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Cellualr Subsets Involved in Protective Immunity to *Babesia rodhaini* Infection in BALB/c Mice

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ABSTRACT: Protective immune responses and the functional role of spleen cells in mice infected with *Babesia rodhaini* were examined with an in vitro proliferation assay systems and by in vivo passive transfer of spleen cells to uninfected mice. Mice that resolved primary babesial infection after chemotherapy (*Babesia* immune mice) had transient and low parasitemia after challenge infection and high rates (75%) of survival. *Babesia* hyperimmune mice, by contrast, had no detectable parasitemia after challenge and 100% survival. Proliferative response of spleen cells to *Babesia* lysate antigen (BLA) were determined for mice from both groups. This proliferative response was inhibited by treatment of spleen cells with anti-T cell serum and monoclonal antibody (MAb) to Lyt l antigen. Spleen cells of hyperimmime mice produced larger amount of IL-2 production than those of immune mice. Transfer of spleen cells from immune mice to nonimmune mice provided protection against babesial infection and recipient mice had high titers of anti-babesial antibody. When these spleen cells were treated with anti-T cell serum or anti-mouse Ig serum, protection against challenge was abolished. By contrast, transfer of hyperimmune spleen cells was capable of protecting recipient mice. Treatment of hyperimmune spleen cells with antiserum to mouse Ig or MAb against Lyt 1 and Lyt 2 antigens did not interfere with their ability to protect recipient mice against infection, even though recipient mice had low levels of antibody production. These results indicate that humoral immune response is important in establishing protection after primary infections while the panicipation of Lyt 1^+ cells and Lyt 2^+ cells and other aspects of the cell-mediated immune response is important in controlling secondary infections.

INTRODUCTION

Babesia rodhaini and *B. microti* have been widely used as experimental models for investigating babesiosis in domestic animals. These two rodent parasites differ widely in pathogenicity. Infections with *B. microti* are non-lethal and self-limiting while *B. rodhaini* are virulent and usually fatal. Mechanisms mediating the clearance of these parasites are not fully understood, but involve both humoral and cell-mediated aspects of immune system.

Adoptive transfer of spleen cells from mice with chronic infections of *B. microti* was effective in reducing parasitemia in recipient mice (Meeusen et al, 1984; Ruebush and Hanson, 1980). In this experiment, B cells were necessary to establish protection during primary infections while T cells played an important role in secondary infections. The role of NK cells in establishing protective immunity to babesial infections is controversial (Eugui and Allison.1980; Wood and Clark, 1982). Mice arc not able to

completely clear primary infections with *B. rodhahni*. However, sensitization of mice with *Toxoplasma* lysate antigen increases survival to as 40%. Numbers of T cells, B cells, and NK cells increase in surviving mice while numbers of thymic Thy-1 cells decrease (Igarashi et al., 1990). After drug treatment, mice could control primary infections (Cox and Young, 1969) and mice were resistant to subsequent challenge infections. Spleen cells Isolated from these animals were capable of transferring protection (Roberts, 1968; Zivkovic el al., 1983). The mice that resolved reinfection with
B. rodhaini were strongly protected against challenge infections. Spleen cells from these mice, however, could not transfer protection to recipient mice (Honda, 1989). These results suggest that subpopulations of spleen cells function at different times during development of infections and play important roles in development of protective immunity to *Babesia*. Protective immune responses and functional role of spleen cells were examined in the present study through use of in vitro proliferation assays and in vivo passive transfer of spleen cells to uninfected mice.

MATERIALS AND METHODS

Animals and Parasites: Female BALB/c mice were bred in our facilities from breeding pairs obtained from CLEA Laboratory (Tokyo) and were 4-5 wk old at the start of experiment. *Babesia rodhaini* of 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. Parasitemias and the course of infections were monitored by examination of Giemsa-stained thin smears of tail blood.

Immunization procedure: Mice were inoculated intraperitoneally (i.p.) with $1x10^5$ parasitized erythrocytes (PE) followed by repeated intramuscular (i.m.) injection of 0.125ml of diminazene diaceturate (Ganaseg, 5 mg/ml in distilled water) over the course of 1 week. Four weeks after inoculation (a.i.), mice did not have detectable PE in the peripheral blood and were considered to be immune. Immune mice were inoculated again with $1x10^4$ PE. Individuals that survived more than 4 wk were classified as hyperimmune (Fig. 1). Uninfected mice of the same age were used as controls.

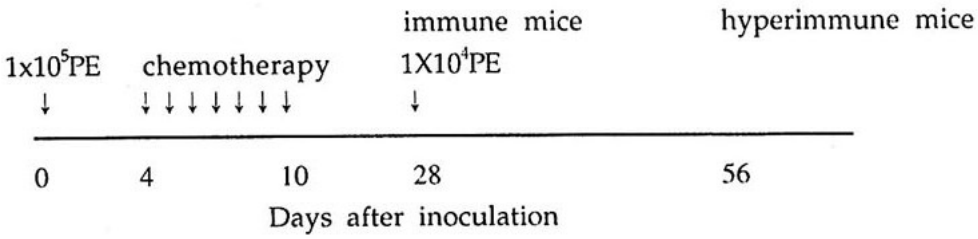

Fig.l. Immunization schedule used in the preparation of *Babesia* immune and hyperimmune mice.

Preparation of Babesia lysate antigen (BLA): Blood with parasitemias exceeding 80% was collected by cardiac puncture in heparinized syringes and was washed three times with saline by centrifugation (400g, 8 min, 4^{O}C). Pelleted PE were subjected to 3 repeated freeze-thaws with liquid nitrogen. The thawed material was centrifuged at 144,000g for 30 min at 4^{O}C. The supernatant was used as BLA.

Preparation of spleen cells: Spleen cells of immune, the hyperimmune and control mice were removed aseptically, minced with scissors, and squeezed between two frosted slides. The cell suspension was filtered through a sterile stainless mesh to remove tissue fragments. Erythrocytes were lysed with warm 0.83% NH_4Cl. After two washes in Hanks balanced salt solution, the cells were suspended in RPMI 1640 (Flow Laboratories, Inc., Irvine, Scotland) supplemented with 12 mM HEPES, 150 µ1 of 2-mercaptoethanol, penicillin G (100 units/ml), and streptomycin sulfate (100 µg/ml).

Antibodies: Antibodies used for in vitro cell depletion included rabbit anti-T cell and rabbit anti-mouse immunoglobulins(Ig) serum and anti-Lyt 1.2 and anti-Lyt 2.2 monoclonal antibodies (MAb). All antibodies as well as low-toxic-M rabbit complement were purchased from Cedarlane Laboratories Limited, Horby, Ontario, Canada. For the depletion in vitro, spleen cells were incubated with antibodies for 60 min at 4^{O}C, washed once, and then incubated for 60 min at 37^{O}C with low-toxic-M rabbit complement.

Proliferative response of spleen cells: Spleen cells were prepared as described above and cell density was adjusted to $1x10^6$cells/ml with RPMI 1640 supplemented with 5% heat-inactivated fetal calf serum (FCS). Aliquots of 0.1 ml of cell suspension were incubated in 96-well flat-bottom tissue culture plate (Falcon 3079, Becton Dickinson, U.S.A.) in a 5%CO_2 incubator at 37^{O}C for 5 days. Wells containing 0.1 ml of medium alone or medium containing BLA at 1:150 dilution were run in triplicate. During last 6-7 h incubation, 0.2 µ Ci of [^{3}H]-thymidine in 50 µl of complete medium was added to each well. Cells were harvested on filler paper with an automatic cell harvester. Incorporated radioactivity was measured in a liquid scintillation spectrometer.

Interleukin 2 activity. Interleukin 2 (IL-2) activity of the immune, the hyperimmune, and control mice was examined by incubating spleen cells in RPMI 1640 containing 10% FCS at a cell density of $2x10^6$ cells/ml. After 24 h incubation, spleen cells were cultured with or without BLA (1:300 dilution) for another 48 h, and supernatant was collected for measurement of IL-2 activity.

IL-2 activity was measured according to the method of Theander et al. (1986). IL-2 dependent CTLL were kindly provided by Prof. Hideo Nariuchi, Institute for Medical Science, University of Tokyo, and cultured in a Celgrosser-H medium (Sumitomo Pharmaceutical Co., Osaka) supplemented with 10% FCS and 5% rat IL-2. Rat IL-2 was prepared by incubating rat spleen cells ($5x10^6$cells/ml) with Concanavalin A (2 µg/ml) for 24h. After incubation, the supernatant was collected and mixed with methyl alpha-D-mannopyranoside (Sigma Chemical Co., St. Louis, U.S.A.) at a concentration of 10-20 mg/ml. This mixture was used for culture of CTLL. CTLL were collected 3 days after subculture and washed 3 times in RPMI 1640. One hundred µl of aliquots of Celgrosser-H medium containing a suspension of CTLL cells at a density of $1x10^5$ cells/ml were incubated for 24h in 96-well culture plate with 100µl serial dilution of standard mouse IL-2 (Inter-cell Technologies Inc., Somerville, New Jersey) or with culture supernatants described earlier. Blastogenic response was measured by the incorporation of [^{3}H]-thymidine as described above. IL-2 activity was expressed with Probit analysis by Gillis et al. (1981).

Adoptive Cell transfer: Spleen cells prepared from the immune mice, the hyperimmune, and the control mice were adjusted to a density of $2.5xl0^7$ cells/ml. Two hundred μl of each cell suspension was transferred intravenously to untreated mice. Following transfer, $1x10^4$ PE were inoculated i.p. The parasitemia, delayed type hypersensitivity and humoral antibody titers were measured.

To determine whether parasites were present in spleen cell suspension, spleen cells from the immune or hyperimmune mice were injected into untreated mice. Blood smears were prepared and examined regularly for more than 20 days to monitor parasitemia, but no evidence of transmission was detected.

Indirect immunofluorescence test: Antibody levels were detected by an indirect fluorescent antibody method (Waki et al., 1974). PE were washed three times in 0.85% saline, applied to 12 spots in 2 rows on individual glass slides, air dried, and stored at -70 ^{O}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^{O}C in a moist chamber. The slide were washed 3 times in phosphate buffered saline (PBS) and drops of fluorescein isothiocyanate-labeled anti-mouse IgM or IgG were incubated on the spots for additional 30 min at 37^{O}C. The slides were then washed as above and coverslipped with glycerin-PBS before examination with a fluorescence microscope.

Measurement of delayed-type hypersensitivity: Delayed-type hypersensitivity (DTH) response was measured as described by Katsura (1975). Six days after cell transfer and challenge infection, all mice were injected subcutaneously with 50 μl of BLA in the right footpad and 50μl of 0.85% saline in the left footpad. Twenty four hours after administration of challenge inoculation, thickness of both footpads was measured. Swelling was quantified with the following formula: Rate of food swelling = 100 x [thickness of footpad 24 h after inoculation (mm) -thickness of footpad before inoculation (mm)] / [thickness of footpad before inoculation (mm)].

RESULTS

Course of challenge infection: Immune and control mice were divided into groups of four and inoculated i.p. with $1x10^4$ PE. Percent parasitemia was monitored in all animals every 2 days(Fig. 2).The control and immune groups had average parasitemia less than 1% by days 8 a.i. By 12 days a.i., mice in the control group had parasitemias that averaged 90.6 ±5.6% while those in the immune group had significantly lower parasitemias of only 10 ± 7.1% ($P<0.001$). All animals in the control group were dead by 14 days a.i. One of 4 immune mice was dead by 14days a.i., but 3 remaining mice survived without subsequent mortality or detectable parasitemias to the end of the experiment. There were no fatalities in the group of hyperimmune mice. Two of these mice had transient parasitemias on the 8th (0.011 ± 0%) and 10th day (0.015 ± 0%) a.i. Differences in parasitemia between immune and hyperimmune mice were significant ($P<0.001$).

Proliferative response to BLA of the spleen cells: Data of proliferative response of spleen cells to the specific antigen BLA are shown in Table 1. Proliferative responses of spleen cells from immune and hyperimmune mice were significantly higher than those of control mice ($P<0.001$). After treatment with anti-T cell serum and anti-Lyt 1.2 MAb, proliferative response of spleen cells from the immune mice was significantly reduced. MAb to Lyt 2.2 antigen, however, had no effect on proliferative response. Incubation of immune spleen cells with antiserum to mouse Ig reduced to mitogenic response by 45%.

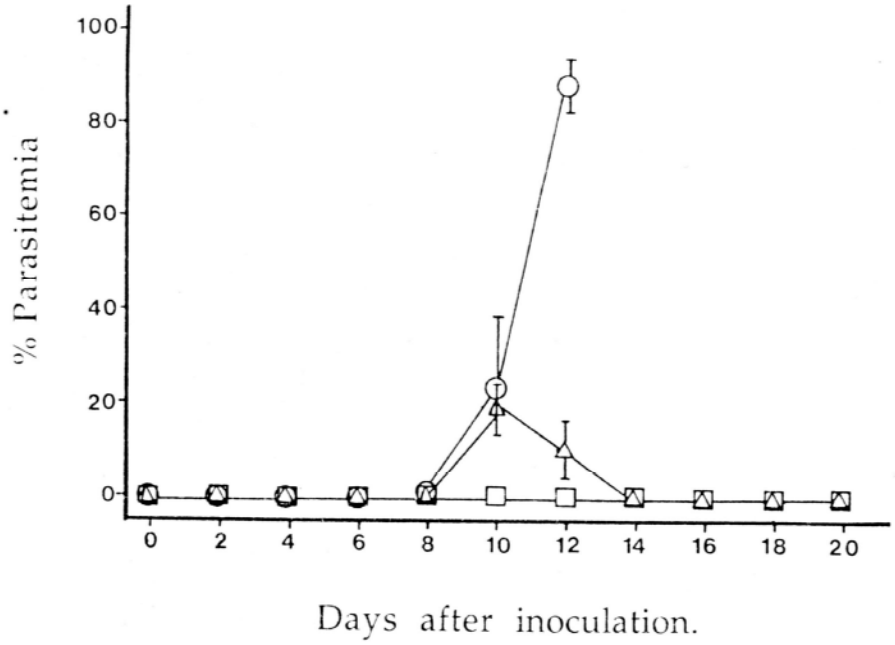

Fig. 2. Progression of parasitemia of *B.rodhaini* infection in control(○), immune (△),and hyperimmune (□) mice (Mean; ⊢⊣ SD, n=4).

Table 1. The effect of antibody treatment on proliferative response of spleen cells to *Babesia* lysate antigen

Spleen cells	Antibody treatment	^{3}H-Thymidine incorporation (Mean cpm ± SD, n=3)	
		medium	BLA
Normal mice		4324±615	3477±545
Immune mice	medium	6798±1044	44570±5425
	anti-mouse Ig	1469±474	21477±4511
	anti-T cell	1461±65	254±115
	anti-Lyt 1.2	1044±501	306±55
	anti-Lyt 2.2	11203±525	53015±2647
Hyperimmune mice	medium	198±22.8	33156±5937
	anti-mouse Ig	238±44.3	34371±3126
	anti-T cell	202±18.5	339±14.3
	anti-Lyt 1.2	185±40	267±20
	anti-Lyt 2.2	524±419	37707±283

Table 2. Interleukin 2 activities in the supernatant of *Babesia* lysate antigen activated spleen cells obtained from normal, immune and hyperimune mice.

	normal	immune	hyperimmune
medium	0.085[a)]	0.11	0.05
BLA	0.098	0.22	0.55

a) Units of IL-2 per ml of cell supernatant

Table 3. Transfer of immunity against *B. rodhaini* infection in recipient mice by injection of subpopulations of spleen cells from immune and hyperimmune mice.

Antibody treatment	Survival rate	Peak * parasitemia	Antibody ** titer IgM	IgG	DTH *** response (%)
Normal mice	0/3	90%≦	1:256	1:4	14.1±4.0
Immune mice					
medium	2/3	42%	1:256	1:4096	39.6±7.9
anti-mouse Ig	0/3	90%≦	1:4	1:64	33.6±3.3
anti-T cell	0/3	90%≦	1:16	1:64	10.2±0.7
Hyperimmune mice					
medium	0/3	90%≦	1:16	1:64	15.4±0.4
anti-mouse Ig	2/3	10%	1:4	1:64	28.0±3.0
anti-T cell	1/3	24%	1:256	1:4096	16.0±4.2
anti-Lyt 1.2	2/3	0%	1:4	1:16	29.8±8.5
anti-Lyt 2.2	2/2	13%	1:16	1:256	43.0±1.4

* Examined on 10 days after inoculation (a.i.).

** Examined by indirect immunofluorescence test at 12 days a.i.

*** Examined on 7 days a.i..

Proliferative response of spleen cells from hyperimmune mice were also reduced significantly when they were treated with antiserum to T cell or MAb to Lyt 1.2 antigen ($P<0.001$). Treatment with antiserum to mouse Ig or MAb to Lyt 2.2 antigen had no effect on proliferative response of hyperimmune mice.

Production of IL-2: Incubation of spleen cells from the immune mice and hyperimmune mice with specific antigen BLA resulted in production of IL-2. The hyperimmune mice produced larger amounts of IL-2 than immune mice (Table 2).

Active transfer of spleen cells from immune mice: Twenty four hours after intravenous transfer of spleen cells from control and immune mice to recipient normal mice, $1x\ 10^4$ PE were inoculated i.p. into the recipients. All recipient mice that received normal mouse spleen cells died by 14 days a.i. (Table 3). By contrast, 2 of 3 recipient mice that received transfers of immune spleen cells survived the challenge infection. These mice had higher titers of IgG antibody and a larger DTH response than control mice. Treatment of immune spleen cells with antiserum to T cells or mouse Ig eliminated their protective ability. A DTH response was present in mice that received spleen cells treated with antiserum to mouse Ig, but did not occur in mice that received T cell depleted spleen cells.

Active transfer of spleen cells from hyperimmune mice: Recipient mice that transferred with hyperimmune spleen cells were unable to survive challenge infections with *Babesia* (Table 3). When hyperimmune spleen cells were treated with antiserum to T cell or mouse Ig, however, one of 3 and 2 of 3 recipient mice survived, respectively. Antibody titers in mice that received spleen cells depleted with anti-mouse Ig had lower titers (1:4 for IgM and 1:64 for IgG) than mice that received T cell-depleted spleen cells (1:256 for IgM and 1:4096 for IgG). Furthermore, spleen cells treated with anti-Lyt 1.2 MAb or anti-Lyt 2.2 MAb could transfer protection to recipient mice (Table 3). However, no increase of antibody titers was observed in these mice. Mice that received spleen cells treated with anti-mouse Ig serum, anti-Lyt 1.2 or anti-Lyt 2.2 MAb had significantly higher DTH responses than that received T cell -depleted spleen cells.

DISCUSSION

The present study was undertaken to examine the differences between the protective mechanisms of immune (able to control primary infections) and hyperimmune (able to survive reinfection) mice. Both immune and hyperimmune mice were resistant to challenge infection, although hyperimmune mice were more strongly protected than immune mice.

Two different in vitro assays were used to measure the functional ability of spleen cells from immune and hyperimmune mice. In malaria infections, the high proliferative responses of immune mice are believed to result from activation of cell-mediated immunity (Weinbaum et al., 1976). In the present study, proliferative responses of spleen cells to BLA were observed in both the immune and hyperimmune mice. The blastogenic response was reduced significantly by the treatment of spleen cells with antibody to T cell or Lyt 1 antigen, but not with antibody to mouse Ig or Lyt2 antigen. These results indicate that proliferative response to babesial antigen is a T cell dependent reaction.

High IL-2 levels was also detected in immune and hyperimmune mice. It has been shown that IL-2 is important in malarial infections (Theanderet al, 1986), but its role in babesial infections is still not clear. Since IL-2 is produced by Lyt 1^+ cells in mice (Miller and Stutman, 1982), its increased production in immune and hyperimmune mice indicates activation of this cell type in infected mice. These in vitro results suggest that T cells, especially Lyt 1^+ cells, are activated by babesial antigen in immune or hyperimmune mice and may play an important role in development of immunity to babesial infections.

To examine this hypothesis, adoptive spleen cell transfer was done. Studies of *B. microti* have shown that transfer of cellular fractions rich in B cells was effective in producing strong resistance to primary infections, while transfer of fractions rich in T cells was effective in producing resistance to reinfection (Meeusen et al, 1984). In the present study, spleen cells from the immune mice could transfer the

protective immunity to recipient mice. The recipient mice produced high liters of IgG antibody. Treatment of spleen cells with antibody to T cell or mouse Ig abolished protection, and mice that received these cells had low antibody titers. These data indicate that antibodies play a major role in the development of protective immunity after primary infection and that cooperation of T cells is essential for the production of antibody by B cells.

Transfer of spleen cells from hyperimmune mice failed to protect uninfected mice from infection even though the cells were more effective than those from immune mice in providing resistance to challenge infections. Hyperimmune spleen cells treated with antibody against T cells or mouse Ig, however, were still able to confer protection to recipient mice after challenge. Mice that received spleen cells treated with antiserum to T cells had higher antibody titers, but lower rates of survival than those that received cells treated with antiserum to mouse Ig. This suggests that T cells are more important than B cells development of defensive mechanisms after secondary infection.

We were unable to identify the subpopulation of T cells from hyperimmune mice that plays the most important role in development of cell-mediated immunity. Spleen cells treated with anti-Lyt 1.2 or anti-Lyt 2.2 MAb could still transfer protection to recipient mice, although there was some difference in degree of protection. Transfer of Lyt 1^+, 2^- (mostly $CD4^+$, $CD8^-$) or Lyt 1^-, 2^+ (mostly $CD4^-$, $CD8^+$) splenic T cells from immune to nonimmune animals provides protection in malarial infection 1-2 wk after spontaneous cure (Mogil et al., 1987). Jayawardena et al. (1982) found that Lyt 1^+, 2^- but not Lyt 1^-, 2^+ T cells could transfer protection 8-10 wk after resolution of infection. Although kinetics of $CD4^{+-}$or $CD8^+$ cells was not examined in the present study, similar changes in splenic T cell populations may occur at different times in mice that are hyperimmune to babesial infections.

DTH response is a well-characterized cell-mediated phenomenon that involves complex interactions between T lymphocytes and macrophages. It is often used as a measure for expression of cell-mediated immunity. Ruebush et al. (1986) found that anti-parasite DTH reactions are correlated with resistance to infection with *B. microti*. Its importance as a protective mechanism in infections with *B. rodhaini* is not clear, however, because the correlation between DTH response and protection was only found when spleen cells from hyperimmune mice were used for transfer. Cher et al. (1987) found recently that the DTH response was mediated by Thl clones, indicating that DTH responses in babesial infections should be correlated with functional ability of Thl cells in future studies. Nonspecific protective effects of tumor necrotic factor (TNF), one of lymphotoxins, have been reported (Clark et al., 1987). In malarial infections, lymphokines such as gamma-interferon (Shofield et al.,1987), TNF (Taverne et al.,1987), and IL-2 (Theander et al.,1986) have also been reported to be important in development of protective immunity. All of these factors are produced by Thl cells (Cherwinski et al., 1987).

The present study suggests that different protective mechanisms occur in immune and hyperimmune mice. T cells provide protective immunity to babesial infections in both types of mice, but have different functions at various stages of infection. The role of specific subsets of T cells should be examined in future studies of protective mechanisms.

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