipients.

iv) Preparation of immunized splenic cells

Splenic cells from immunized and intact mice were obtained aseptically, minced with scissors, and squeezed between two frosted slide glasses. The cell suspension was filtered through a sterile stainless mesh to remove tissue debris. Erythrocytes were

lysed with warm 0.83% NH_4Cl solution. After washing 2 times in Hank's balanced salt solution (HBSS), the cells were suspended in RPMI 1640 (containing 12 mM HEPES, 150 µl of 2-mercaptoethanol, 100 unit/ml penicillin G, and 100 µg/ml streptomycin sulfate, pH 7.2).

v) Pretreatment of immunized splenic cells

Rabbit anti-mouse T cell and rabbit anti-mouse immunoglobulin (Ig) sera, and low-toxic-M rabbit complement were purchased from Cedarlane Laboratories Limited (Horby, Ontario, Canada). Splenic cells collected were incubated with antibodies in RPMI 1640 containing 0.3% of BSA at an optimal concentration for 60 min at 4 °C. Then, cells were washed once and incubated with complement in the same medium at an optimal concentration for 60 min at 37 °C. After incubation, treated cells were washed 3 times with HBSS and resuspended in RPMI 1640.

vi) Adoptive cell transfer

The untreated or anti-serum treated splenic cells were adjusted to the concentration of $2.5x10^7$ cells/ml with HBSS. The 200 µl of each cell suspension was transferred intravenously to 3 intact 8 weeks aged mice. Mice were divided into 4 groups according to the splenic cells transferred: splenic cells from intact mice (control), un-treated splenic cells from immunized mice (un-treated), T cell depleted splenic cells from immunized mice (D-T cell), and surface Ig positive cells depleted splenic cells from immunized mice (D-Ig cell). And 3 non-transferred mice were also examined as intact control (non-transferred). After transfer, all mice in 5 groups were inoculated i.p. with $1x10^4$ PE.

vii) *Babesia* lysate antigen (BLA)

The blood with parasitemia exceeding 80% was collected by cardiac puncture in heparinized syringe and washed 3 times with physiological saline by centrifugation (400 xg, 8 min, 4 °C). The PE pellet were freezed and thawed with liquid nitrogen for 3 times. The materials was finally centrifuged at 144,000 xg for 30 min at 4 °C. The supernatant was used as BLA for anti-parasite DTH response analysis.

viii) Anti-parasite DTH response

At day 6 ai, all mice were injected subcutaneously with 50 μl of BLA in the right footpad and equal volume of physiological saline in the left footpad after the measurement of their thickness. At 24 hr after the treatment, thickness of both footpads were measured. Swelling rate was quantified with the following formula: Footpad swelling rate (%) = (thickness of right footpad 24 hr after injection - thickness of right footpad before injection) x 100/ thickness of right footpad before injection.

ix) Anti-parasite antibody titer

Serum of each mouse was obtained from orbital plexus venosus using Pasteur pipettes at day 12 ai. Anti-parasite antibody titers were detected by an indirect fluorescent antibody method. Briefly, PE were washed 3 times in physiological saline, applied onto 12 spots in 2 rows on individual glass slides, air dried, and stored at -70 °C until use. Drops of sera at dilutions ranging from 1:4 to 1:4096 were placed over antigen spots and incubated for 30 min at 37 °C in a humid chamber. The slides were washed 3 times in phosphate buffered saline (PBS, 0.01 M, pH 7.2), and drops of fluorescein isothiocyanate-conjugated (FITC) anti-mouse IgM and IgG (Cappel, Westchester, Penna., U.S.A.) were incubated on the

spots for additional 30 min at 37 °C in a humid chamber. Then, the slides were washed as described above and examined under a fluorescence microscope.

Results

i) Parasitemia and Mortality

In control and un-treated groups, remarkably high parasitemias were detected at day 10 and 12 ai and all mice died by day 12 ai (Fig. 1). In D-T cell group, 2 of the 3 mice died at day 12 ai and one mouse survived until the end of the experiment. In D-Ig cell group, 2 of the 3 mice survived and revealed remarkably low parasitemia throughout the experiment (peak parasitemia; 10%).

ii) Anti-parasite DTH response

No significant difference in anti-parasite DTH response was observed among non-transferred, control, and un-treated groups (non-transferred; 14.6±2.0, control; 14.1±4.0, un-treated; 15.4±0.2, Fig. 2). D- T cell group showed almost the same swelling rate (16.0±4.2), whereas D-Ig cell group showed significantly higher rate (28.0±4.2) than that in other groups.

iii) Anti-parasite antibody titers

No difference in both IgM and IgG titers against BR at day 12 ai was observed between non-transferred and control groups (Fig. 3). In un-treated group, both titers (IgM; 1:16, IgG; 1:64) were lower than those in non-transferred and control groups. D-T cell group revealed the same IgM and higher IgG titer (1:4096) as compared to those in non-transferred and control groups. D-Ig group showed lower IgM (1:4) and IgG (1:64) titers as compared to non-transferred and control groups.

Discussion

In this experiment, control group showed about the same course of infection as non-transferred mice. The anti-parasite DTH response and antibody titer in this group were also similar to those in non-transferred group. It was suggested that intact splenic cells transferred had no effect on the course of infection and immunological responses in the recipient mice. However, un-treated group showed similar anti-parasite DTH response and lower anti-parasite antibody titer as compared to non-transferred and control groups. It seemed that the anti-parasite antibody production in the recipient mice might be inhibited by the immunized splenic cell.

On the other hand, one of the 3 mice in D-T cell group survived inspite of higher anti-parasite antibody titer as compared to non-transferred and control groups. Meeusen et al. [27] reported that the mice transferred with immune serum showed significantly lower parasitemia in the course of BM infection as compared to non-transferred intact mice and a significant reduction of parasitemia was observed in mice transferred with B cell enriched immunized spleen cells. However, Cavacini et al. [4] demonstrated that the course of BM infection in B cell-deficient mice was not different from that in immunologically intact mice. Several investigators [1, 12, 26, 28, 31, 34] also reported that hyper immune serum administration could not protect the mice from lethal BR infection. In this experiment, the mortality of D-Ig cell group, which showed lower antibody titers in comparison to non-transferred and control groups was lower than that of D-T cell group. Thus, splenic B cells and anti-parasite antibody production

were considered to play a minor role in protection against BR infection.

In D-Ig cell group, 2 of the 3 mice survived and revealed significantly higher anti-parasite DTH response as compared to those in other 4 groups. The mortality of this group was the lowest among 5 groups. Ruebush et al. [40, 41] reported that the enhancement of anti-parasite DTH response, the well-characterized cell-mediated immune response, was in accordance with the resistance of mice against BM infection. Zivkovic et al. [54, 56] suggested that T cells and macrophages related to the expression of DTH response were required for complete protection against BR infection. Therefore, cell-mediated immune response was considered to play an major role in protection against BR infection.

From these results, it was indicated that splenic T cells played an important role in protective mechanism against BR infection.

Conclusion

To examine the role of splenic cells in protection against BR infection, mortality, anti-parasite DTH response, and anti-parasite antibody titers after BR infection were determined in mice transferred with immunized splenic cells.

Mice were divided into 4 groups according to the splenic cells transferred: splenic cells from intact mice (control), un-treated splenic cells from immunized mice (un-treated), T cell depleted splenic cells from immunized mice (D-T cell), and surface Ig positive cells depleted splenic cells from immunized mice (D-Ig cell). And 3 non-transferred mice were also examined as intact control (non-transferred).

No difference was observed in mortality by day 12 ai among non-transferred, control, and un-treated groups. One of the 3 mice survived in D-T cell group, which revealed a high anti-parasite antibody titer as compared to other 4 groups, whereas 2 of the 3 mice survived in D-Ig cell group, which revealed the lowest anti-parasite antibody titer. These results suggested that splenic B cells and anti-parasite antibody production played a minor role in protection against BR infection. On the other hand, D-Ig cell group showed significantly higher DTH response as compared to other 4 groups. These results indicated that cell-mediated immune response by splenic T cells play a major role in protection against BR infection.

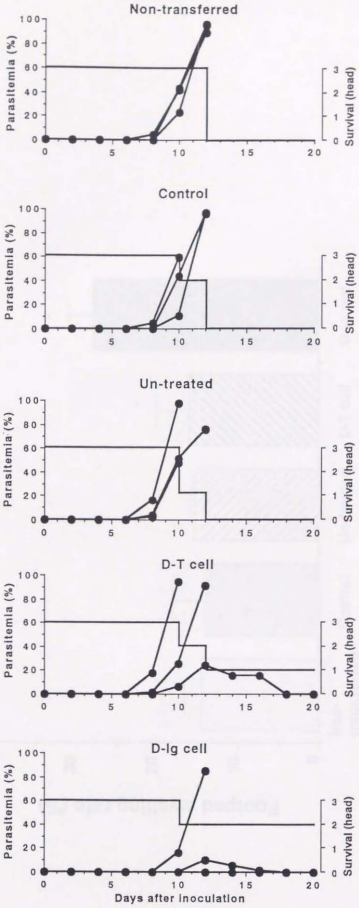

Fig. 1. Change of parasitemia and survival in mice transferred with immunized splenic cells after *B. rodhaini* inoculation. (Non-transferred: no transferred, Control: splenic cells from intact mice, Un-treated: splenic cells from immunized mice, D-T cell: T cell depleted splenic cells from immunized mice, D-Ig cell: surface immunoglobulin positive cell depleted splenic cells from immunized mice.)

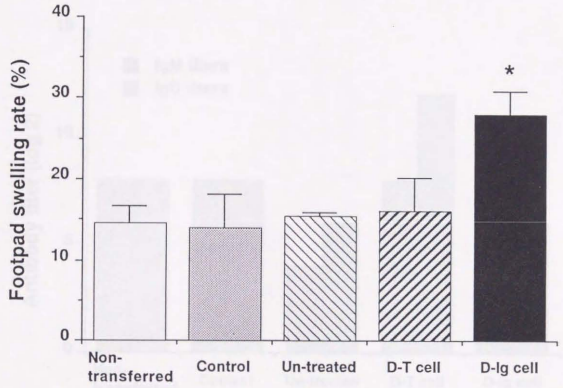

Fig. 2. Delayed-type hypersensitivity response to *B. rodhaini* antigen in mice transferred with immunized splenic cells after *B. rodhaini* inoculation. (Non-transferred: no transferred, Control: splenic cells from intact mice, Un-treated: splenic cells from immunized mice, D-T cell: T cell depleted splenic cells from immunized mice, D-Ig cell: surface immunoglobulin positive cell depleted splenic cells from immunized mice.)
*: significant difference between D-Ig cell group and each of other 4 groups ($p < 0.01$).

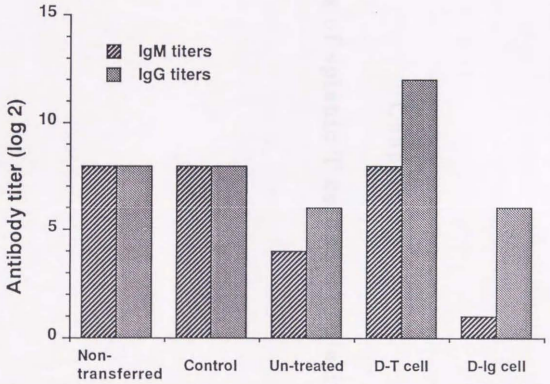

Fig. 3. Antibody titers against *B. rodhaini* in mice transferred with immunized splenic cells at day 12 after *B. rodhaini* inoculation. (Non-transferred: no transferred, Control: splenic cells from intact mice, Un-treated: splenic cells from immunized mice, D-T cell: T cell depleted splenic cells from immunized mice, D-Ig cell: surface immunoglobulin positive cell depleted splenic cells from immunized mice.)

Chapter 3

Changes of splenic T cell subpopulations

From the results in chapter 2, splenic T cells were considered to play an important role in protection against BR infection. Ruebush et al. [40, 41] suggested that the enhancement of anti-parasite DTH response by T cells closely related with resistance to BM infection. Therefore, it is presumed that one of the cause of difference observed in the course of infection with BM and BR was dependent on splenic T cell activities that enhance anti-parasite DTH response. On the other hand, splenic cytotoxic/suppressor (Lyt-2 positive) T cell number increased in BR infected mice [18, 19]. Zivkovic et al. [54] also reported that the resistance of intact mice to BR infection was brought about by the treatment of mice with cyclophosphamide, probably by eliminating suppressor T cells, that inhibit cell-mediated immune response. However, there are few informations available on the dominant populations in splenic T cells providing protection against BM and BR infection.

Therefore, changes of splenic T cell subpopulations and some immunological responses were examined in mice inoculated with BM and BR.

Materials and Methods

i) Mice

Male BALB/c mice aged 4 and 8 weeks were supplied from Nippon SLC Inc. (Shizuoka, Japan).

ii) Parasites

Munich strain of BM and Australian strain of BR were maintained in our laboratory by serial passages of parasitized blood to BALB/c mice.

iii) Immunization and inoculation

Preimmunized mice with BR were prepared as follows: $1x10^5$ PE were injected intraperitoneally to intact mice. When the percentage of parasitemia exceeded 1%, 4-4' diazoamino-dibezamidine diaceturate (Ganaseg, E, R, Squibb & Sons Inc., Manila, Philippines) was administrated intramuscularly (i.m.) at a dose of 0.75 mg/head successively for 5 days. The mice were examined randomly for parasitemia. Mice showing no parasitemia at 6 weeks after inoculation were used as immunized mice. The experimental mice were divided into 3 groups: BM inoculated mice (BM group), BR inoculated mice (BR group), and BR inoculated mice preimmunized with homologous parasite (BR immunized group) as another non-lethal control. Inoculations were performed by a peritoneal injection with $1x10^4$ PE.

iv) Parasitemia and packed cell volume

Parasitemia and packed cell volume (PCV) were examined in 3 mice from each group at an interval of 3 days ai.

v) Anti-parasite DTH response

DTH response was determined in 3 mice from each group at day 6 ai as described in Chapter 2.

vi) Anti-parasite antibody titer

Anti-parasite antibody titers were examined in each mice at an interval of 3 days ai as described in Chapter 2.

vi) Total splenic cell and splenic subpopulation cell number

Total splenic cell and splenic subpopulation cell numbers were determined in 3 mice from each group at an interval of 3 days ai. Splenic cells were prepared as described in chapter 2 and total splenic cell numbers were counted with a hemocytometer. Splenic cells were incubated with various optimally diluted FITC or phycoerythrin (PE)-conjugated antibodies, such as anti-Thy-1.2 monoclonal antibody (mAb), anti-L3T4 mAb, and anti-Lyt-2 mAb (Becton Dickinson Immunocytometry System, California, U.S.A.), anti-IgM Ab, and anti-IgG Ab (Cappel, Westchester, Penna., U.S.A.), for 30 min on ice. After incubation, splenic cells stained were washed 3 times with cold PBS (0.01 M, pH 7.2). Then, each sample was resuspended with PBS and the percents of Thy-1, L3T4, Lyt-2, IgM, and IgG positive ($Thy\text{-}1^+$, $L3T4^+$, $Lyt\text{-}2^+$, IgM^+, and IgG^+, respectively) cells were determined by a Flow Cytometer (Nihon-Bunko Inc., Tokyo, Japan). Absolute numbers of positive cells of each phenotype were calculated by the following formula: cell number = Total splenic cell number x percent of positive cells of phenotype / 100.

Results

i) Parasitemia and PCV

The parasitemia in BM group increased from day 12 ai, to the maximum value of approximately 60% at day 18 ai, and then decreased. The parasitemia in BR group increased from day 9 resulting in the death of all the mice by day 12 ai (Figure 4). The appearance of parasitemia in BR group was earlier than that in BM group. In BR immunized group, no parasitemia was observed after the challenge with homologous parasite. The PCV in BM group decreased, to the minimum value of about 30% at day 18 ai and then increased (Figure 5). In BR group, the PCV decreased from day 9 until the death of the mice. Decrease of PCV started earlier in BR group than that in BM group.

ii) Anti-parasite DTH response

Footpad swelling rate in BM group (36.7 ± 10) was significantly higher than that in BR group (18.3 ± 2.0, $p < 0.01$, Figure 6). Furthermore, the significantly higher rate (42.6 ± 8.0) was observed in BR immunized group as compared to that in BR group ($p < 0.01$).

iii) Anti-parasite antibody titer

Antibody responses against homologous parasites of 3 groups were shown in Table 1. The IgM and IgG titers in BM group increased from day 6 to 12 ai, while those titers in BR group increased from day 3 to 12 ai. In BR immunized group, no change of both titers was observed in the course of infection.

iv) Total splenic cell number

Total splenic cell numbers in both BM and BR groups increased gradually to the maximum value of approximately 2.3×10^8 cells at days 12 and 15 ai, respectively (Figure 7). The BR immunized mice

showed no change in total splenic cell numbers during the course of infection.

v) Splenic subpopulation cell number

Numbers of the IgM^+ cells in both BM and BR groups increased to the maximum value of approximately $1.1x10^8$ at days 10 and 12 ai, respectively (Figure 8). Numbers of the IgG^+ cells also increased to the maximum value of approximately $1.4x10^8$ cells at days 12 and 15 ai, respectively. The maximum number of IgM^+ and IgG^+ cells in both BM and BR groups were identical, although the difference was observed in the period of onset of the increase (at days 6 and 9 ai, respectively). The cell numbers in BR immunized group was similar to the maximum number in BM and BR groups, and did not change throughout the experiment.

The Thy-1^+ cell numbers in BM group increased from day 9 to 12 ai, and retained its maximum value of approximately $6.5x10^7$ cells (Figure 9). The Thy-1^+ cell numbers in BR group increased from day 6 to 9 ai, and retained its maximum value of approximately $5.5x10^7$ cells until the mice died. The maximum number of Thy-1^+ cells in BM group was significantly higher than that in BR group ($p < 0.01$). No change of Thy-1^+ cell numbers was observed in BR immunized group throughout the experiment. Note that this number, approximately $6.5x10^7$, is identical to that observed in BM group.

The ratio of L3T4^+ cell to Lyt-2^+ cell in BM group initially increased at day 3 ai but decreased from day 6 to 15 ai, and then increased again gradually until day 21 ai (Figure 10). The ratio in BR group decreased and showed a minimum value at day 6 ai, and then increased until the mice died. The patterns observed in both BM and BR groups were similar to each other, except for the presence of initial increasing phase in BM group and for the period

at which the minimum value was seen. The ratio in BR immunized group increased to day 6 ai and then decreased gradually until day 12 ai. The pattern in BR immunized group was quite similar to that in BM group.

Discussion

The patterns of changes in parasitemia, PCV, and antibody response observed in this experiment were identical to the results previously reported [10, 21]. Inchley et al. [20] presented that the spleen enlargement after BM infection mainly depend on splenic B cell proliferation. However, Igarashi et al. [18] reported that no differences in both total splenic cell numbers and B cell numbers was observed between BR infected mice and BR immunized mice. In this experiment, no difference was observed in the maximum numbers of total splenic cells and IgM^+ and IgG^+ cells between BM and BR group, and also BR immunized group with the only exception in the period of onset of the increase. The period of onset in BR group was earlier than those in the other groups, which was also observed in the antibody titers. These results suggested that the B cell proliferation or antibody production make little contribution to the difference of resistance and/or parasitemia observed between these 2 species.

On the other hand, significantly higher anti-parasite DTH response were observed in both BM group and BR immunized groups as compared to BR group. The $Thy\text{-}1^+$ cell numbers in both BM group and BR immunized group were also significantly higher than that in BR group. In addition, $L3T4^+$ cell ratio to $Lyt\text{-}2^+$ cell increased at the initial phase of infection in BM group and BR immunized group, while it decreased in BR group. Inchley et al. [21] suggested that early death observed in BR infected mice was closely related to the lack of reduction in T cell proliferation in the early phase. Gross et al. [14] reported that the decrease of $Lyt\text{-}2^-$ T cell/$Lyt\text{-}2^+$ T cell ratio was observed in lethal *Plasmodium berghei*

infected mice and also detected an inhibitory effect of splenic Lyt-2+ T cells on proliferative response of splenic T cells to T cell mitogen. Zivkovic et al. [54] also reported that 8 mice out of 14 pretreated with cyclophosphamide at a suppressive dose for immunoglobulin production survived against BR infection and suggested that the protective effect of cyclophosphamide was caused by elimination of suppressor T cells. Therefore, the decrease of L3T4+ cell/Lyt-2+ cell ratio at the initial phase of infection observed in this experiment, was closely related to the difference in the course of infection between BM and BR infected mice.

Conclusion

To make a comparative investigations in the response of splenic T cell subpopulations between BM and BR infected mice, BM inoculated mice (BM group), BR inoculated mice (BR group), and BR inoculated mice preimmunized with homologous parasite (BR immunized group) as another non-lethal control were examined in the changes of splenic T cell subpopulations and some immunological responses.

BM group and BR immunized group showed significantly higher anti-parasite DTH response as compared to BR groups. The Thy-1^+ cell numbers in both BM group and BR immunized group were significantly higher than that in BR group. In addition, $L3T4^+$ cell ratio to Lyt-2^+ cell increased at the initial phase of infection in BM group and BR immunized group, while in BR group it decreased. These results indicated that the suppression of splenic Lyt-2^+ T cells at the initial phase of infection did not develop in BM and BR immunized groups.

From these results, it was suggested that the splenic T cell induced immunological response, especially the suppressor activity of Lyt-2^+ T cells, at the initial phase of infection was closely related to the difference in the course of infection between BM and BR infected mice.

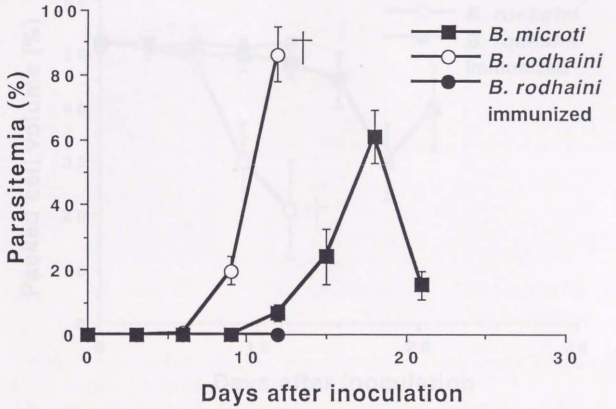

Fig. 4. Changes of parasitemia in mice inoculated with *B. microti* and *B. rodhaini*.

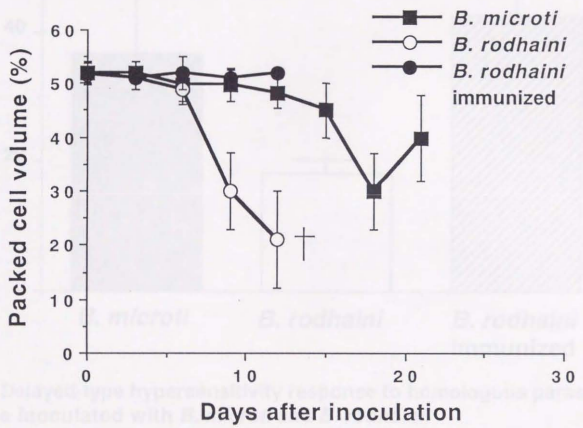

Fig. 5. Changes of packed cell volume in mice inoculated with *B. microti* and *B. rodhaini*.

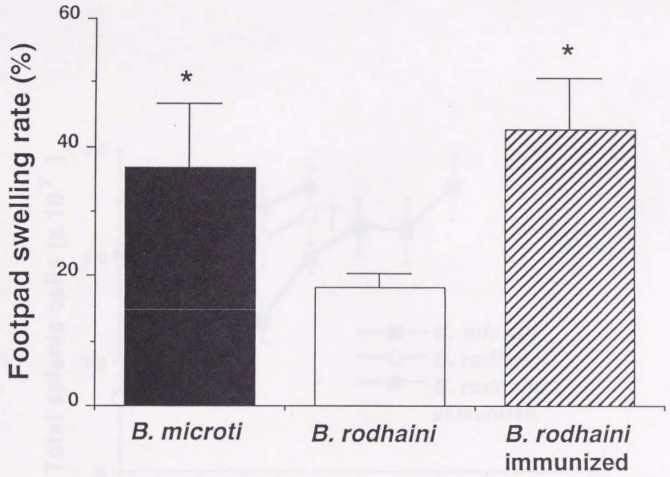

Fig. 6. Delayed-type hypersensitivity response to homologous parasite antigens in mice inoculated with *B. microti* and *B. rodhaini*.
*: significant difference from *B. rodhaini* inoculated mice ($p < 0.01$).

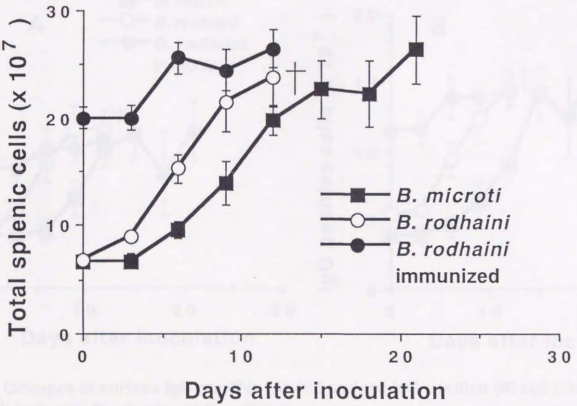

Fig. 7. Changes of total splenic cell numbers in mice inoculated with *B. microti* and *B. rodhaini*.

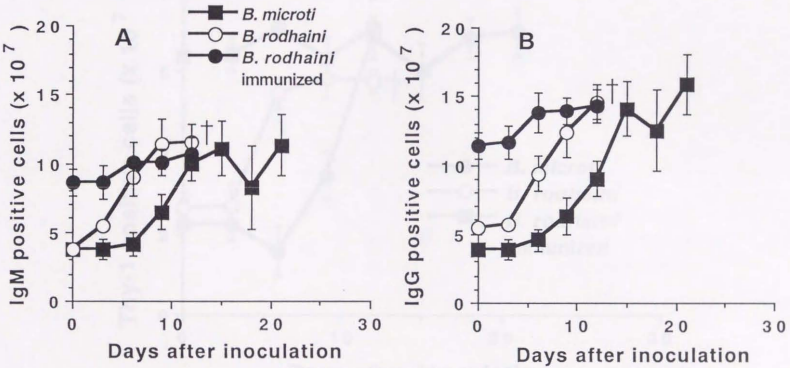

Fig. 8. Changes of surface IgM positive (A) and surface IgG positive (B) cell numbers in mice inoculated with *B. microti* and *B. rodhaini*.

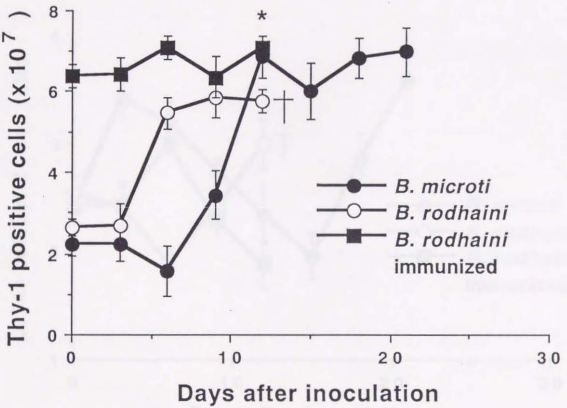

Fig. 9. Change of splenic Thy-1 positive cell numbers in mice inoculated with *B. microti* and *B. rodhaini*.
*: significant difference from *B. rodhaini* inoculated mice ($p < 0.01$).

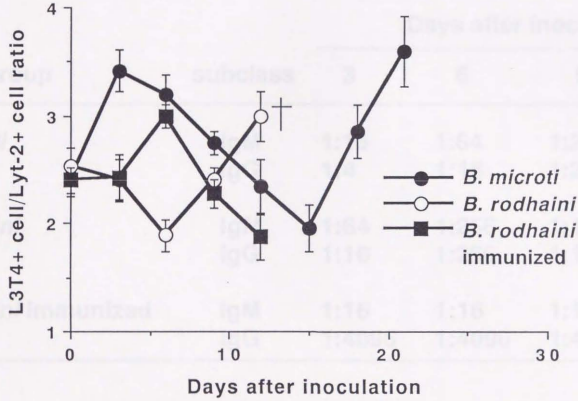

Fig. 10. Change of splenic L3T4 positive cell/ Lyt-2 positive cell ratios in mice inoculated with *B. microti* and *B. rodhaini*.

Table 1. Antibody titers against homologous parasites in mice inoculated with *B. microti* and *B. rodhaini*

Group	subclass	Days after inoculation			
		3	6	9	12
B. micorti	IgM	1:16	1:64	1:256	1:256
	IgG	1:4	1:16	1:256	1:256
B. rodhaini	IgM	1:64	1:256	1:1024	1:256
	IgG	1:16	1:256	1:1024	1:1024
B. rodhaini immunized	IgM	1:16	1:16	1:16	1:16
	IgG	1:4096	1:4096	1:4096	1:4096

Chapter 4

Effects of depletion of T cell subpopulations on *Babesia microti* and *Babesia rodhaini* infection

From the results in chapter 3, the difference in the course of infection between BM and BR infected mice was considered to depend on cellular immunity, especially splenic Lyt-2^+ T cell mediated response at the initial phase of infection. However, Stevenson et al. [48] reported that splenic $CD4^+$ ($L3T4^+$) T cells in susceptible mice suppressed cell-mediated immune response at the initial phase of *Plasmodium chabaudi* AS infection. Moreover, $L3T4^+$ T cells in susceptible mice inhibited anti-parasite DTH response at the initial phase of *Leishmania major* infection [3, 6, 25, 30, 47, 48]. To elucidate the cause of difference in the course of infection of BM and BR, it is necessary to examine the role of T cell subpopulations, Lyt-2^+ and $L3T4^+$ T cells, in the protective mechanism against these two *Babesia* species.

Therefore, changes in the course of infection, anti-parasite DTH response, and anti-parasite antibody titer were investigated in Lyt-2^+ T cell- or $L3T4^+$ T cell-depleted mice after BM or BR inoculation.

Materials and Methods

i) Mice, parasites, and inoculation

Male BALB/c mice aged 7 weeks were used. Both BM and BR were prepared as described in chapter 3 and inoculated by a peritoneal injection of $1x10^4$ PE.

ii) Depletion of Lyt-2^+ and L3T4^+ cells in vivo

Monoclonal antibodies (mAb) of rat anti-L3T4 and rat anti-Lyt-2, and control antibody, rat IgG, were obtained from BIOSYS S.A. (France) and Cappel Inc. (Westchester Penna., U.S.A.), respectively. Lyt-2^+ and L3T4^+ T cell depleted mice (D-Lyt-2 and D-L3T4, respectively) were prepared by intraperitoneal administration with mAb (500 μg at 5 days, and 250 μg at 4 days and 1 day before PE inoculation). Intact and rat IgG injected (Rat IgG) mice were used as controls. In the preliminary study by flow cytometric analysis splenic Lyt-2^+ T cell number in D-Lyt-2 mice and splenic L3T4^+ T cell number in D-L3T4 mice were significantly reduced as compared to those in controls at day 6 after BM or BR inoculation (Table 2).

iii) Parasitemia and PCV

Parasitemia and PCV were determined as described in chapter 3 at an interval of 3 and 6 days ai, respectively.

iv) anti-parasite DTH response

DTH response was determined in each mice at day 6 ai as described in chapter 2.

v) Anti-parasite antibody titer

Anti-parasite antibody titers were determined in each mice at an interval of 6 days ai as described in chapter 2.

Results

i) Parasitemia and PCV

In BM inoculated D-Lyt-2 mice (BM D-Lyt-2), parasitemia at day 15 ai was significantly lower than those in BM inoculated intact and Rat IgG mice (BM controls) ($p < 0.01$). PCV at day 15 ai in BM D-Lyt-2 was also significantly higher than those in BM controls ($p < 0.01$). In contrast, BR inoculated D-Lyt-2 mice (BR D-Lyt-2) showed higher parasitemia than those of BR inoculated controls (BR controls) at day 9 ai. At day 10 ai, PCV in these mice revealed significantly lower than those of BR controls ($p < 0.05$). All BR D-Lyt-2 died at day 10 ai, 2 days earlier as compared to BR controls (Fig. 11).

On the other hand, BM inoculated D-L3T4 mice (BM D-L3T4) showed significantly higher parasitemia than those of BM controls at day 9 ai ($p < 0.05$) and a persistence of high parasitemia until day 21 ai, while no parasitemia was detected in BM controls at day 21 ai. In BM D-L3T4, PCV was significantly lower than those in controls ($p < 0.05$) at day 21 ai. In BR inoculated D-L3T4 mice (BR D-L3T4), significantly lower parasitemia and higher PCV were observed at day 10 ai as compared to those in BR controls (Fig. 12).

ii) DTH response

On footpad swelling rate, BM D-Lyt-2 showed significantly higher, while BM D-L3T4 significantly lower than BM controls ($p < 0.05$, $p < 0.01$, respectively). On the other hand, no significant difference on the swelling rate was observed in both BR D-Lyt-2 and BR D-L3T4 compared to BR controls (Fig. 13).

iii) Anti-parasite antibody titer

Although IgM and IgG titers in BM D-Lyt-2 showed the same values as those in BM controls at day 6 ai, they showed lower values than those in BM controls at day 12 ai. In IgM and IgG titers of BR D-Lyt-2, no difference was observed as compared with those of BR controls at day 6 to 10 ai. On the other hand, both IgM and IgG titers of BM and BR D-L3T4 revealed low at day 6 and 12 ai as compared to each controls (Fig. 14).

Discussion

Depletion of Lyt-2^+ T cells induced strong resistance in mice against BM infection, whereas it increased the susceptibility to BR infection. In addition, depletion of L3T4^+ T cells increased the susceptibility to BM infection in mice, while it induced resistance against BR infection. From the results in chapter 3, it was suggested that the difference in the course of infection between BM and BR infected mice was closely related to the increase of suppressive activity of splenic Lyt-2^+ T cells at the initial phase of infection. However, many investigators [6, 16, 25, 43, 45, 47] demonstrated that pretreatment with anti-L3T4 monoclonal antibody induced exacerbation of infection in resistant strain mice, and also brought about resistance in susceptible strain mice against *Leishmania major* infection. Therefore, the difference in the course of infection between BM and BR infected mice might depend on the respective response of Lyt-2^+ T cells to BM and L3T4^+ T cells to BR at the initial phase of infection.

Depletion of Lyt-2^+ T cells significantly enhanced, while depletion of L3T4^+ T cells significantly reduced anti-parasite DTH response in BM infected mice. However, depletions of either of the cells showed no effect on anti-parasite DTH response in BR infected mice. Lyt-2^+ T cells in BM infected mice, but not in BR infected mice, were considered to suppress anti-parasite cell-mediated immunity at the initial phase of infection. Moreover, L3T4^+ T cells in BM infected mice, but not in BR infected mice, were considered to enhance anti-parasite cell-mediated immunity at the initial phase of infection. Therefore, it was suggested that the suppressive effect of Lyt-2^+ T cells on cell-mediated immunity enhanced susceptibility

to BM infection in mice, while L3T4+ T cells did not enhance cell-mediated immunity, resulting in the exacerbation of BR infection in mice.

On the other hand, Stevenson et al. [48] reported that Lyt-2^+ T cells in *Plasmodium chabaudi* AS infected mice suppressed cell-mediated immunity and enhanced antibody production at the initial phase of infection. In this study, depletion of Lyt-2^+ T cells induced a decrease of anti-parasite antibody production in BM infected mice, whereas no change was observed in BR infected mice. It appeared that Lyt-2^+ T cells in BM infected mice, but not in BR infected mice, enhanced anti-parasite antibody production at the initial phase of infection.

From these results, roles of splenic Lyt-2^+ T cells and/or L3T4$^+$ T cells in the protective immune mechanism in BM infected mice were consider to differ from those in BR infected mice, resulting in the difference in the course of infection between BM and BR infected mice.

Conclusion

To examine the roles of T cell subpopulations in the course of infection, anti-parasite DTH response, and anti-parasite antibody titers were measured in Lyt-2^+ T cell and L3T4^+ T cell depleted mice after BM and BR inoculation.

Depletion of Lyt-2^+ T cells induced strong resistance to BM infection in mice, whereas it increased susceptibility to BR infection. In contrast, depletion of L3T4^+ T cells increased susceptibility to BM infection, while it induced resistance against BR infection in mice. The anti-parasite DTH response in BM infected mice was significantly enhanced by depletion of Lyt-2^+ T cells, while significantly reduced by depletion of L3T4^+ T cells. On the contrary, no effects was observed in either depletion of Lyt-2^+ or L3T4^+ T cells on anti-parasite DTH response in BR infected mice. Lyt-2^+ T cells in BM infected mice, but not in BR infected mice, were considered to suppress anti-parasite cell-mediated immunity at the initial phase of infection. Moreover, L3T4^+ T cells in BM infected mice, but not in BR infected mice, were considered to enhance anti-parasite cell-mediated immunity at the initial phase of infection.

From these results, roles of splenic Lyt-2^+ T cells and/or L3T4^+ T cells in the protective immune mechanism in BM infected mice were considered to differ from those in BR infected mice, resulting in the difference in course of infection between BM and BR infected mice.

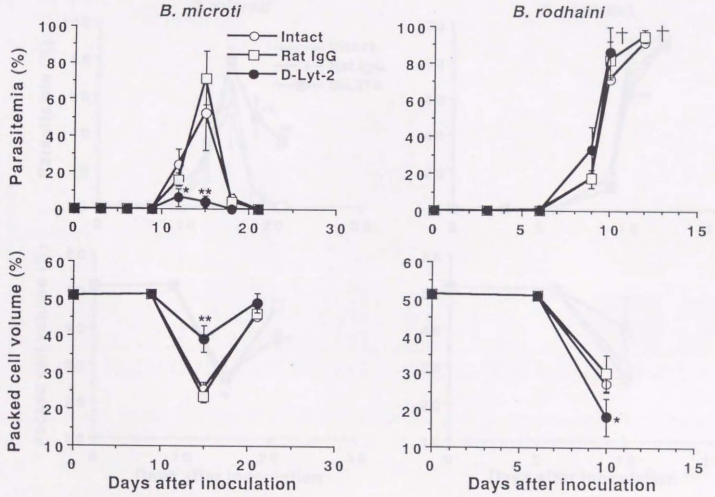

Fig. 11. Change of parasitemia and packed cell volume in Lyt-2 positive cell depleted mice after *B. microti* and *B. rodhaini* inoculation. ○—○: intact mice, □—□: Rat IgG injected mice, ●—●: Lyt-2 positive cell depleted mice. * and **: Significant difference between intact and Lyt-2 positive cell depleted mice. (*: $p < 0.05$, **: $p < 0.01$)

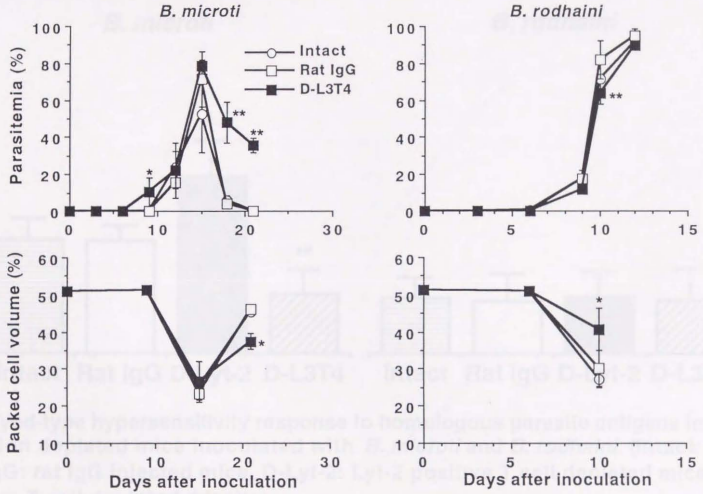

Fig. 12. Change of parasitemia and packed cell volume in L3T4 positive cell depleted mice after *B. microti* and *B. rodhaini* inoculation. ○—○: intact mice, □—□: Rat IgG injected mice, ■—■: L3T4 positive cell depleted mice. * and **: Significant difference between intact and L3T4 positive cell depleted mice. (*: $p < 0.05$, **: $p < 0.01$)

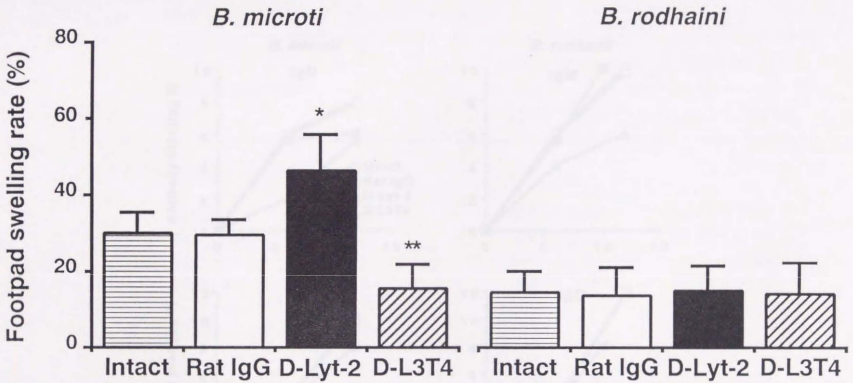

Fig. 13. Delayed-type hypersensitivity response to homologous parasite antigens in T cell subpopulation depleted mice inoculated with *B. microti* and *B. rodhaini*. (Intact: intact mice, Rat IgG: rat IgG injected mice, D-Lyt-2: Lyt-2 positive T cell depleted mice, D-L3T4: L3T4 positive T cell depleted mice)
* and **: Significant difference between intact and T cell subpopulation depleted mice. (*: $p < 0.05$, **: $p < 0.01$)

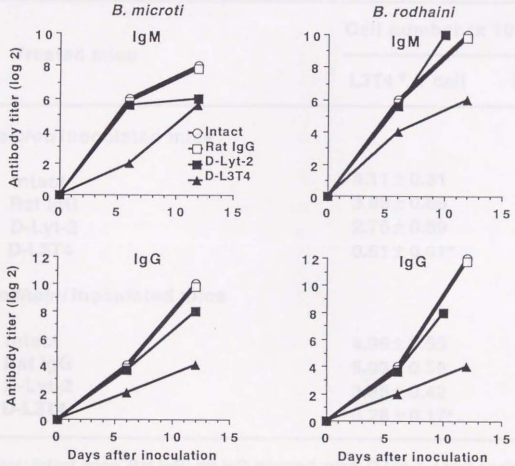

Fig. 14. Change of antibody titers against homologous parasites in T cell subpopulation depleted mice after *B. microti* and *B. rodhaini* inoculation. ○—○: intact mice, □—□: Rat IgG injected mice, ■—■: Lyt-2 positive cell depleted mice, ▲—▲: L3T4 positive cell depleted mice.

Table 2. Cell numbers of splenic T cell subpopulations in T cell subpopulation depleted mice at day 6 after *B. microti* and *B. rodhaini* inoculation

Treated mice	Cell number (x 10^7, mean±SD)	
	L3T4$^+$ T cell	Lyt-2$^+$ T cell
B. microti inoculated mice		
Intact	4.11±0.31	0.91±0.12
Rat IgG	3.98±0.65	0.87±0.15
D-Lyt-2	2.75±0.59	0.15±0.07*
D-L3T4	0.61±0.01*	0.30±0.01
B. rodhaini inoculated mice		
Intact	4.96±0.55	2.62±0.33
Rat IgG	5.03±0.59	2.87±0.27
D-Lyt-2	3.85±0.42	0.37±0.09*
D-L3T4	0.28±0.17*	1.37±0.34

Intact: intact mice, Rat IgG: rat IgG injected mice, D-Lyt-2: Lyt-2 positive T cell depleted mice, D-L3T4: L3T4 positive T cell depleted mice.
*: Significant difference compared with intact mice ($p < 0.001$)

Chapter 5

Expressions of gamma-interferon and interleukin-4 messenger RNA in splenic L3T4 positive T cells

The results in chapter 4 indicated that the difference in the course of infection between BM and BR infected mice resulted from the difference in roles of splenic Lyt-2$^+$ T cell and/or L3T4$^+$ T cell in protective immune mechanism at the initial phase of BM and BR infection. L3T4$^+$ T cells in BM infected mice might regulate cell-mediated immunity, whereas these cells in BR infected mice might regulate anti-parasite antibody production at the initial phase of infection.

It has been well-established that L3T4$^+$ T cells can be divided into two subsets, helper T cell type-1 (Th1 cell) and type-2 (Th2 cell), on the basis of their cytokine production and regulation of immune responses [11, 13, 29, 33, 36, 37, 44, 46, 51]. Briefly, the former cells produced γ-interferon (γ-IFN) and interleukin (IL)-2, and regulated cell-mediated immune response, while the latter cells produced IL-4, IL-5, and IL-6, and regulated humoral one [11, 13, 29, 33, 36, 37, 44, 46, 51]. In addition, the contribution of Th1 and Th2 cells to resolution or progression of infection have been investigated in several parasite infections [5-7, 11, 16, 22-25, 29, 30, 43, 45-49, 51].

Therefore, to clarify the immune response regulated by splenic L3T4$^+$ T cells at the initial phase of BM and BR infected mice, expressions of γ-IFN and IL-4 mRNA in splenic L3T4$^+$ T cells of BM and BR infected mice were examined by reverse transcriptase-polymerase chain reaction (RT-PCR) technique.

Materials and Methods

i) Mice, parasites, and inoculation

Male BALB/c mice aged 8 weeks were used. Parasites, BM and BR, were prepared as described in chapter 3 and inoculated by a peritoneal injection with $1x10^4$ PE.

ii) Monoclonal antibody

The hybridoma producing anti-L3T4 (GK 1.5, IgG_{2b}) mAb were kindly provided from Dr. R. Lelchuk (Wellcome Research Laboratories, Beckenham, Kent, U.K.). Antibodies were partially purified from ascites by the twice precipitation in 50% saturated ammonium sulfate solution. Purified antibodies were used for the collection of $L3T4^+$ T cells.

iii) Collection of $L3T4^+$ T cells

Splenic cells were prepared at an interval of 3 days until day 12 ai as described in Chapter 2. Collection of $L3T4^+$ T cells from the prepared splenic cells were performed by panning method [53]. Briefly, the whole area of a polystyrene Petri dish (bacteriological grade) was covered with 5 ml of anti-L3T4 mAb solution (50 µg/ml in 0.01 M PBS, pH 7.4). The dish was stood overnight at room temperature (RT). After mAb coating, supernatant on the dish was discarded and to block any residual protein-absorbing sites on the dish, the whole area of the dish was covered with 0.2% bovine serum albumin in PBS (0.2% BSA-PBS) for 30 min at RT. After blocking, 4 ml of $5x10^6$ cells/ml splenic cell suspension in 0.2% BSA-PBS were placed on the dish and incubated for 60 min at 4 °C. After incubation, the dish was washed 3 times with PBS to remove non-adherent cells. Adherent cells were collected by scraping the bottom of the dish with a rubber policeman. The percent of $L3T4^+$ T

cells in the adherent cells was determined by flow cytometric analysis as described in Chapter 3. The adherent cells of which $L3T4^+$ T cell percent was over 90% were used in RT-PCR assay.

iv) RNA extraction

Total RNA in the samples was prepared by guanidium thiocyanate-phenol-chloroform extraction method. Briefly, pelleted cells were solubilized in solution-D (containing 4 M guanidinium thiocyanate, 25 mM sodium citrate at pH 7.0, 0.5% sarkosyl, and 0.1 M 2-mercaptoethanol). RNA was isolated by phenol/ chloroform/ isoamyl extraction and precipitated by isopropyl alcohol. After precipitation, RNA was washed with ethyl alcohol, solubilized with solution-D, and reprecipitated by isopropyl alcohol. After washing with ethyl alcohol, RNA was resuspended in diethylpyrocarbonate-treated water. RNA concentrations were determined by absorbance at 260 nm.

v) RT-PCR

Strand synthesis of cDNA was performed by incubating total RNA with 0.125 μM oligo $d(T)_{16}$, 5 U of cloned moloney murine leukemia virus reverse transcriptase (United states Biochemical Corp., Cleveland, OH), 4 mM dNTP, 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1 mM dithiothreitol, and 1 U of RNase inhibitor (Toyobo Inc., Tokyo, Japan) for 20 min at 42 °C. The cDNA product was then amplified in 2.5 U of Taq DNA polymerase (Perkin Elmer Cetus Crop., Norwalk, CT), 5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, and 0.5 pmol/μl of each up/downstream primer using a Program Temp Control System PC-700 (Astec Inc., Tokyo, Japan). The following primer pairs were used:

γ-IFN; upstream primer 5'-CGCTACACACTGCATCTTGG-3' and

downstream primer 5'-GGCTGGATTCCGGCAACA-3'

IL-4; upstream primer 5'-GTCACAGTTTTCAGCTGTATAGGG-3' and
downstream primer 5'-AACACCACAGAGAGTGAGCTCGTCT-3'
The program consisted of one cycle of 95 °C for 2 min, followed by 30 cycles of 95 °C for 1.5 min, 30 cycles of 60 °C for 1.5 min, and one cycle of 60 °C for 1 min. The PCR products were analyzed by 2% agarose gel electrophoresis and visualized by ethidium bromide staining.

In BM infected mice, although the expression of both γ-IFN and IL-4 mRNA were detected at day 3 ai, the former expression was stronger than the latter one. Splenic L3T4$^+$ T cells at days 6 and 9 ai only showed the expression of γ-IFN mRNA. These cells at day 12 ai expressed both γ-IFN and IL-4 mRNA without difference in their intensity. On the other hand, in BR infected mice expression of both γ-IFN and IL-4 mRNA were detected at day 3 and 6 ai in which the latter one was much stronger than the former one. Splenic L3T4$^+$ T cells at day 9 and 12 ai expressed only γ-IFN mRNA (Fig. 15 and 16).

Discussion

In BM infected mice, the expression of both γ-IFN and IL-4 mRNAs were detected at day 3 ai. The expression was stronger in the former than in the latter. Th1 and Th2 cells were reported to differentiate from Th0 cells that possesses the cytokine profiles of both Th1 and Th2 cells [29]. Thus, the subset of splenic $L3T4^+$ T cells in BM infected mice at day 3 ai might be Th0 cell. At days 6 and 9 ai, the expression of only γ-IFN gene was observed, which indicated that Th1 cells were activated in the spleen at the initial phase of BM infection. Some investigators suggested that the subset of splenic $CD4^+$ ($L3T4^+$) T cells was switched from Th1 and Th2 cells during the course of *Plasmodium chabaudi* AS infection [48, 49]. In this study, both cytokine mRNAs were expressed again at day 12 ai. Therefore, The subset of splenic $L3T4^+$ T cells might be switched from Th1 to Th2 cells at day 12 ai in BM infection.

On the other hand, in BR infected mice, the strong expression of IL-4 mRNA were observed at days 3 and 6 ai, although the weak expression of γ-IFN mRNA were also detected at these periods. This indicated that Th2 cells were already activated in the spleen at the initial phase of BR infection, as supported by the observation that $L3T4^+$ T cells from susceptible mice to *Leishmania major* infection produced large amount of IL-4 within 3 days after inoculation [25, 45, 47]. Additionally, the expressions of only γ-IFN mRNA were detected at days 9 and 12 ai, indicating the activation of Th1 cells in the spleen after BR infection.

These results indicated that the subset of splenic $L3T4^+$ T cells activated at the initial phase of infection was Th1 cells in BM infected mice, while it was Th2 cells in BR infected mice. In

Leishmania major infection, Th1 cells were activated in resistant mice at the initial phase of infection, whereas Th2 cells were activated in susceptible mice [6, 16, 25, 30, 43, 45-49, 51]. Furthermore, Stevenson et al. [48] suggested that induction of a strong Th2 response in the initial phase of infection lead to a severe and lethal course of malaria.

Thus, it was suggested that the different course of infection between non-lethal BM and lethal BR infection in mice was resulted from the activation of different subset of splenic $L3T4^+$ T cells at the initial phase of infection.

Conclusion

To elucidate the immune response regulated by splenic L3T4$^+$ T cell at the initial phase of BM and BR infection, expressions of γ-IFN and IL-4 mRNA in splenic L3T4$^+$ T cells of BM and BR infected mice were examined.

In BM infected mice, the strong expression of γ-IFN mRNA was observed at day 3 ai. Splenic L3T4$^+$ T cells expressed only γ-IFN gene at days 6 and 9 ai. On the other hand, in BR infected mice, the strong expressions of IL-4 mRNA were detected at days 3 and 6 ai.

These results indicated that the subset of splenic L3T4$^+$ T cells at the initial phase of BM infection was Th1 cells, whereas it was Th2 cells in BR infection. Therefore, it was suggested that the different course of infection between non-lethal BM and lethal BR infection in mice was resulted from the activation of different subset of splenic L3T4$^+$ T cells at the initial phase of infection.

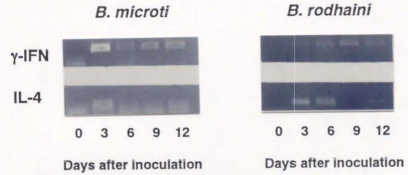

Fig. 15. Expressions of γ-IFN and IL-4 mRNA in splenic L3T4 positive T cells after *B. microti* and *B. rodhaini* inoculation.

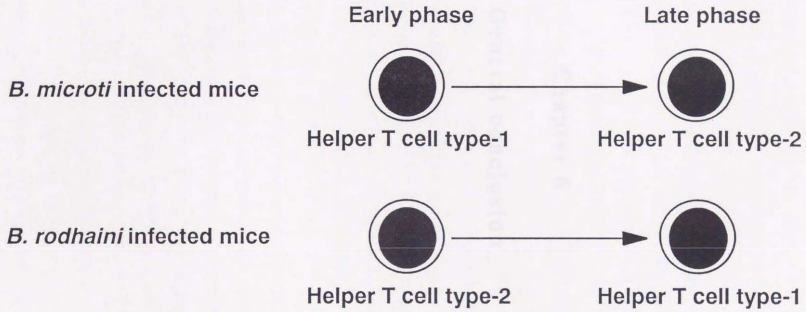

Fig. 16. Activation of helper T cell subsets in course of *B. microti* and *B. rodhaini* infection.

Chapter 6

General conclusion

Intra-erythrocytic protozoa *Babesia* species, *Babesia microti* (BM) and *Babesia rodhaini* (BR) are causative agents of murine babesiosis. It is well documented that these 2 species cause different course of infection in mice. Briefly, BM infection is chronic and non-lethal, whereas BR infection is acute and lethal. Many investigators suggested that this difference was mainly dependent on immunological responses in host. Since splenectomized mice were found to be more susceptible to BM and BR infection as compared to intact mice, splenic cells may play important roles in protective immunity against BM and BR infection.

It was suggested that antibody-independent, T cell mediated immune response regulated the protective mechanism against BM infection. The development of anti-parasite delayed-type hypersensitivity (DTH) response also occurred in accordance with the resistance against BM infection. However, it is uncertain whether the dominant splenic cell subpopulation providing protection against BR infection is T or B cells. Additionally, there are few informations on the role of splenic cells in protective immunity against BM and BR infection.

In this study, the effects of adoptive transfer of immunized splenic cells on lethal BR infection, the changes of splenic subpopulations following BM and BR infection, the effects of Lyt-2 positive (Lyt-2^+) and L3T4 positive ($L3T4^+$) T cell depletion on immunological responses after BM and BR infection, and the expressions of γ-interferon and interleukin-4 mRNA in splenic $L3T4^+$ T cells were examined in BM and BR infected mice.

In chapter 2, in order to elucidate the protective mechanism by splenic cells in BR infection, mortality, anti-parasite DTH response (at day 7 ai), and anti-parasite antibody titers (at day 12 ai) after

BR infection were examined in mice transferred with immunized splenic cells. Mice were divided into 4 groups according to the splenic cells transferred: splenic cells from intact mice (control), un-treated splenic cells from immunized mice (un-treated), T cell depleted splenic cells from immunized mice (D-T cell), and surface Ig positive cell depleted splenic cells from immunized mice (D-Ig cell). Non-transferred 3 mice were also examined as intact control (non-transferred).

In non-transferred, control, and un-treated groups, all of the 3 mice died by BR infection. D-T cell group showed higher IgG titer as compared to other 4 groups, whereas only 1 out of the 3 mice survived in this group. Contrary, in D-Ig cell group which showed the lowest mortality (2 of the 3 mice), the antibody titers revealed to be lowest among 5 groups. Furthermore, this group mounted significantly higher anti-parasite DTH response as compared to the other groups. These results indicated that cell-mediated immune response by splenic T cells play a major role in protection against BR infection.

In chapter 3, changes in the numbers of splenic T cell subpopulations, $L3T4^+$ and $Lyt\text{-}2^+$ cells, the $L3T4^+$ cell/$Lyt\text{-}2^+$ cell ratio in the course of infection, and additionally anti-parasite DTH response (at day 7 ai) were examined in BM inoculated mice (BM group), BR inoculated mice (BR group), and also BR inoculated mice preimmunized with BR (BR immunized group) as a resistant control.

The rapid increase of splenic Thy-1 positive ($Thy\text{-}1^+$) cell numbers after inoculation were observed in both BM and BR groups. However, maximum cell number observed in BM group was significantly higher than that in BR group. BR immunized group showed the similar maximum $Thy\text{-}1^+$ cell number as BM group,

although no change in the cell numbers was observed during the experiment in this group. Furthermore, BM group and BR immunized group revealed the significantly higher anti-parasite DTH response as compared to BR group. On the other hand, the splenic L3T4$^+$ cell/Lyt-2$^+$ cell ratios in both BM group and BR immunized group increased at the initial phase and then decreased gradually, while the ratio in BR group decreased at the initial phase of infection. Therefore, the splenic Lyt-2$^+$ T cells in BR group was considered to activate earlier than those in BM group. The lower anti-parasite DTH response in BR group appeared to reflect the enhancement of suppressive activity of Lyt-2$^+$ T cells. From these results, it was suggested that immune response induced by splenic T cell subpopulations, especially the suppressive activity of Lyt-2$^+$ T cells, at the initial phase of infection was closely related to the difference in the course of infection with BM and BR.

In chapter 4, to clarify the distinct roles of Lyt-2$^+$ and L3T4$^+$ T cells at the initial phase of immune response against BM and BR infection, the course of infection, anti-parasite DTH response (at day 7 ai), and antibody titers (at days 6 and 12 ai) were examined after BM or BR inoculation in mice of which Lyt-2$^+$ T cells or L3T4$^+$ T cells were depleted by administration of monoclonal antibody against each T cell subpopulation.

Depletion of Lyt-2$^+$ T cells induced strong resistance to BM infection in mice, whereas it increased susceptibility to BR infection. In contrast, depletion of L3T4$^+$ T cells increased susceptibility to BM infection, while it induced resistance against BR infection in mice. The anti-parasite DTH response in BM infected mice was significantly enhanced by depletion of Lyt-2$^+$ T cells, while significantly reduced by depletion of L3T4$^+$ T cells. On the contrary,

no effects was observed in the depletion of either Lyt-2$^+$ or L3T4$^+$ T cells on anti-parasite DTH response in BR infected mice. Therefore, Lyt-2$^+$ T cells in BM infected mice was considered to suppress anti-parasite cell-mediated immune response, while these cells in BR infected mice was not. Additionally, L3T4$^+$ T cells in BM infected mice was considered to enhance cell-mediated immune response, while these cells in BR infected mice was not. From these results, it was suggested that the roles of Lyt-2$^+$ and L3T4$^+$ T cells at the initial phase on immune response differed between BM and BR infected mice. These difference were presumed to be related to the difference in the course of infection with BM and BR.

It is well known that L3T4$^+$ T cells are divided into two subsets, helper T cell type-1 (Th1 cell) and type-2 (Th2 cell). Th1 cells produce γ-interferon (γ-IFN) and interleukin (IL)-2, and regulate cell-mediated immune response, while Th2 cells produce IL-4, IL-5, and IL-6, and regulate humoral one. Therefore, in chapter 5, the expressions of γ-IFN and IL-4 mRNA in splenic L3T4$^+$ T cells at the initial phase of infection were examined in BM and BR infected mice by Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) technique.

In splenic L3T4$^+$ T cells from BM infected mice, the strong expression of γ-IFN mRNA was observed at day 3 ai and the expressions of only γ-IFN mRNA were detected at days 6 and 9 ai. At day 12 ai, the gene expressions of both γ-IFN and IL-4 mRNA were observed. On the other hand, in the cells from BR infected mice, the strong expressions of IL-4 mRNA were observed at days 3 and 6 ai. Only γ-IFN mRNA were expressed at days 9 and 12 ai. Therefore, the subset of L3T4$^+$ T cell induced in spleen at the initial phase of infection was Th1 cells in BM infected mice, while it was

Th2 cells in BR infected mice. These results indicated that the host's protective mechanism against BM infection primarily consisted of cell-mediated immune response, whereas that against BR infection primarily consisted of humoral one.

In conclusion, it was suggested that the difference in the course of infection between BM infected mice, which showed chronic and non-lethal infection, and BR infected mice, which revealed acute and lethal infection, was attributed to the difference of immune responses at the initial phase of infection. That is, protective immune response against BM infection primarily consisted of cell-mediated one induced by a $L3T4^+$ T cell subset, helper T cell type-1, with slight suppression by $Lyt\text{-}2^+$ T cells, whereas protective immune response against BR infection preferably consisted of humoral one induced by another $L3T4^+$ T cell subset, helper T cell type-2.

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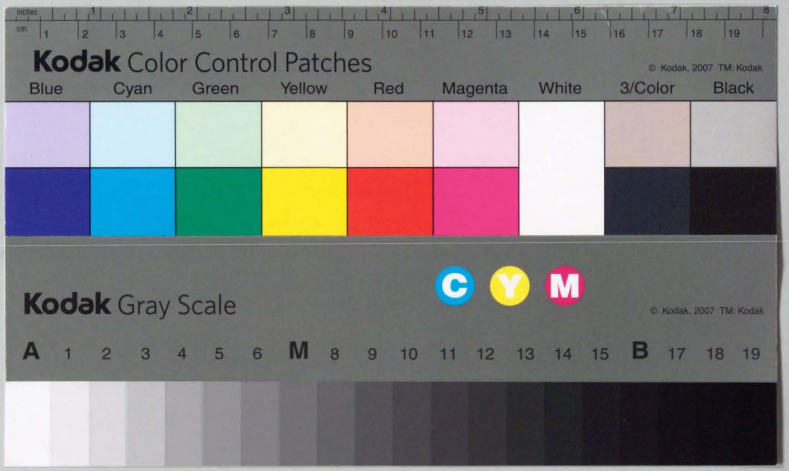
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