Changes of Splenic Lymphocyte Subpopulation in Mice inoculated with *Babesia microti* and *Babesia rodhaini*

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ABSTRACT. Changes of splenic lymphocyte subpopulation after *Babesia microti* and *Babesia rodhaini* inoculation in mice were examined by flow cytometric analysis. The *B. microti* inoculated mice showed a longer period of time from inoculation to the onset of increase or decrease parasitaemia (%), packed cell volume, total spleen cell numbers and surface immunoglobulin positive splenic cell numbers than respective periods in *B. rodhaini* inoculated mice. The Thy-1 positive cell numbers in *B. microti* inoculated mice and *B. rodhaini* inoculated mice pre-immunized with homologous parasites were significantly higher than that of *B. rodhaini* inoculated mice. The ratio of L3T4 positive cell/Lyt-2 positive cell after inoculation with *B. microti* was quite similar to that in *B. rodhaini* mice pre-immunized. However, the ratio in *B. rodhaini* inoculated mice revealed a lack of an increasing phase. These results suggested that the T-cell dependent early immune response, especially suppressor activity, was closely related to the difference in the course of infection between the non-lethal *B. microti* and the lethal *B. rodhaini* infection in mice.—KEY WORDS: *Babesia microti, Babesia rodhaini,* mouse, splenic lymphocyte, subpopulation.

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It has been widely accepted that the enlargement of the spleen is always observed in mice infected with the intraerythrocytic protozoan parasite, *Babesia* spp. [1, 4–7]. Since splenectomized mice were found to be more susceptible to *Babesia* infection than mice with intact spleens [8], the spleen cells were considered to play important roles on the protective mechanism. The transfer of spleen cells obtained from immunized mice showed a protective activity [5, 9–13], and induced significantly lower parasitemia compared with the transfer of immunized lymph node cells [12].

There are 2 major species of *Babesia* in rodents and it is well documented that these 2 species show a different course of infection [1, 7]. Briefly, the *B. microti* infection is non-lethal and chronic, whereas *B. rodhaini* is lethal in mice. These differences in infection were considered to be mainly dependent on the immune response of host against infectious organisms [13, 14]. However, there are few reports of the dominant cell population in splenic cells providing protection from *B. microti* or *B. rodhaini* infection.

This paper consequently deals with the changes of splenic lymphocyte subpopulation after *B. microti* and *B. rodhaini* inoculations in mice by flow cytometric analysis.

MATERIALS AND METHODS

Inoculation and immunization: Male BALB/c mice aged 8 weeks were supplied from Nippon SLC Inc. (Shizuoka, Japan). *Babesia microti* (Munich strain: BM) and *B. rodhaini* (Australia strain: BR) were maintained in our laboratory by serial passages of parasitized blood to the BALB/c mice. Inoculations were performed by a peritoneal injection with 1×10^4 parasitized erythrocytes (PRBC) in 0.2 m*l* of physiological saline. The experimental mice were divided into 3 groups: mice inoculated with *B. microti* (BM mice), mice inoculated with *B. rodhaini* (BR mice), and mice inoculated with *B. rodhaini* and preimmunized with homologous parasite (BR immunized mice) as another non-lethal control. The immunization was carried out by the method described previously [5]; that is, approximately 1×10^5 *B. rodhaini* PRBC were injected intraperitoneally to normal mice. If the percentage of parasitaemia was more than 1% in peripheral blood, 4–4' diazoaminodibezamidine diaceturate (Ganasec, E, R, Squibb & Sons Inc., Manila, Philippines) was administered intramuscularly at a dose of 0.75 mg/head for 5 days. The mice were examined randomly for parasitaemia. Mice with no parasitaemia at 6 weeks after inoculation qualified as immunized mice.

Analysis: Parasitaemia (%, calculated by Giemsa's staining blood smear), packed cell volume

(PCV), total spleen cell numbers, and splenic lymphocyte subpopulation were examined in 3 mice from each group at an interval of 3 days after inoculation (ai). The antibody titer against homologous parasites inoculated was also measured by the indirect fluorescent antibody technique [14]. The spleen cell preparation for total spleen cell counts and flow cytometric analysis was carried out according to the method described by Inchley [6]. Briefly, the spleen was removed from each mouse, cut into small piecies, and forced through a 200 stainless screen mesh with phosphate buffered saline (PBS, 0.01 M, pH 7.2). The cell suspension was mixed with 0.83% NH_4Cl solution for lysing erythrocytes and centrifuged at 800 G for 7 min. Spleen cells were collected and washed 3 times with PBS and counted with a hemocytometer.

A suspension of splenic cells was prepared at a concentration of 2×10^6 cells/m*l* and stained with various optimally diluted fluorescein-isothiocyanate- or phycoerythrin-conjugated antibodies, such as anti-Thy-1.2 monoclonal antibody (mAb), anti-L3T4 mAb, 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.). After incubation for 30 min in ice, splenic cells stained were washed 3 times with cold PBS. Then, each sample was resuspended with 1 m*l* of PBS and the surface phenotype was examined with the use of a Flow Cytometer (Nihon-Bunko Inc., Tokyo, Japan).

RESULTS

Parasitaemia, PCV and antibody response: The parasitaemia in BM mice increased from day 12, showing a maximum value of 60% at day 18, and then decreased. The parasitaemia in BR mice increased from days 9 to 12, inducing the death of hosts (Fig. 1). The appearance of parasitaemia in BR mice was earlier than that in BM mice. In BR immunized mice, no parasitaemia was observed after the challenge with homologous parasite. The PCV in BM mice decreased, showing a minimum value of 30% at day 18 and then increased. In BR mice, the PCV decreased from days 9 to 12 ai (Fig. 2). Decrease in PCV was seen earlier in BR mice than that in BM mice. Antibody responses against homologous parasites of 3 groups are shown in Table 1. The antibody titer in BM mice increased from days 6 to 12 ai, while in BR mice increased from days 3 to 12 ai.

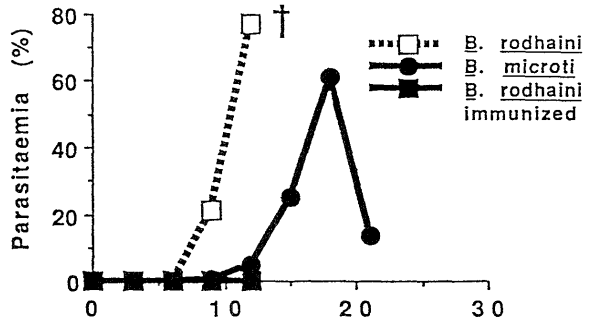

Fig. 1. Changes of parasitaemia in mice inoculated with *Babesia rodhaini* and *Babesia microti*.
---□---: inoculated with *B. rodhaini*;
—●—: inoculated with *B. microti*;
—■—: inoculated with *B. rodhaini* and pre-immunized.
Each point represents the mean value of 3 mice in each group.

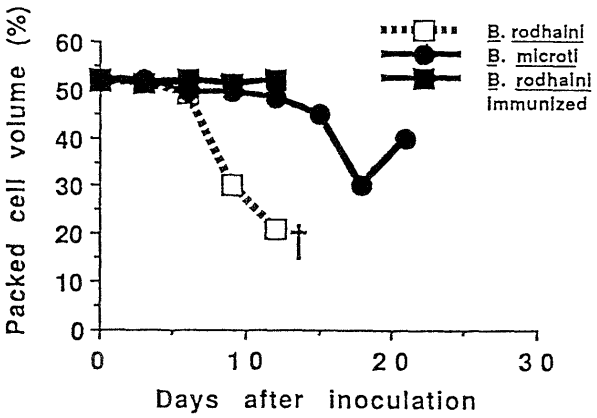

Fig. 2. Changes of packed cell volume in mice inoculated with *Babesia rodhaini* and *Babesia microti*.
---□---: inoculated with *B. rodhaini*;
—●—: inoculated with *B. microti*;
—■—: inoculated with *B. rodhaini* and pre-immunized.
Each point represents the mean value of 3 mice in each group.

Total spleen cell numbers: Total spleen cell numbers in both BM mice and BR mice increased gradually in a peak value of approximately 2.3×10^8 cells at days 12 and 15 ai, respectively (Fig. 3). The BR immunized mice showed no changes of total spleen cell numbers during the challenge inoculation.

Splenic lymphocyte subpopulation : Numbers of the IgM positive cells in both BM mice and BR mice increased to the maximum value of approximately 1.1×10^8 at days 10 and 12 ai, respectively. Numbers of the IgG positive cells also increased to the

Table 1. Antibody response against homologous parasites

Group	Subclass	Days after inoculation 3	6	9	12
B. microti inoculated mice	IgM	1:16	1:64	1:256	1:256
	IgG	1:4	1:16	1:256	1:256
B. rodhaini inoculated mice	IgM	1:64	1:256	1:1024	1:256
	IgG	1:16	1:256	1:1024	1:1024
B. rodhaini immunized mice	IgM	1:16	1:16	1:16	1:16
	IgG	1:4096	1:4096	1:4096	1:4096

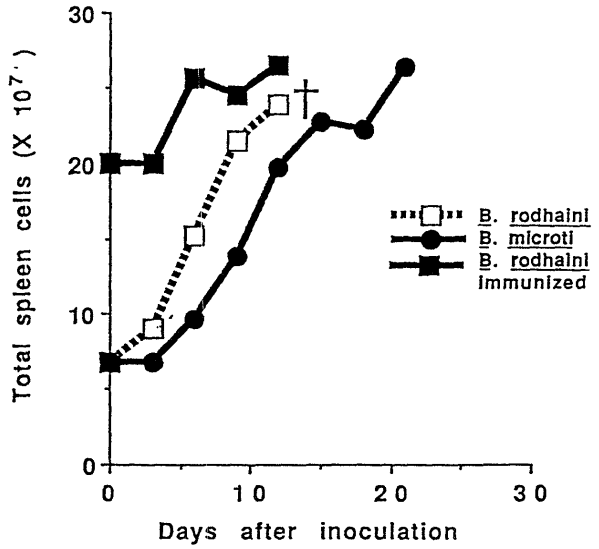

Fig. 3. Changes of total splenic cell numbers in mice inoculated with *Babesia rodhaini* and *Babesia microti*.
---□---: inoculated with *B. rodhaini*;
—●—: inoculated with *B. microti*;
—■—: inoculated with *B. rodhaini* and pre-immunized. Each point represents the mean value of 3 mice in each group.

maximum value of approximately 1.4×10^8 cells at days 12 and 15, respectively (Fig. 4). The peak number of IgM and IgG positive cells in both inoculated mice were similar to each other, the only exception of the difference of the day of onset for increasing cell numbers (at days 6 and 9 ai, respectively). The cell numbers in BR immunized mice were same at the initial onset of the experiment as they did throughout the rest of the experiment. The Thy-1 positive cell numbers in BM mice increased from days 9 to 12 ai, and continued at a value of 6.5×10^7 cells (Fig. 5). The Thy-1 positive cell numbers in BR mice increased from days 6 to 9, keeping at a value of 5.5×10^7 cells. The peak number of Thy-1 positive cells in BM mice was significantly higher than that in BR mice. No change of Thy-1 positive cell numbers was observed in BR immunized mice during the experiment, showing a value of 6.5×10^7 cells. Note that this is the similar cell numbers to that found in BM mice. The ratio of L3T4 positive cells to Lyt-2 positive cells in BM mice

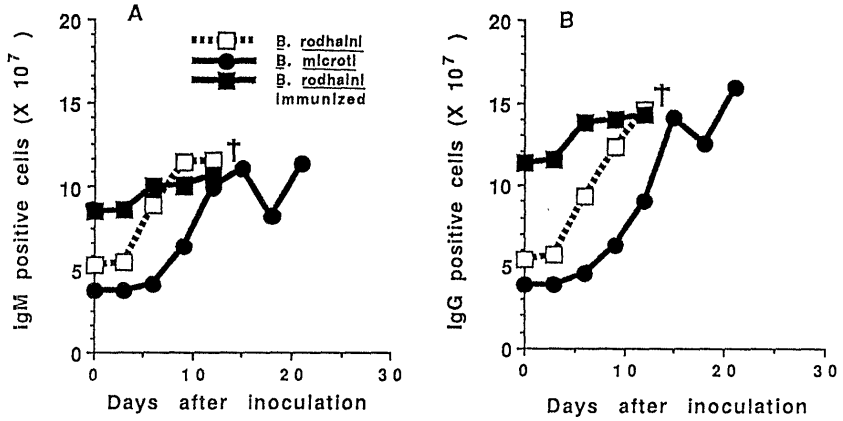

Fig. 4. Changes of splenic surface IgM positive (A) and surface IgG positive (B) cell numbers in mice inoculated with *Babesia rodhaini* and *Babesia microti*.
---□---: inoculated with *B. rodhaini*;
—●—: inoculated with *B. microti*;
—■—: inoculated with *B. rodhaini* and pre-immunized. Each point represents the mean value of 3 mice in each group.

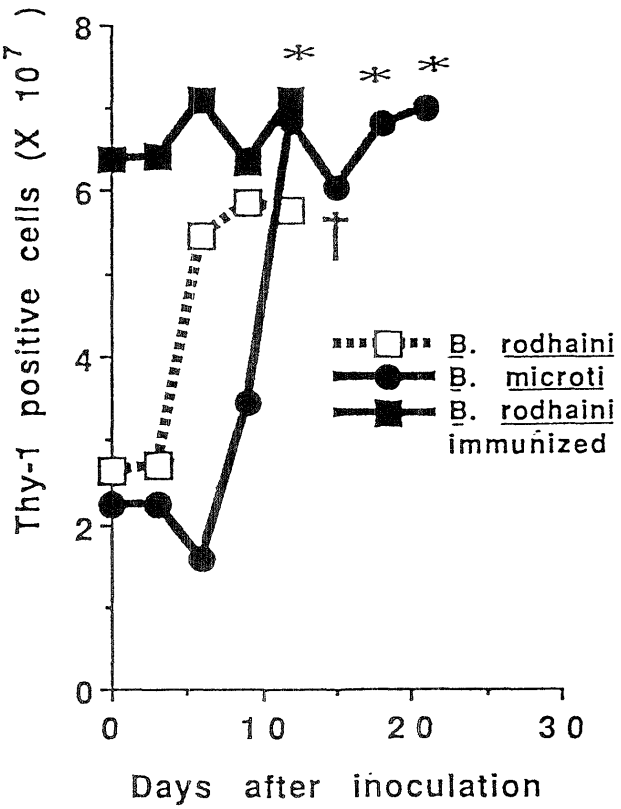

Fig. 5. Changes of splenic Thy-1 positive cell numbers in mice inoculated with *Babesia rodhaini* and *Babesia microti*.
---□---: inoculated with *B. rodhaini*;
—●—: inoculated with *B. microti*;
—■—: inoculated with *B. rodhaini* and pre-immunized.
Each point represents the mean value of 3 mice in each group. *: $p<0.001$

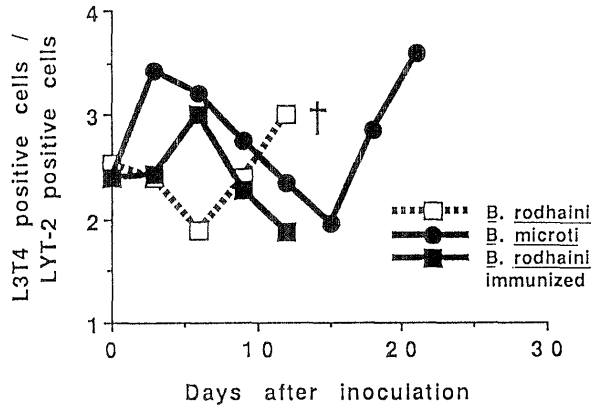

Fig. 6. Changes of L3T4 positive cell/Lyt-2 positive cell ratio in mice inoculated with *Babesia rodhaini* and *Babesia microti*.
---□---: inoculated with *B. rodhaini*;
—●—: inoculated with *B. microti*;
—■—: inoculated with *B. rodhaini* and pre-immunized.
Each point represents the mean value of 3 mice in each group.

initially increased at day 3, decreased from days 6 to 15, and then increased gradually until day 21 ai (Fig. 6). The ratio in BR mice decreased from days 3 to 6, showing a minimum value, and then increased from days 9 to 12 ai. The patterns in both BM mice and BR mice were similar to each other, with exceptions of their initial increasing phase observed in BM mice and the day at which they showed a minimum value. The ratio in BR immunized mice increased to day 6 and then decreased gradually until day 12 ai. The pattern in BR immunized mice was quite similar to that in BM mice.

DISCUSSION

The data presented in this paper showed the differences of splenic lymphocyte subpopulation between lethal and non-lethal *Babesia* infection. The increasing or decreasing patterns of parasitaemia, packed cell volume, and antibody response observed in this study were similar to the results previously reported [1, 7]. It was also shown that the onset day of increasing or decreasing cell volume in BM mice was later than that in BR mice [7]. Many investigators have suggested that the spleen cells play important roles in the protective mechanisms against *Babesia* infection [1, 4, 8, 9, 11]. It has also been suggested that the spleen cells have an effect on the course of *Babesia* infection, being non-lethal in the case of BM mice and lethal in BR mice. Inchley *et al.* [7] reported that the enlargement of the spleen observed in BM mice was later than that in BR mice. The spleen enlargement after BM inoculation resulted from B-cell proliferation [6]. However, Igarashi *et al.* [5] reported that no differences in both total spleen cell numbers and B-cell numbers was observed between BR mice and BR immunized mice. In this experiment, no difference was observed in the peak numbers of total spleen cells and of IgM and IgG positive cells among BM and BR mice, and also BR immunized mice with the only exception being the initial day of the onset for increase in cell numbers. The onset day in BR mice was earlier than those in others, like as shown in the antibody response. These results suggested that the B-cell proliferation might be a minor reason for the different course of the resistance and/or parasitaemia observed in these 2 species.

Ruebush and Hanson [12] reported that the splenic T-cells had a major effect on the protective

mechanisms against *Babesia* infection. They showed that the protective effects of the tranferred spleen cells was abrogated by pretreatment with anti-T-cell serum. It was also noted that athymic nude mice were more susceptible to *Babesia* infection [5, 12]. These observations led to the conclusion that the splenic T-cells might be reason for the difference in the course of infection between BM and BR infected mice. In this study, the Thy-1 positive cell numbers in both BM mice and BR immunized mice were significantly higher than that in BR mice. In addition, L3T4 positive cell ratios to Lyt-2 positive cells increased at the initial phase in BM mice and BR immunized mice, while in BR mice they decreased. These results indicate that the immuno suppressive response did not occur in the early phase of inoculation in BM mice and BR immunized mice. Inchley [6] suggested that early death observed in BR infected mice was closely related to the lack of early phase reduction in T-cell proliferation. Zivkovic *et al.* [13] also reported that 8 mice out of 14 pretreated with cyclophosphamide as a complete 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 difference in the course of infection between BM mice non-lethal and BR mice lethal was considered to be mainly dependent upon the onset of suppressor T- cell proliferation after infections.

On the other hand, Habicht *et al.* [3] reported that the incidence of *Babesia* spp. infection was remarkably enhanced in an immunocompromised host. The variation of immunosuppressive activities between BM mice and BR mice might be related to the difference in the respective course of infection, since Gray and Phillips [2] reported that the BM infection developed immunosuppressive effects on host immune system. Further studies are necessary for the understanding of *Babesia* spp. infection on the immunosuppressive phenomena, as well as on the initiation and memory of immunity, especially macrophage responses suggested by Inchley [7].

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REFERENCES

1. Cox, F. E. G. and Young, A. S. 1969. Acquired immunity to *Babesia microti* and *Babesia rodhaini* in mice. *Parasitology* 59: 257–269.
2. Gray, G. D. and Phillips, R. S. 1983. Suppression of primary and secondary antibody responses and inhibition of antigen priming during *Babesia microti* infection in mice. *Parasite Immunol.* 5: 123–134.
3. Habicht, G., Benanch, J., Leichling, K. D., Gocinski, B. L., and Coleman, J. L. 1983. The effect of age on the infection and immunoresponseness of mice to *Babesia microti*. *Mech. Ageing Dev.* 23: 357–369.
4. Hussen, H. S. 1979. *Babesia microti* and *Babesia hylomysei*: Spleen and phagocytosis in infected mice. *Exp. Parasitol.* 47: 1–12.
5. Igarashi, I., Shimada, T., Omata, Y., Claveria, F. G., Saito, A., Ono, K., and Suzuki, N. 1990. Defference in resistance between hetero and nude Rowell rats and BALB/c mice to *Babesia rodhaini* infection. *Allergy and Immunol.* 9: 95–103.
6. Inchley, C. J. 1987. The contribution of B cell proliferation to spleen enlargement in *Babesia microti* infected mice. *Immunology* 60: 57–60.
7. Inchley, C. J., Greieve, E. M., and Preston, P. M. 1987. The proliferative response of mouse lymphoid tissues during infections with *Babesia microti* or *Babesia rodhaini*. *Int. J. Parasitol.* 17: 945–950.
8. Ivin, A. D., Young, E. R., Osborn, G. D., and Francis, L. M. A. 1981. A comparison of Babesia infections in intact, surgically splectomized, and congenitally asplenic (Dh/f) mice. *Int. J. Parasitol.* 11: 251–255.
9. Meeusen, E., Lloyd, S., and Soulsby, E. J. L. 1984. *Babesia microti* in mice. Subpopulations of cells involved in the adaptive transfer of immunity with immune spleen cells. *Aust. J. Exp. Biol. Med. Sci.* 62: 567–575.
10. Meeusen, E., Lioyd, S., and Sousby, E. J. L. 1984. *Babesia microti* in mice. Adaptive transfer of immunity with serum and cells. *Aust. J. Exp. Biol. Med. Sci.* 62: 551–566.
11. Roberts, J. A. 1968. Adoptive transfer of immunity to *Babesia rodhaini* by spleen cells from immune rats. *Aust. J. Exp. Biol. Med. Sci.* 46: 807–808.
12. Ruebush, M. J. and Hanson, W. L. 1980. Transfer of immunity to *Babesia microti* of Human Origin using T lymphocytes in mice. *Cell. Immunol.* 52: 255–265.
13. Zivkovic, D., Seinen, W., Kuil, H., Albers-van Bemmel, C. M. G., and Spekshijder, J. E. 1983. Immunity to Babesia in mice. Adaptive transfer of immunity to *Babesia rodhaini* with immune spleen cell. *Vet. Immunol. Immunopathol.* 5: 343–358.
14. Zivkovic, D., Speksnijder, J. E., Kuil, H., and Seinen, W. 1983. Immunity to *Babesia* in mice. Cross protection between various *Babesia* and *Plasmodium* species and its relevance to the nature of *Babesia* immunity. *Vet. Immunol. Immunopathol.* 5: 359–368.