実験的赤血球内寄生原虫症の感染防御に関する基礎的研究

研究課題番号　０３０６６０３１８

平成５年度科学研究費補助金（一般研究Ｃ）研究成果報告書

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目　次

はしがき

赤血球内寄生原虫性疾患であるピロプラズマ病は家畜に大きな被害を与えている。しかしながら、薬剤耐性や毒血接種における白血病ウィルス感染等の問題があり、現在のところ効果的な治療及び予防法は確立されていない。このため有効で安全なワクチンの開発が強く切望されており、そのためには、感染防御機構を解明することが極めて重要と考えられる。本研究は実験的マウスバベシア病を用いて防御免疫を担う細胞とその機構を明かにすることを目的として計画された。また、腎臓障害について臨床病理及び免疫病理学見地から検討した。

致死性の*B.rodhaini*感染において初感染と再感染に耐過したマウスでは抵抗性および関与する細胞が異なることが明かになった。また、非致死性の*B.microti*感染において、Ｔ細胞群のなかのＣＤ４陽性細胞が原虫排除に重要な役割を果すことが判明し、この細胞群によって産生されるサイトカインの一つであるIFN-γが*B.microti*感染の原虫の排除の初期に部分的に関与することが明かとなった。さらにバベシア免疫マウスより得られたモノクローナル抗体は、７０,３０kDaの抗原を認識し、マウスに投与すると、*B.microti*及び*B.rodhaini*感染における原虫血症の遅延が認められた。また、ネコを終宿主とする胞子虫類*Isospora felis*が*B.microti*初感染に対して非特異的感染防御能を賦与することが示された。バベシア原虫の宿主への障害性について検討した所、致死性の*B.rodhaini*は非致死性の*B.microti*よりも肝臓、腎臓に強い障害性があることが示された。

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研究成果の概要

1）*B. rodhaini* 感染における防御機構

B. rodhaini は致死性の原虫で、マウスに感染させると全例死亡するが、ガナゼックで治療することにより、感染死を防御することができる。これらのマウスを用い、再感染防御能について検討した。感染後化学治療を行ったマウスに再感染を行うと、低い原虫血症を示した後７５％のマウスが生残し、抗体が再感染防御に重要であることが示された。一方、同様の経過を経て再感染に生残したマウスにもう１度感染させると、殆ど原虫血症を示さずに１００％生残し、Ｔ細胞が重要な役割をはたしていることが明かとなった。

2）*B. microti* 感染における防御機構

B. microti は非致死性の原虫で、原虫感染により高い一過性の原虫血症を示した後生残耐過する。しかし、ヌードマウスおよびSCIDマウスにおける感染では高い原虫血症が持続した。次にＴ細胞のどのサブセットがバベシア原虫排除に関与しているか検討するために、BALB/cマウスに抗CD4モノクローナル抗体（mAb）、抗CD8mAbを投与し*B. microti* を感染させたところ、抗CD４mAb処理マウスが高い原虫血症を示した。これらの結果は、*B. microti* 初感染においてCD4陽性Ｔ細胞が原虫の排除に重要な役割を果していることを示している。

次にCD4陽性細胞がどのような機構でバベシア原虫の排除に関与しているのか検討するために、*B. microti* 感染マウスの脾臓細胞をバベシア抗原と共に培養し、培養上清中のサイトカインの産生を測定した。その結果、非処理および抗ＣＤ８モノクローナル抗体処理マウスの培養上清に高いIFN-γが認められたが、抗ＣＤ４モノクローナル抗体処理マウスでは認められなかった。またIL-4活性は何れのマウスの培養上清中にも認められなかった。更に抗IFN-γモノクローナル抗体と抗IL-4モノクローナル抗体で感染マウスを処理したところ、抗IFNγモノクローナル抗体処理マウスにおいて高い原虫血症を認めた。これらの結果より、ＣＤ４陽性細胞によって産生されるIFN-γが*B. microti* 感染の原虫の排除の初期に部分

的に関与することが明かとなった。今後、IFN-γ以外のサイトカインの役割について検討する必要がある。

3）バベシア感染を防御する特異的・非特異的抗原

*B.microti*感染耐過マウスに*B.microti*および*B.rodhaini*で攻撃感染を行ったところ、*B.microti*感染に対してばかりでなく、*B.rodhaini*攻撃感染マウスも全例生残し、強い抵抗性が認められた。また、このようにして得られた免疫マウスよりモノクローナル抗体を作成し、その性質につき検討した。得られたモノクローナル抗体は、７０,３０kDaの抗原を認識し、マウスに投与することにより、*B.microti*及び*B.rodhaini*感染における原虫血症の遅延が認められた。

ＢＣＧや*Corynebacterium parvum*が非特異的にバベシア感染を防御することが知られているが、ネコを終宿主とする胞子虫類*Isospora felis*の*B. microti*初感染に対する感染防御能について検討した。*I. felis*オーシスト接種マウスにおける*B.microti*感染に対する防御効果は*I. felis*接種後の時間的経過に伴い亢進し、接種後４週間後に顕著な抵抗性を示した。*I. felis*接種マウスに抗ＣＤ４あるいは抗ＣＤ８モノクローナル抗体を投与し、*B. microti*に対する感染抵抗性を検討したところ、抗ＣＤ４抗体を投与した群において抵抗性が減弱し、ＣＤ４陽性細胞の重要性が示唆された。

4）宿主への障害性

バベシア原虫の宿主への障害性の機序を解明するため、致死性の*B. rodhaini*及び非致死性の*B .microti*のマウスに対する障害性を肝臓、腎臓につき臨床病理、病理学的に比較検討した。*B.rodhaini*感染では*B.microti*感染に比較し障害の程度が高く、ヘモグロビン尿症、高分子の蛋白質尿が顕著であった。光・電顕的検索では高電子密度の沈着が認められ、原虫抗原、抗体、補体の存在により免疫複合体であることが推察された。肝臓では散在性の壊死巣、細胞浸潤がみとめられたたが、*B. microti*感染では変化が認められないかあるいは認められても軽度であった。

研究成果

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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 Lyt1 antigen. Spleen cells of hyperimmune 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 participation 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 are not able to completely clear primary infections with *B. rodhaini*. 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 et 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 s pleen 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 $1x10^1$ PE. Individuals that survived more than 4 wk were classified as hyperimmune (Fig. 1). Uninfected mice of the same age were used as controls.

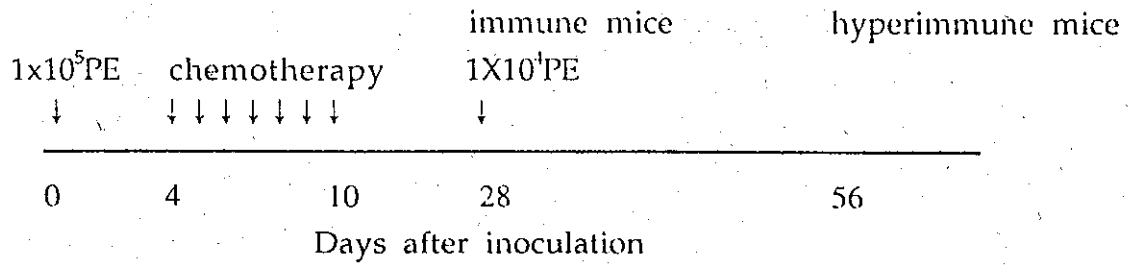

Fig.1. Immunization schedule used in the preparation of *Babesia* immune and hyperimmune mice.

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℃). 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 ℃. 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 μl 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-Lyt1.2 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, splenn cells were incubated with antibodies for 60 min at 4℃, washed once, and then incubated for 60 min at 37℃ 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℃ 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 filter 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 24 h. 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 μ l of aliquots of Celgrosser-H medium containing a suspension of CTLL cells at a density of $1x10^5$ cells/ml were incubated for 24 h in 96-well culture plate with 100 μl 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 $2.5x10^7$ 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 ℃ 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℃ 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℃. 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 50 µl 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 14 days 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

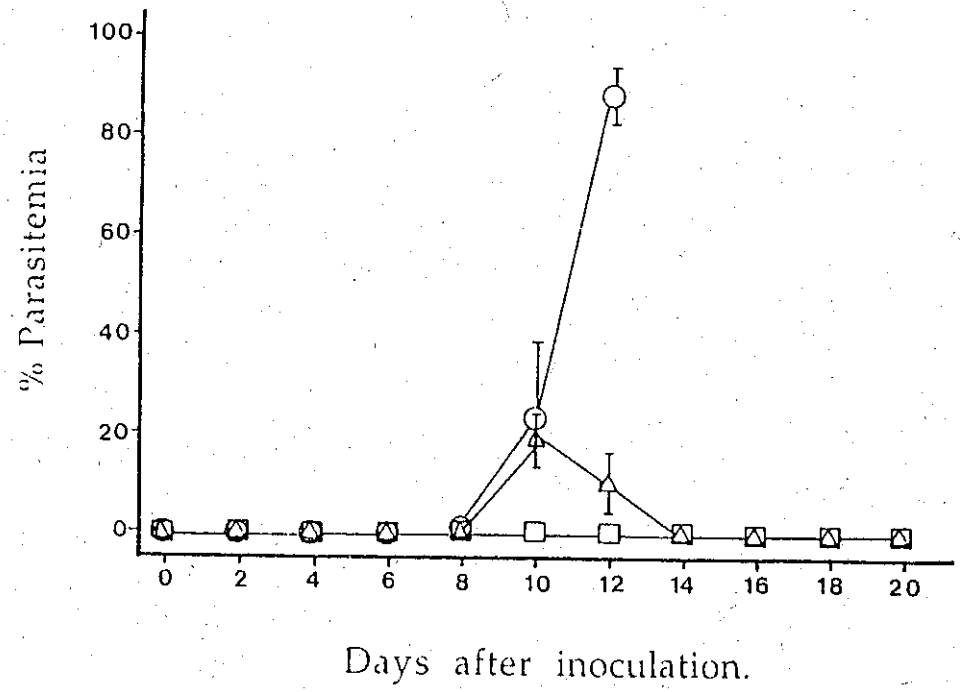

Fig. 2. Progression of parasitemia of *B.rodhaini* infection in control(○), immune (△),and hyperimmune (□) mice (Mean; ⊢⊣ SD, n=4).

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..

immune spleen cells with antiserum to mouse Ig reduced to mitogenic response by 45%. 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, $1x10^4$ 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 hyperimmun 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 Lyt 2 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 (Theander et 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 titers 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 Th1 clones, indicating that DTH responses in babesial infections should be correlated with functional ability of Th1 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 Th1 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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Comparison of Damage to Kidneys and Liver Caused by Lethal *Babesia rodhaini* Infection and Non-lethal *Babesia microti* Infection in Mice

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Key words: *Babesia rodhaini*, *Babesia microti*, kidneys, liver, mice infection

ABSTRACT

Two mouse babesioses, lethal *Babesia rodhaini* and non-lethal *Babesia microti* infections, were examined to determine if damage to kidneys and liver is correlated with the prognosis of these infections. All *B. rodhaini*-infected mice died after a sudden increase of parasitemia, severe hemolysis, and excretion of reddish hemoglobinuria. All *B. microti*-infected mice survived after a temporary moderate increase of parasitemia, moderate hemolysis, and excretion of greenish urine. *B. rodhaini*-infected mice showed immune complex-induced mesangiopathic glomerulonephropathy, moderate renal tubular necrosis, and extensive liver necrosis. In the glomerulonephropathy, electron microscopy showed electron-dense deposits in the mesangial matrix and along the glomerular basement membrane. Biochemical analysis of blood and urine from these mice confirmed renal damage in terms of increased BUN and of proteinuria that contained albumin and protein of more than 200kDa molecular weight, and hepatic damage in terms of an increase in serum direct bilirubin. *B. microti*-infected mice had relatively mild immune complex-induced mesangiopathic glomerulonephropathy, mild renal tubular necrosis, and focal liver necrosis. BUN and serum direct bilirubin showed no increase, and proteinuria contained no detectable proteins of more than 200kDa. These data suggest that the severity of damage in the kidneys and liver is correlated with the prognosis of the two *Babesia* infections.

INTRODUCTION

Babesia is a species of hemoprotozoan parasite that induces babesiosis in domestic and experimental animals. *Babesia* infection induces hemolytic anemia, hemoglobinemia, and subsequent disorder of kidneys and liver. Damage of kidneys and liver is the most conspicuous pathological change in *Babesia* infections. In the kidneys, pathological changes include renal tubular necrosis induced by hemoglobinemia (Habela et al. 1991, Hussein 1977b, Liddel et al. 1980) and/or glomerulonephritis (Maegraith et al. 1957, Rogers 1971, Hussein 1977b) induced by immune

complexes(Annable and Ward 1974). Pathological changes in the liver encompass focal, mid-zonal or centrolobular necrosis of the parenchyma (Habela et al.1991, Liddel et al. 1980, Hussein 1977a, Maegraith et al. 1957, Rogers 1971, Paget et al. 1962). In mouse babesiosis, *B. rodhaini* and *B. microti* infections show different prognoses. *B. rodhaini* is lethal to mice whereas *B. microti* generally is not lethal (Clark and Allison 1974). We compared these two mouse babesioses that have different clinical outcomes with regard to pathological changes occurring in kidneys and liver, and with regard to analysis of blood and urine that reflect the functional conditions of these organs.

MATERIALS AND METHODS

Animals and Parasites: Male and Female BALB/c mice, 6-7 weeks old, were used in these experiments. *Babesia rodhaini* (Australia strain) and *Babesia microti* (Munich strain) were provided by the Kyushu Branch of National Institute of Animal Health (Ministry of Agriculture, Forestry and Fishery, Kagoshima, Japan) and by Prof. O.A. Heydorn (Free University of Berlin, Berlin, Germany) respectively, and maintained by blood passage in BALB/c mice. Mice were inoculated intraperitoneally (i.p.) with $1x10^7$ parasitized erythrocytes (PRBC). The percentage of parasitemia was determined by counting the number of PRBC in tail blood smears stained with Giemsa. Hematocrit of the blood, which was obtained from the infraorbital venous plexus of mice, was measured by the microhematocrit method. Blood serum and urine samples were collected daily and stored at -20℃ until use.

Biochemical analysis of blood serum: Blood sera were obtained from centrifuged blood collected from mouse hearts under ether anesthesia, and examined for serum bilirubin and blood urea nitrogen (BUN). Serum bilirubin concentration was measured with the alkaline-azobilirubin method (Bilirubin B II-test Wako, Wako Pure Chemical Industries,Ltd., Tokyo, Japan). BUN was measured with a commercial kit (Unikitrate BUN, Kanto Chemical, Tokyo, Japan).

Urinary protein analysis: Urine was obtained from mice by forced urination. Urinary protein concentration was measured with the Bradford method with bovine serum albumin as standard. For detection of hemoglobin in the urine, urine and hemoglobin solutions were examined with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), loading samples on a 12% gel concentration with a Mini-PROTEAN II cell (Bio-Rad Laboratories, CA, U.S.A.). The hemoglobin solution was obtained from BALB/c mouse blood. The blood was washed three times with physiological saline, frozen and thawed twice, diluted with double-distilled water, and centrifuged 13000xg for 20 min. The supernatant was used as a hemoglobin solution against which urine samples were compared. After electrophoresis, the gels were stained with o-dianisidine solution for hemoglobin detection. To estimate the molecular weight of protein in the urine, samples were loaded on a 7.5% gel concentration and proteins were stained with 0.1% Coomassie brilliant blue R-250 after electrophoresis.

Tissue processing and staining: Tissue samples were collected when infected mice had high parasitemia and excreted discolored urine. Small pieces of the kidneys and liver specimens were processed for histological, immunohistological, and electron microscopic studies. For histology, tissues were fixed in 10% phosphate-buffered formalin, dehydrated in alcohol and xylene, embedded in paraffin, and sectioned at 4μm. Sections were stained with hematoxylin and eosin (H&E), periodic-acid-Schiff

(PAS), and methenamine silver. For electron microscopy, renal tissues were fixed in 2.5% glutaraldehyde in 0.05M phosphate buffer (pH 7.4) and 4% sucrose for 2 hr, postfixed in 1% osmium tetroxide for 2 hr, dehydrated in increasing concentrations of ethyl alcohol and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate, and examined with a JEOL 100 CX electron microscope. For immunofluorescent study, small blocks of kidney tissue were embedded in O.C.T. compound (Tissue-Tek, Division of Miles Laboratories Inc., Naperville, U.S.A.), frozen in dry ice-acetone, cut with a cryostat (Leitz 1720 digital, Wetzlar, Germany), dried, and stained with immune sera.

Antibodies: Antibodies to *B. rodhaini* and *B. microti* were obtained from rats immunized with mouse erythrocytes infected with the respective parasites. Rats were inoculated i.p. with $1x10^8$ PRBC, and after 3 weeks rats were inoculated i.p. with a booster of same dose of PRBC. Four weeks after booster inoculation, sera were taken from the rats. Indirect fluorescent antibody test showed that the titer of both anti-*B. rodhaini* and anti-*B. microti* was 1:4096 against *B.rodhaini*-infected and *B. microti*-infected RBC respectively. These sera were used as primary antibodies for detection of *Babesia* antigens in kidneys. FITC-conjugated IgG fraction of goat anti-rat IgG (Organon Teknika Corp., West Chester, U.S.A.) was used secondary antibody. FITC-conjugated (Fab') goat anti-mouse IgG or anti-mouse IgM and FITC-conjugated goat IgG fraction anti-mouse C3 were obtained from TAGO Inc. (CA, U.S.A.) and Organon Teknika Corp. (West Chester, U.S.A.), respectively.

Immunostaining: Deposition in glomeruli of IgG, IgM, C3, and Babesia antigens was examined by direct or indirect fluorescent antibody staining.

Direct immunofluorescent antibody staining: IgG, IgM, and C3 in glomeruli were examined by direct immunofluorescent antibody staining. Kidney sections were treated with blocking solution (2% skimmed milk- 5% normal goat serum in PBS) at 37℃ for 30 min and incubated with FITC-conjugated goat anti-mouse IgG, FITC-conjugated goat anti-mouse IgM, or FITC-conjugated goat IgG fraction to mouse C3 at 37℃ for 1 hr, washed in PBS, and examined with a fluorescence microscope (Optiphot-2, Nikon, Tokyo, Japan).

Indirect immunofluorescent antibody staining: Kidney sections were treated with blocking solution at 37℃ for 30 min and incubated with either rat anti-*B. rodhaini* or *B. microti* serum or normal rat serum as control at 37℃ for 30 min. These sections were washed in PBS, incubated FITC-conjugated IgG fraction of goat anti-rat IgG at 37℃ for 30 min, washed in PBS, and examined with a fluorescence microscope.

RESULTS

1) *B. rodhaini* infection in mice.

a)Clinical data

Parasitemia appeared on the 2nd day of infection and progressively increased until the 7th day when all mice died (Fig. 1). The hematocrit decreased after the infection and fell 30% (normal value 48-49%) (Fig. 2). By the 4th day of infection, all mice excreted greenish urine, which at days 5-7 became reddish. The appearance of discolored urine corresponded to a high parasitemia. SDS-PAGE analysis confirmed the reddish urine was hemoglobinuria (Fig. 3). *B. rodhaini* infection induced severe hemolysis in the mice before death.

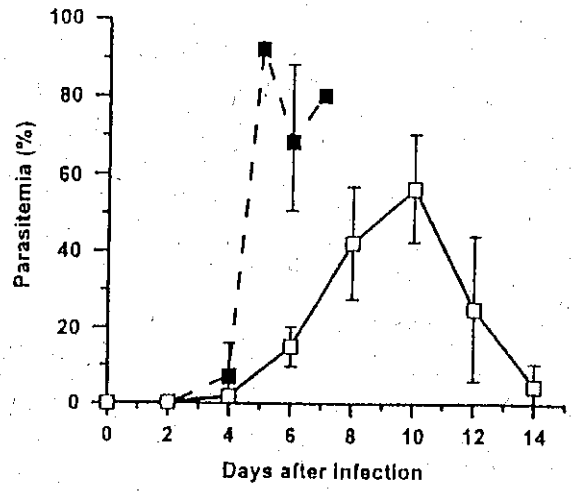


Fig. 1. Changes of parasitemias in *Babesia*-infected mice. *B. rodhaini*-infected mice (-■-) showed sudden increase of parasitemia until the 7th day when all mice died. *B. microti*-infected mice (-□-).

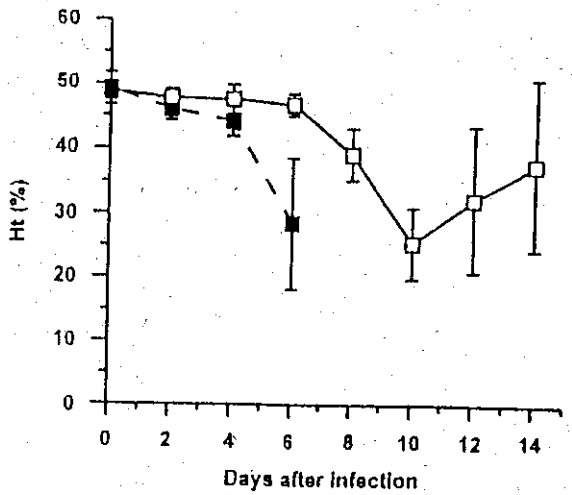


Fig. 2. Decrease of hematocrit in Babesia-infected mice. *B. rodhaini* infection (-■-). *B. microti* infection (-□-).

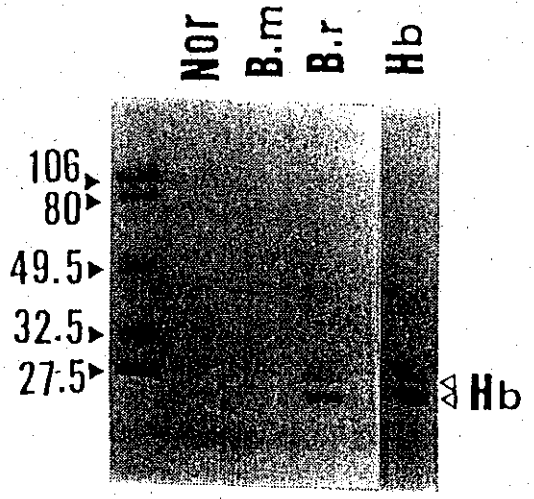


Fig. 3. Hemoglobin-stained SDS-PAGE (12% acrylamide) of mouse urine samples and mouse hemoglobin solution: Normal mice urine (lane Nor); *B. microti*-infected mice urine (lane B.m); *B. rodhaini*-infected mice urine (lane B.r); Hemoglobin solution (lane Hb). Standard urinary proteins of known molecular weight are shown (left lane). Arrow heads on the right margin indicate the position of the hemoglobin. Sizes on the left margin are in kilodaltons.

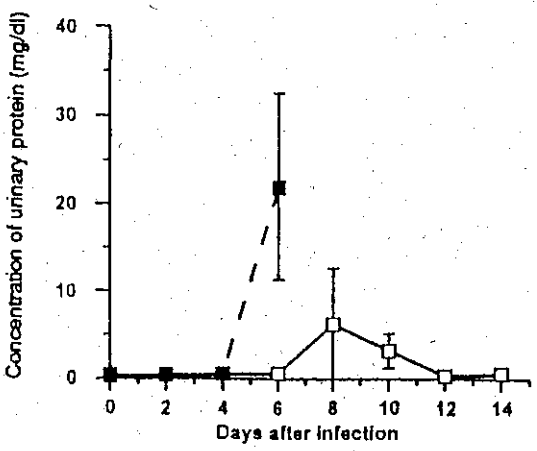


Fig. 4. Changes of proteinuria of mice infected with *B. rodhaini* (-■-) and *B. microti* (-□-).

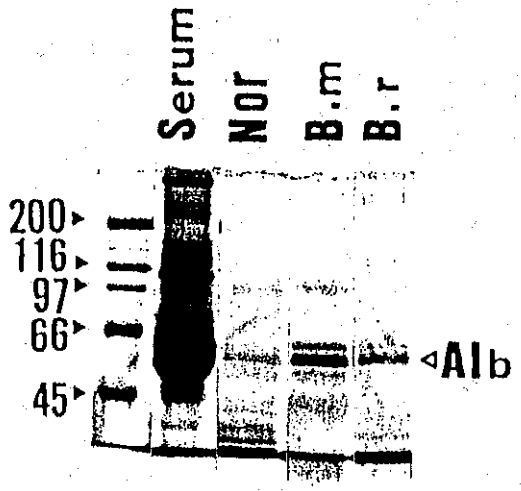


Fig. 5. SDS-PAGE (7.5% acrylamide) of mouse urine samples stained with Coomassie brilliant blue R-250. Lanes Nor. B.m, and B.r are same as in Fig. 3. Normal mouse serum (lane Serum) and standard serum proteins of known molecular weight (left lane) are shown. An arrow head on the right margin indicates the position of the albumin. Sizes on the left margin are in kilodaltons.

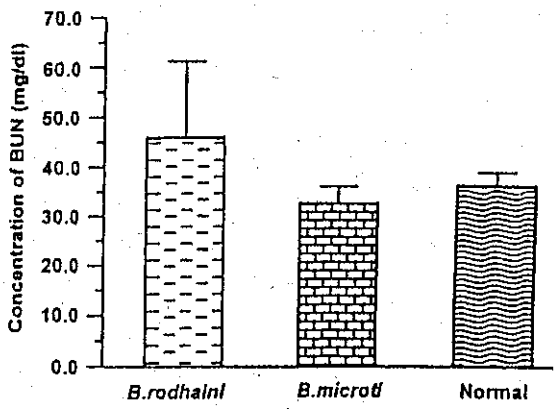


Fig. 6. BUN concentration in *Babesia*-infected m Significant increases are seen in *B. rodhaini*-infec mice ($p<0.05$).

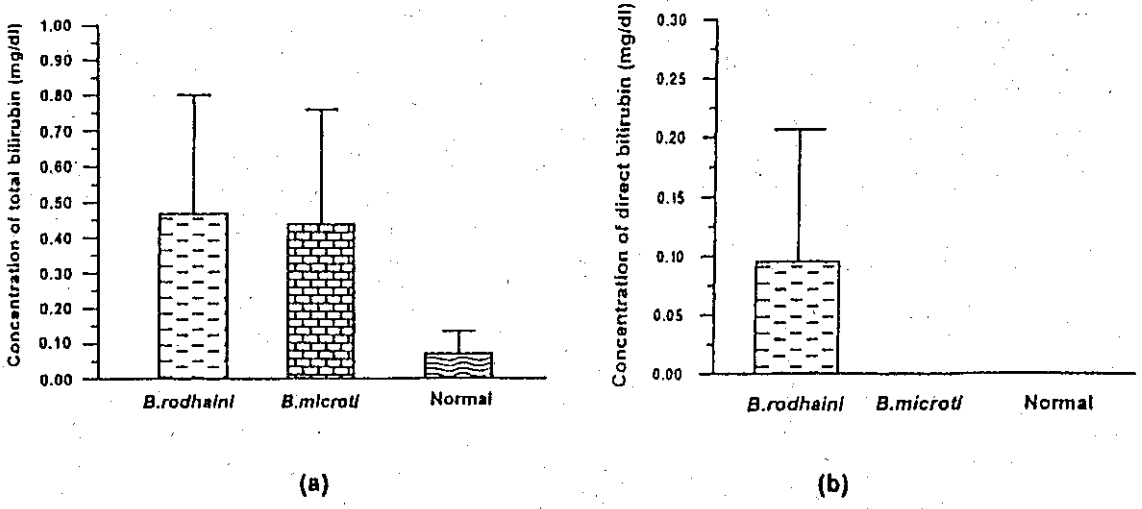


Fig. 7. Serum bilirubin concentration in *Babesia*-infected mice. (a) Both *B. rodhaini* and *B. microti*-infected mice showed increase of total bilirubin. Values are significantly different between *B. rodhaini*-infected mice and normal mice ($p<0.05$). (b) Significant increase of direct bilirubin in *B. rodhaini*-infected mice ($p<0.05$).

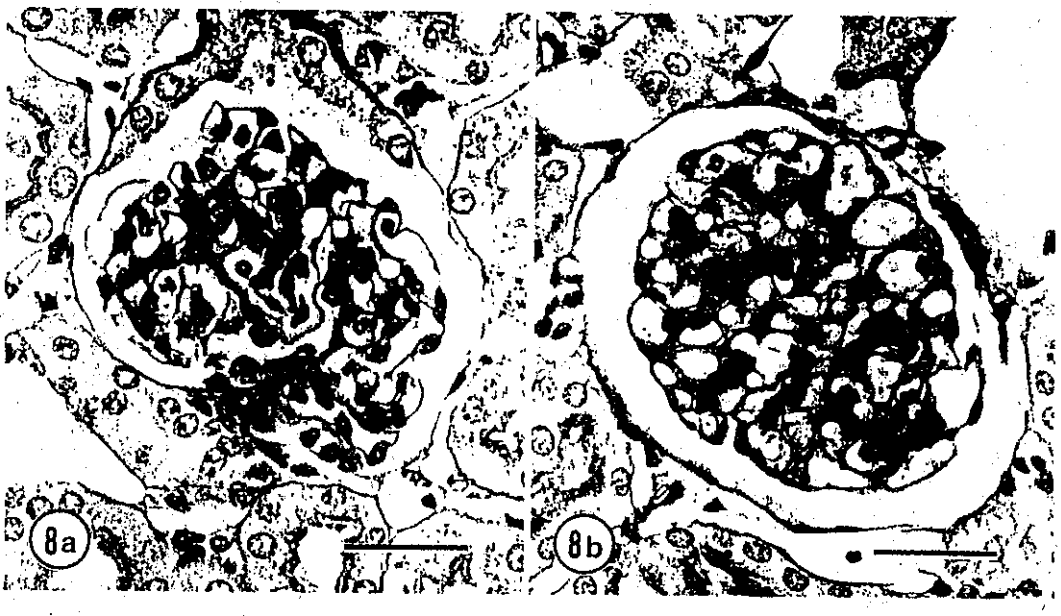


Fig. 8. PAS stained sections showing an increase in number of mesangial cells and mesangial matrix. **a**, *B. rodhaini*-infected mouse kidney. Bar= 10 μ m. **b**, B. *microti*-infected mouse kidney. Bar=10 μ m.

Biochemical analysis of the urine indicated functional damage to the kidneys. Proteinuria was detected by the 2nd day after infection and markedly increased to 24.6 $\pm$9.5mg/ml immediately before death (Fig. 4). SDS-PAGE analysis of the urine (Boesken et al. 1973) showed not only a prominent band of albumin but also several conspicuous bands of larger proteins that were more than 200 kDa molecular weight (Fig. 5). Biochemical tests of the blood serum showed an increase in BUN (Fig. 6) and bilirubin concentration (Fig. 7a and b) before the mice died. BUN, which is a useful serum marker to estimate renal function, reached 46.1$\pm$15.3mg/dl (control: 36.4$\pm$2.7mg/dl). Total bilirubin concentration was 0.47$\pm$0.33mg/dl (control: 0.07$\pm$0.06mg/dl), and direct bilirubin concentration was 0.10$\pm$0.11mg/dl (control: 0.01$\pm$0.03mg/dl). Total bilirubin dominant bilirubinemia corresponded to severe hemolysis in *B. rodhaini*-infected mice. The increase of direct bilirubin suggests functional damage of liver tissue.

b) Pathology of the kidneys and liver

When the mice had the highest parasitemia and were excreting reddish urine, we examined the microscopic appearance of kidneys and livers. Mesangiopathic glomerulonephropathy was the main histopathological change in the kidneys and was characterized by endocapillary proliferation, increase of mesangial matrix, and mild thickening of the basement membrane (Fig. 8a). Additionally, tubular necrosis sometimes was observed (not shown).

Electron microscopy showed an increase in mesangial cells and matrix, as well as electron-dense deposits, in the mesangial matrix and along the glomerular basement membrane (Fig. 9a and c). The basement membrane was somewhat thickened and

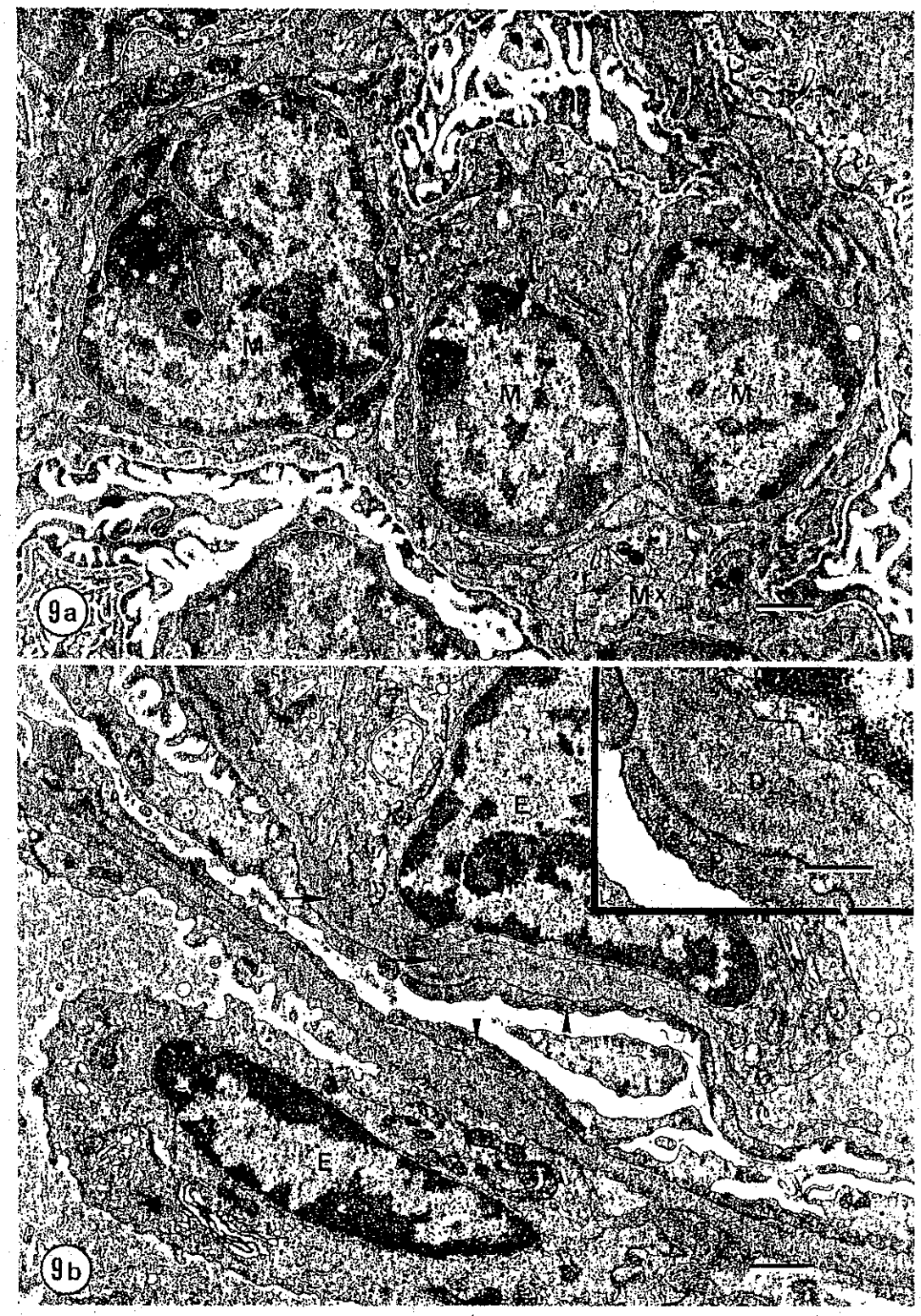
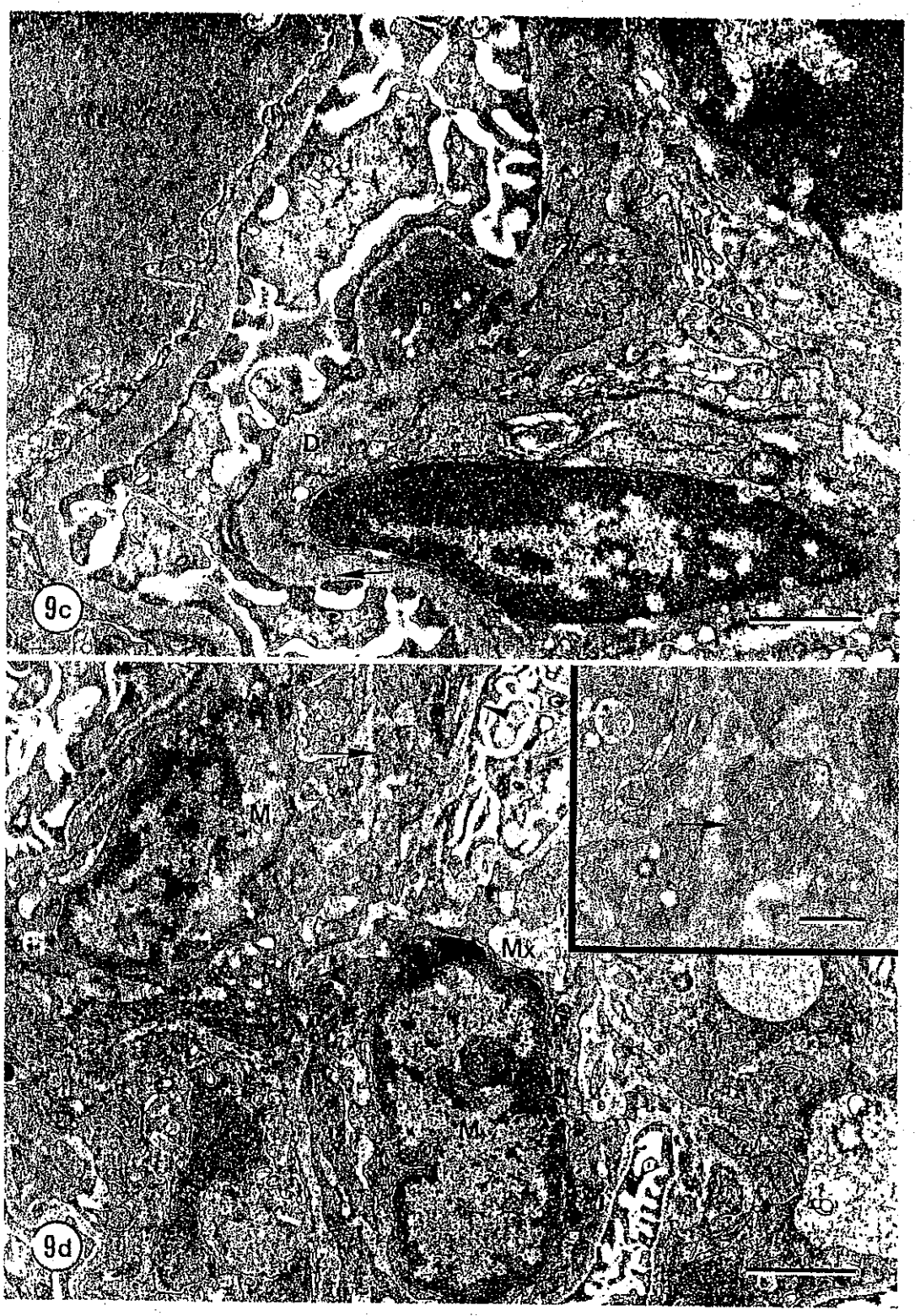


Fig.9. Electron micrograph of glomerulus in *B. rodhaini* (**a**,**b**,**c**) and *B. microti* infection (**d**). **a**, Mesangial expansion with an increase of cells (M) and matrix (Mx). Bar=1 μ m. **b**, Fusion of foot processes (arrow heads) and electron-dense deposits (arrows) along the basement membrane. Endothelial cells (E). Bar=1 μ m. Inset: High magnification of electron-dense deposits (D) and fused foot



processes (P).Bar=0.5 μ m. **c**, Electron-dense deposits (D) along the basement membrane (arrows). Bar=1 μ m. **d**, Mesangial expansion with increase in cells (M) and matrix (Mx). Electron-dense deposits (arrow). Fusion of foot processes (arrow head). Bar=2 μ m. Inset: High magnification of electron-dense deposits in the mesangial matrix (arrow). Bar=0.5 μ m.

foot processes of podocytes were focally fused (Fig. 9b). Immunohistochemistry revealed deposits of IgG (Fig. 10a), IgM, C3 and *Babesia* antigens in the mesangium regions and along the basement membrane.

Light microscopy of the liver showed extensive mid-zonal and centrolobular necrosis, with leukocyte infiltration around the necrotic areas (Fig. 11a). Many leukocytes including lymphocytes, monocytes, and macrophages were in the sinusoids and dilated central veins and interlobular veins.

2) *B. microti* infection in mice

a) Clinical data

Parasitemia appeared on the 2nd day after infection, reached its peak (51.8 ± 4.9%) by the 10th day, and gradually decreased by the 14th day when mice recovered from the infection(Fig. 1). Their hematocrit decreased after infection and fell to 36.8 ± 9.0% at the 10th day (Fig. 2). Greenish urine appeared by the 8-10th day and gradually disappeared as mice recovered from the infection. Proteinuria was found at the time high parasitemia. SDS-PAGE analysis of the urine showed a band which corresponded to albumin (Fig. 5). Hemoglobin and proteins larger than 200kDa were not detected in the greenish urine. Biochemical tests of the blood serum showed an increase in bilirubin concentration (Fig. 7). Total bilirubin concentration was 0.44 ± 0.32 mg/ dl; however, direct bilirubin concentration was within normal limits. The increase of indirect bilirubin suggested that greenish urine was bilirubinuria. The BUN concentration was not increased (Fig. 6).

b) Pathology of the kidneys and liver

Kidneys showed mesangiopathic glomerulonephropathy with an increase in mesangial cells and mesangial matrix (Fig. 8b). Immunohistochemical observations showed deposits of IgG, IgM, C3 and *B. microti* antigens in the mesangial regions and along the basement membrane (Fig. 10b). Electron microscopy showed electron-dense deposits in the mesangial matrix and along the basement membrane, and fusion of

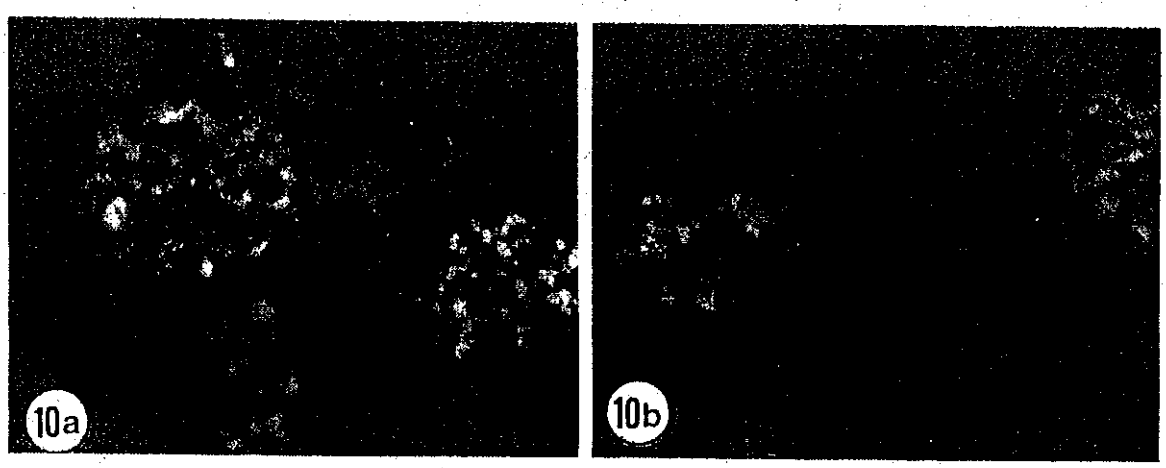


Fig.10. **a**, Granular deposition of IgG in the mesangium of *B. rodhaini*-infected mice kidney. **b**, Granular deposition of IgM in the mesangium of *B. microti*-infected mice kidney.

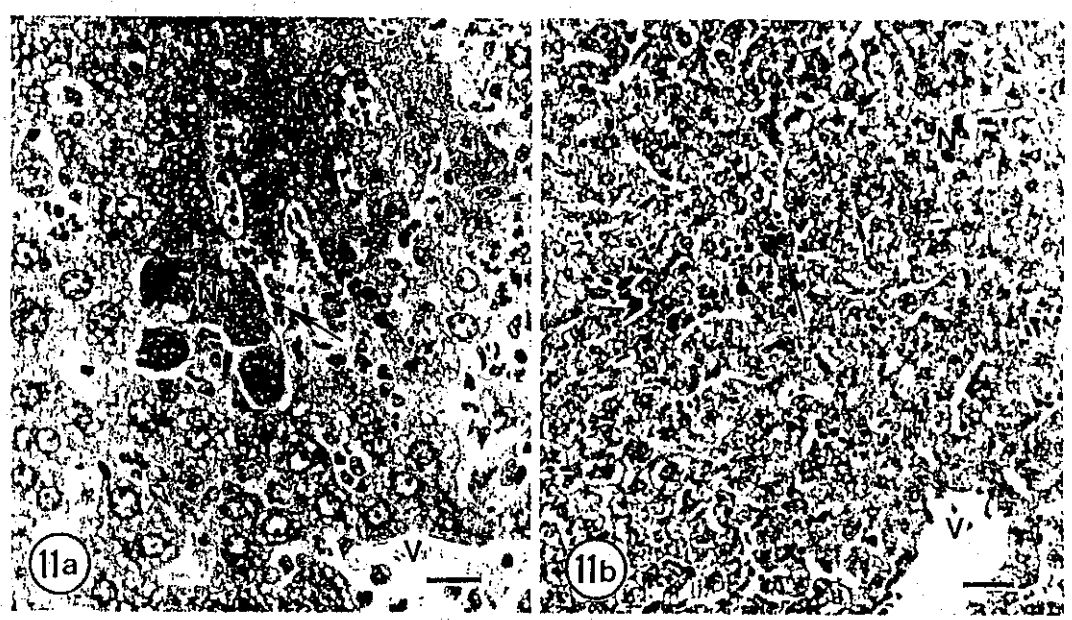


Fig.11. H&E stained sections. **a**, Mid-zonal necrosis(N) of the liver parenchyma in *B. rodhaini* infection. Leukocyte infiltration (arrow) around the necrotic area. Central vein(V). Bar=10 μ m. **b**, Focal necrosis (N) of the liver parenchyma in *B. microti* infection. Leukocyte infiltration (arrow). Central vein(V).Bar=10 μ m.

podocytes foot processes (Fig. 9d). However, these ultrastructural changes were relatively mild in *B. microti* -infected mice compared to *B. rodhaini*-infected mice. Renal tubular damage was mild (not shown).

Liver tissue showed focal necrosis and some leukocyte infiltration in the mid-zone of liver lobules (Fig. 11b). These necrotic changes were less severe in *B. microti*-infected mice than in *B. rodhaini*-infected mice.

DISCUSSION

B. rodhaini infection was different from *B. microti* infection in its clinical course. All *B. rodhaini*-infected mice died after the sudden increase of parasitemia and excretion of reddish hemoglobinuria. In contrast, all *B. microti*-infected mice survived after a temporary moderate increase in parasitemia and in excretion of greenish urine. The decrease of hematocrit and the increase of indirect bilirubin confirmed intravascular hemolysis, which caused discolored urine in both *Babesia* infections. The excretion of hemoglobinuria suggested that serum hemoglobin concentration exceeded serum haptoglobins, which capture and bind free hemoglobin molecules and prevent their excretion. These date indicate that the intravascular hemolysis was more severe in *B. rodhaini*-infected mice than *B. microti*-infected mice.

Biochemical analysis of blood and urine showed functional conditions of kidneys in these *Babesia* infections. Both types of the infected mice excreted proteinuria , which contained albumin. However, the specific data (such as the increase of BUN and

proteinuria containing large molecular weight proteins that were more than 200kDa) suggested that *B. rodhaini*-infected mice had more severe functional damage of kidneys than did *B. microti*-infected mice. BUN is a useful parameter of diagnosis of the renal involvement in *Babesia* infections (Hussein 1977b, Maegraith et a.l 1957, Rogers 1971, Wright 1972). The excretion of large proteins indicates severe disorder of the filter function of kidneys.

Glomerulonephropathy was a characteristic pathological change in both babesioses. The deposition of IgG, IgM, C3 and Babesia antigens in glomerulus strongly suggested that babesioses caused the immune complex-induced mesangiopathic glomerulonephropathy. Similar immune complex-induced glomerulonephropathy was reported in rat babesiosis (Annable and Ward 1974) and in *Plasmodium* infections (Aikawa et al. 1988, Allison et al. 1969, Dixon 1966, Ward and Kibukamusoke 1969, Hartenbower et al. 1972, Bhamarapravati and Boonpucknavig 1973, Boonpucknavig and Sitprija 1979). In babesiosis, the deposition of electron-dense materials was first manifested in the mesangial area and along basement membranes; these densities may correspond to the immune complex deposits. It should be noted that although such deposits were immediately obvious by electron microscopy, they were not discernible by conventional light microscopy. With electron microscopy, these deposits were ubiquitous in the glomerular basement membranes of *B. rodhaini*-infected mice, but sporadic in the *B. microti*-infected animals. These ultrastructural changes in *B. rodhaini*-infected kidneys corresponded to the relatively severe functional damage incurred by these organs.

The kidneys of the infected mice also had mild tubular damage. It has been postulated that excessive hemoglobin in concert with tissue hypoxia is toxic to renal tubules (Braun et al. 1970). This is consistent with the fact that renal tubular necrosis was more severe in *B. rodhaini*-infected mice with relatively severe hemolysis and low hematocrit than in *B. microti*-infected mice.

The extensive liver necrosis and increase of direct bilirubin in *B. rodhaini*-infected mice showed that liver damage was relatively severe in *B. rodhaini* infection. Babesiosis induces the characteristic focal, mid-zonal, and/or centrolobular liver necrosis in various animals (Habela et al. 1991, Liddel et al. 1981, Hussein 1977a, Maegraith et al. 1954, Rogers 1971). Clark et al. (1987) suggested an excessive release of tumor necrotic factor could account for mid-zonal liver damage in *Plasmodium* infection. However, the mechanism of the liver necrosis has not yet been fully elucidated.

We showed that severity of the damage to kidneys and liver under the conditions of high parasitemia and discolored urine excretion is correlated with the prognosis of these two *Babesia* infections. The damage to kidneys and liver, as well as severe hemolytic anemia, in *B. rodhaini* infection seemed to cause failing of the mice, eventually leading to their death.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the USPHS(Grant AI-10645) and Grant-Aid for Scientific Research Ministry of Education, Science and Culture, Japan. We acknowledge the excellent technical assistance of Kiet Dan Luc and Ana Milosavljevic , and Dr. B. Tandler for help with the manuscript.

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Babesia rodhaini and *Babesia microti*: Cross-Immunity and Cross-Antigens

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ABSTRACT

Cross reactivity of *Babesia rodhaini* (*B. rodhaini*) and *Babesia microti* (*B. microti*) antigens against *Babesia*-chronically infected mice sera was examined using indirect fluorescent antibody (IFA) technique and westernblotting. The effect of monoclonal antibody #7 (mAb #7), which recognized both *B. microti* and *B. rodhaini* antigens, was also studied against *Babesia*-infections. Passive immunization with mAb #7 was carried out to clarify the relationship between cross-reactive antigens and progression of *Babesia* infection. Results of IFA assay showed cross reactivity in *B. microti* and *B. rodhaini* antisera at a dilutions of 1:512 and 1:128, respectively. Each type of antiserum exhibited parasite specific fluorescence. Immunoblotting demonstrated the reactivity of *B. microti* and *B. rodhaini* antisera with the 70 and 32 kilodaltons (kDa) *B. rodhaini* antigens, and with the 70 kDa B. microti antigen, respectively. These results suggest a stronger cross reactivity of *B. microti* antiserum compared to *B. rodhaini* antiserum. Monoclonal antibody #7 reacted with 70 and 30 kDa antigens of *B. microti*, and with 70 and 32 kDa antigens of *B. rodhaini*. Passive immunization using mAb #7 showed a delayed development in high levels of parasitemia in both *Babesia* spp., compared to the control groups. These results demonstrate the apparent role of mAb #7 in suppressing the onset of parasitemia.

INTRODUCTION

B. microti and *B. rodhaini* are rodent forms of *Babesia*, the former causing chronic babesiosis, and the latter species being fatal in the absence of treatment (Cox et al. 1969). Several authors have reported host immune response against *B. microti* and *B. rodhaini* during initial, as well as in cases of re-infection. Among *B. rodhaini*-infected and *B. microti* immune mice, Simada et al. (1992) noted a decrease, and an increase in the proportion or ratio of $L3T4^+$ to $Lyt2^+$ cells, in the spleen, respectively. They hypothesized that in immune mice, the suppressor cells may have been activated during the initial stage of infection, effecting suppression, thereafter. While, there are

reports on the adoptive transfer of protective immunity with *B. rodhaini* immune spleen cells (Roberts et al., 1968; Zivkovic et al., 1984), and of mice protected against babesiosis *rodhaini* using anti-*B. rodhaini* monoclonal antibodies (Claveria et al., 1992), detailed information about cross-immunitiy between *B. rodhaini* and *B. microti* is still wanting. In the present study, we analyzed the cross-reactive antigens of *B. rodhaini* and *B. microti*, and determined the protective effect of these antigens through passive immunization.

MATERIALS AND METHODS

Animals and Parasites: BALB/c and ICR mouse strains, ages 4 weeks which were bred in the Animal Facility of the Department of Veterinary Physiology, Obihiro University were used. The Munich strain of *B. microti* and Australian strain of *B. rodhaini* were maintained in mice through intraperitoneal (IP) injection of infected blood.

***Babesia* Immune mice and sera preparation:** Immune mice were prepared following the method of Simada et al. (1992).

Preparation of *Babesia* spp. antigen: Blood from severely infected mice was obtained through cardiac puncture using heparinized syringe. To remove the supernatant and buffy coat, blood was centrifuged at 1,000 xg, 4°C for 10 minutes. The cell sediment was washed three times with phosphate buffered saline (PBS), resuspended in 50 mM Tris-HCl (pH 7.4), 0.1 M NaCl, 1 mM PMSF, and then frozen (-80°C) and thawed (37°C), three times. The sample was centrifuged at 100,000 xg for 30 minutes at 4°C. The supernatant was kept at -80°C until use.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western-blotting: Equal volume of each *Babesia* spp. prepared antigen and sample buffer (0.125 M Tris-HCl (pH 6.8), 20% glycerol, 10% 2-mercaptoethanol, 4%(w/v) SDS, 0.1%(w/v) bromophenol blue) were mixed, then heated to 95°C for 4 minutes in a waterbath. The sample was centrifuged at 15,000 xg, 25°C for 10 minutes. The supernatant was electrophoresed in a 10% SDS-polyacrylamide gel. Separate proteins were transblotted into a PVDF membrane (0.22 μm, GVHP; Millipore, USA), and the transblots were stained with amido black solution (0.1% amido black 10B, 7% acetic acid), and then washed with double distilled water (D.D.W.). The membrane was fixed in acetic acid : methanol : D.D.W. (1 : 5 : 5) for 5 minutes, and then washed with PBS + 0.05% Tween 20, three times, 5 minutes each time. Blocking was carried out overnight in PBS + 10% skim milk at 4°C, and each membrane was reacted with *B. microti*, *B. rodhaini*. antiserum or anti-*Babesia* spp. mAb #7 in PBS + 0.5% skim milk for two hours at room temperature. The membrane were reacted with peroxidase-conjugated anti-mouse IgG (Blotting Grade Affinity Purified Goat Anti- Mouse IgG (H+L) Horseradish Peroxidase Conjugate; BIO-RAD, USA) for another two hours, and the bands were visualized using 0.1 M Tris-HCl (pH 7.4), 3,3'-diaminobenzidine 4HCl salt and 0.03% H_2O_2 solution or chemiluminescence reagent (RENAISSANCE™; DUPONT NEN, USA).

Immunoprecipitation: *Babesia* prepared antigen was mixed x2 concentration of NET solution (300 mM NaCl, 10 mM EDTA, 100 mM Tris-HCl (pH 8.0)) and 10-50 μl *B. microti*, *B. rodhaini* antiserum or 20-50 μg mAb #7, and kept overnight at 4°C. Thereafter, 50 μl of protein A-Toyopearl 650M (Tsk-gel protein A-Toyopearl 650M;

TOSO, Japan) suspended in NET (Protein A-Toyopearl : NET = 1 : 2) was added and incubated for one hour at room temperature. The immunoprecipitate was centrifuged at 120 xg, at 25°C for five minutes, and then washed three times with solution I (0.05% NP-40, 1 mg/ml BSA in NET), and another three washing with solution II (300 mM NaCl, 0.1% SDS, 0.05% NP-40, 50 mM Tris-HCl (pH 8.6)). To the immunoprecipitate, an equal volume of 2x concentration of SDS-PAGE sample buffer, then heated to 95°C for 4 minutes in waterbath, centrifuged at 500 xg for 10 minutes. The supernatant was kept at -80°C until use.

Indirect fluorescence antibody test (IFAT): *Babesia* PRBC were coated into wells on a glass slide and if necessary, the preparations were fixed with cold acetone or cold methanol for 10 minutes. Antigens on wells were incubated with antisera, supernatant from hybridomas, or normal sera (2^{-1} to 2^{-11} dilution in PBS) for 30 minutes at 37°C, followed with three times washing with PBS. The wells were then incubated with fluorescein isothiocyanate conjugated anti-mouse IgG (Goat $F(ab')^2$ Anti-Mouse IgG (H&L)-FITC; TAGO, Inc., USA) for 30 minutes at 37°C, washed three times with PBS, and mounted in glycerol buffer (90% glycerol in PBS) for examination using fluorescence microscope.

Production of monoclonal antibodies (mAb): BALB/c mice were primed through IP inoculation of $1x10^6$ *B. microti* PRBC, and one month later they were challenged with $1x10^6$ *B. rodhaini* PRBC, IP. At exactly three days post-challenge, immune spleen cells were obtained and processed for hybridoma production following the methods of Vernon et al. (1980) and Galfre et al. (1977). Briefly, immune spleen cells suspended in RPMI 1640 were fused with SP2/0-Ag14, a BALB/c myeloma cell line using 50% polyethylene glycol 1500 (Boehringer Mannheim, Germany). Hybridized cells were suspended in S-Clone (S-Clone CM-B; Sankyo Junyaku Co., Ltd., Japan) + 0.2% hypoxanthine, aminopterin and thymidine (HAT) medium (HAT Media supplement 500x; Boehringer Mannheim, Germany), and were plated into 96-wells tissue plates ($1x10^6$ cells/0.1ml/well), and incubated at 37°C. Wells with healthy growth cell colonies were checked for antibody production using IFAT. Those that were positive colonies were cultivated to generate specific and relevant anti-*Babesia* spp. monoclonal antibodies.

Positive clones were expanded in vivo using BALB/c mice which were IP-injected incomplete Freund's adjuvant 7-10 days prior to IP-inoculation of $1x10^7$ hybridoma cells. MAb diluted in PBS were precipitated from mouse ascitic fluid, using 50% saturated ammonium sulfate and dialyzed in three changes of PBS at 4°C. Protein concentration was estimated using a spectrophotometer. Isotyping of mAb was done with the mAb isotyping kit (Amersham, UK).

Passive Immunization with mAb: Sixteen 5 weeks old female BALB/c mice were divided into groups namely: G-1, G-2, G-3 and G-4. Mice were exposed IP to $1x10^6$ *B. microti* (G-3), and *B. rodhaini* (G-4) PRBC which were incubated with 100 μg mAb #7 in PBS at 37°C for 30 minutes, prior to inoculation. Thereafter, in G-3, two mice were intravenously injected 100 μg mAb/mouse daily, starting the day prior to exposure to PRBC, until the 14th day post-infection (PI), and the other two mice until the 21st day PI. All mice in G-4 were treated similarly as those in G-3, and two mice were mAb-treated until the 10th day PI, and the remaining two mice were passively immunized until prior to onset of death. Mice comprising control groups (G-1 and G-2)

were exposed to $1x10^6$ *B. microti* and *B. rodhaini* PRBC, respectively, which were incubated with PBS at 37°C for 30 minutes. Parasitemia was assessed every two days interval by counting the number PRBC divided by the total erythrocyte count.

RESULTS AND DISCUSSION

Antisera obtained from *B. rodhaini* and *B. microti* chronically infected mice showed cross reactivity with the heterologous species (Fig. 1). With westernblotting (Fig. 2), *B. microti* antiserum reacted strongly with *B. rodhaini* antigens of molecular weights 70, 32 kilodaltons (kDa) and less strongly with 46 and 39 kDa antigens. Interestingly, *B. rodhaini* antiserum reacted only with the 70 kDa *B. microti* antigen. Our findings demonstrate a higher cross-reactivity of anti-*B. microti* serum.

One monoclonal antibody designated as mAb #7, isotype IgG2b was isolated from immune spleen cells obtained from *B. microti*-chronically infected mice challenged with *B. rodhaini*. Monoclonal antibody #7, exhibited specific reactivity with unfixed *Babesia* spp. PRBC (Fig. 3), but showed no reactivity against either acetone or methanol-fixed PRBC. Using electrophoresed *Babesia* spp. antigens, mAb #7 did not recognize any of the *Babesia* spp. antigens used in the study (Fig. 4). However, westernblot analysis of immunoprecipitated antigens using mAb #7, yielded antigen bands of molecular weights 70 and 30 kDa of *B. microti*, 70 and 32 kDa of *B. rodhaini* reactive with anti-*B. microti* serum, and only with the 70 kDa *B. microti* and 32 kDa *B. rodhaini* antigens using anti-*B. rodhaini* serum.

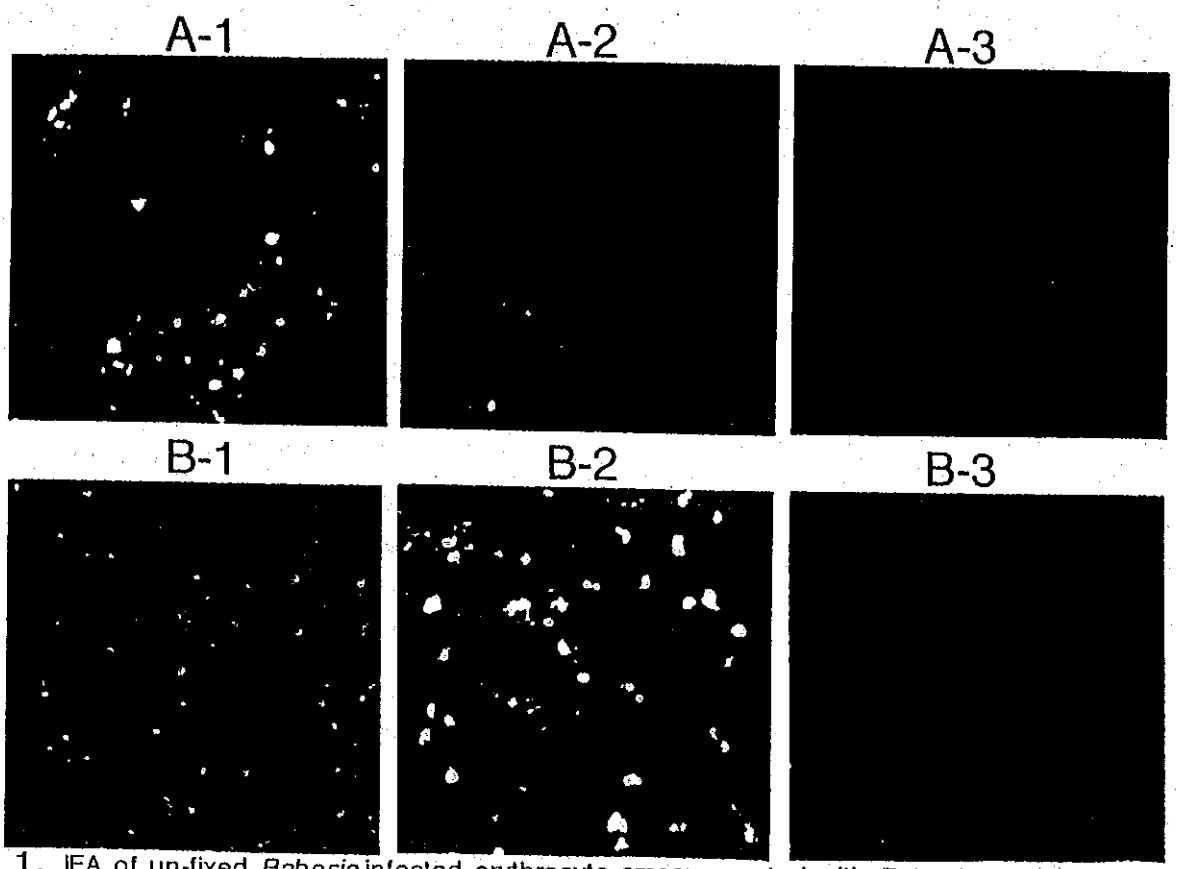

Fig. 1, IFA of un-fixed *Babesia*-infected erythrocyte smears reacted with *Babesia*-antisera. *Babesia microti* against *B. microti* (A-1) and *B. rodhaini* (A-2) antisera, or normal serum (A-3). *Babesia rodhaini* against *B. microti* (B-1) and *B. rodhaini* (B-2) antisera, or normal serum (B-3). x400

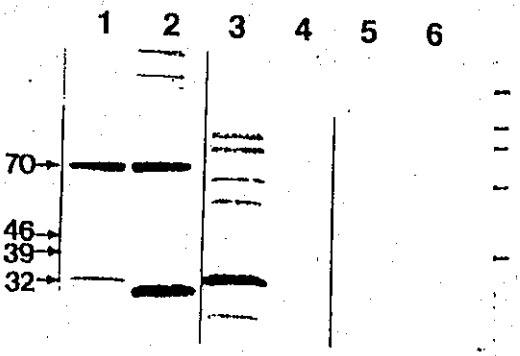

Fig. 2, Westernblot analysis of *Babesia* antigens. *Babesia rodhaini* (lane 1) and *B. microti* (lane 2) antigens detected using *B. microti anitserum. Babesia rodhaini* (lane 3) and *B. microti* (lane 4) antigens detected using *B. rodhaini* antiserum. *Babesia rodhaini* (lane 5) and *B. microti* (lane 6) antigens detected using normal mouse serum. Molecular weight markers (-) correspond to 200, 116.25, 97.4, 66.2 and 45 kilodaltons.

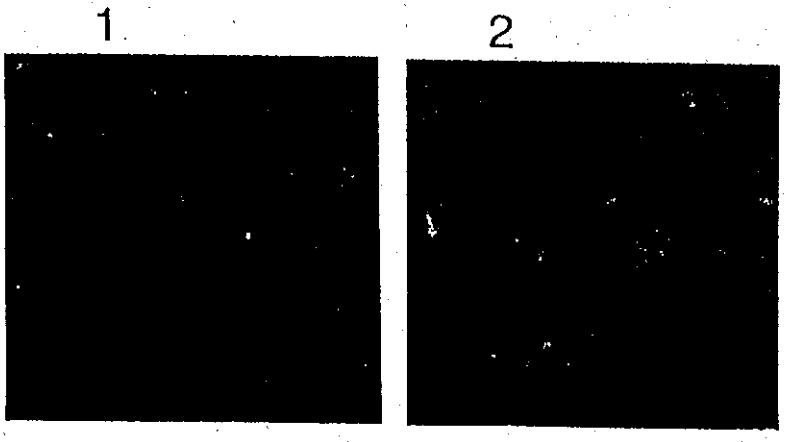

Fig. 3, IFA of un-fixed *Babesia* infected erythrocyte smears reacted with monoclonal anitbody #7. *Babesia microti* (1) and *B. rodhaini* (2). x400

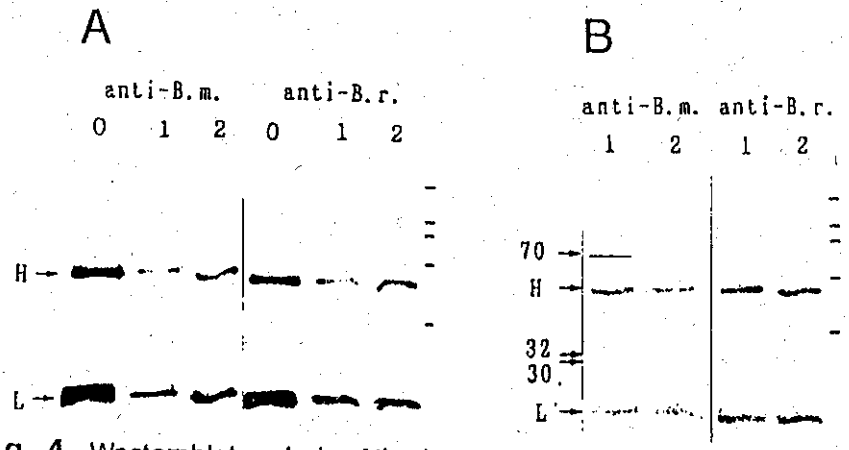

Fig. 4, Westernblot analysis of the immunoprecipitate materials from *Babesia* antigens with mAb #7 (A; lane 0, B; lane 1 and 2) or anti-*Toxoplasma gondii* mAb (A; lane 1 and 2). Normal erythrocyte (lane 0), *B. microti* (lane 1) and *B. rodhaini* (lane 2) antigens detected with *B. microti* (anti-B. m.) and *B. rodhaini* (anti-B. r.) antisera. Molecular weight markers (-) correspond to 200, 116.25, 97.4, 66.2 and 45 kilodaltons.

Figures 5 and 6 summarize the effect of mAb #7 against progression of parasitemia in both control and experimental groups. A 100% motality was noted among non-treated *B. rodhaini* infected mice within 10 days post-exposure, with percent parasitemia ranging between 82.0-86.8%. In the experimental group, all treated-*B. rodhaini*-infected mice exhibited about 1% parasitemia at 8 days PI, however two mice succumbed to death on the 13th day PI, and the remaining two mice died between the 17th-19th days.

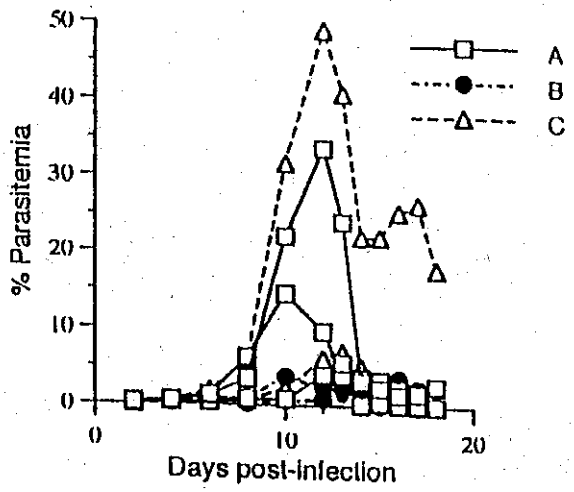

Fig. 5, progression of parasitemia in *B. microti*-exposed mice, given injections of mAb #7. (A): Injected 0.1 ml PBS i. v. from the day prior to exposure until the 14th day post-infection. (B): Injected 0.1 ml (100 μg) mAb #7 i. v. from the day prior to exposure until the 14th day post-infection. (C): Injected 0.1 ml (100 μg) mAb #7 i. v. from the day prior to exposure until the 21st day post-infection.

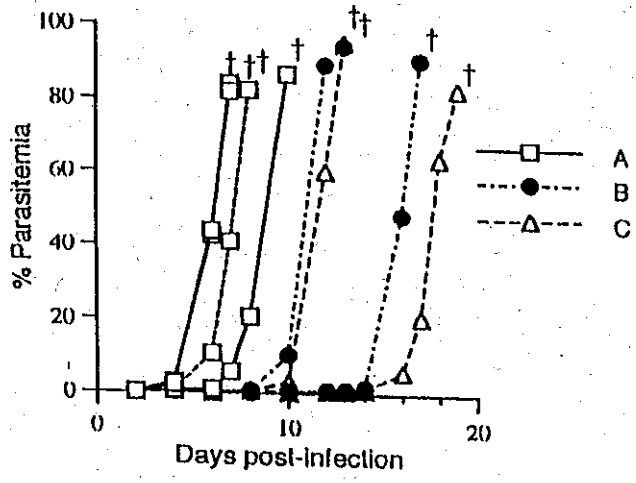

Fig. 6, progression of parasitemia in *B. rodhaini*-exposed mice, given injections of mAb #7. (A): Injected 0.1 ml PBS i. v. from the day prior to onset of death. (B): Injected 0.1 ml (100 μg) mAb #7 i. v. from the day prior to exposure until the 10th day post-infection. (C): Injected 0.1 ml (100 μg) mAb #7 i. v. from the day prior to exposure until prior to onset of death.

Passive immunization of infected mice with mAb #7 clearly showed a significant delay in the progression of both *B. microti* and *B. rodhaini* infection. Such effect could be attributed to the killing of extracellular parasites via complement and/or inhibition of parasite entry into erythrocytes. In the present study, the cross-reactive antigens of molecular weights 70 kDa could have provided significant protection to mice against severe infection with *B. microti,* a conjecture that is supported by the reports of Bautista et al. (1979), Kurtzhals et al. (1988) and Meeusen et al. (1984). While all mAb #7 treated *B. rodhaini* infected mice died, there was a delay in the progress of infection and subsequent prolongation of survival, a finding that is consistent with those of Roberts et al. (1968, 1975).

In summary, our findings demonstrate that mAb #7 recognizes cross-reactive antigens of *B. microti* and *B. rodhaini.* Thus, further clarification of the difference in the cross reactivity of anti-*B. microti* and anti-*B. rodhaini* sera, and the analysis of the nature of the antigens recognized by mAb #7, warrant further investigation.

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We greatly acknowledge Dr. M. Shinagawa, Dr. K. Shimazaki and Mr. Y. Maki for their help and advice during the course of this research work. This work was supported by a Grant-Aid for Scientific Research (C), Ministry of Education, Science and Culture, Japan.

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ROLE OF CD4 $^{+}$ T CELLS IN THE CONTROL OF PRIMARY INFECTION WITH *BABESIA MICROTI* IN MICE

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ABSTRACT

The role of T cells for the resolution of acute primary infection with *Babesia microti* was investigated in the present study. BALB/c mice exhibited a peak parasitemia of approximately 40% of parasitemia and subsequently recovered naturally from their primary infection. Nude mice, however, could not resolved primary infection and developed persistent high parasitemia. Mice depleted of CD4+ T cells with monoclonal antibody (mAb) had high parasitemia and failed to control the infection. However, depletion of $CD4^+$ cells one week after infection did not affect the course of infection. Depletion of $CD8^+$ T cells showed no apparent effect on the course of infection. High concentration of IFN-γ was demonstrated in the culture supernatant of spleen cells from untreated and anti-CD8 treated mice, but not from anti-CD4 treated mice. Mice treated with anti-IFN-γ mAb showed higher peak parasitemia and remained above 10% of parasitemia until days 26 after infection. These results suggest that CD 4^+ cells play an essential role in the resolution of *B. microti* acute primary infection and that IFN-γ produced by $CD4^+$ T cells is partially responsible for control of early stage of acute infection with *B. microti*.

INTRODUCTION

Babesia, a tick-transmitted hemoprotozoan parasite, causes enormous economic losses in domestic animals throughout the world (MaCosker 1981). *Babesia microti*, a species that parasitizes rodents, produces transient high parasitemia in mice and they recover naturally from the acute infection (Ruebusin and Hanson 1979). The importance of cell-mediated immunity in *B. microti* infection has been reported (Irvin et al. 1981; Eugui and Allison 1980). Congenitally athymic nude mice (Clark and Allison 1974), lethally irradiated, thymectomized mice reconstituted with anti-theta serum-treated bone marrow cells (Ruebusin and Hanson 1980) or the administration of anti-lymphocyte serum in hamsters (Wolf 1974) fail to suppress infection with *B. microti*. Likewise, B cell-dificient mice acutely infected with *B. microti* undergo remission with similar kinetics of parasitemia as immunologically intact mice (Cavacini et al. 1990). These results suggest that T cell-mediated immunity play a more significant role than the antibody-dependent immunity in the resolution of acute *B. microti* infection. However, the mechanism of mediating control of the primary infection with *B. microti* has not been fully understood.

In the present study, we focused on role of T cells in the control of primary infection with *B. microti* using immunocompetent BALB/c mice, and immunodeficient athymic nude mice and SCID mice. Specifically, we sought to identify T cell subpopulation(s), and effect of in vivo depletion of T cell subpopulation(s) in the course of infection using anti-CD4 and anti-CD8 monoclonal antibodies (mAb). Also, the T cell subsets controlling the course of infection were identified through cytokine production in spleen cells and by monitoring the effect of administration of mAb against cytokines produced by T cells.

MATERIALS AND METHODS

Mice and parasite: Female BALB/c mice used in the study were purchased from CLEA (Tokyo, Japan). Histocompatible female BALB/c nu/nu, and nu/+ mice were obtained from CLEA. Female mutant mice having severe combined immune deficiency (SCID), and background CB-17 mice were purchased from CLEA, or were kindly provided by Central Laboratory for Experimental Institute (Kawasaki, Japan). All mice used ranged between 5-7 wks old at the start of the experiment. *B. microti* (Munich strain) was kindly provided by Prof. A. O. Heydorn of the Institute of Parasitology and Tropical Veterinary Medicine, Free University Of Berlin, Germany. Mice were inoculated intraperitoneally (IP) with $1x10^7$ parasitized erythrocytes (PRBC). Parasitemia was estimated by counting PRBC of Giemsa-stained mouse blood smears, and was monitored at 2 days interval.

Monoclonal Antibodies (mAb): GK1.5 and 53-6.72 mAb were used against mouse CD4 and CD8, respectively, while XMG1.2 and 11B1 were used against IFN-γ and IL-4, respectively (Waki et al. 1992). MAb were obtained from mouse ascites (Mueller et al. 1986) and were purified by 50% ammonium sulfate precipitation and subsequently dialized against phosphate-buffered saline (PBS). Protein concentration was determined with Bradford method (Bradford 1976) using bovine serum albumin, as standard.

Treatment with mAb: Mice were depleted of T cell subsets by IP injection with 0.5 mg of anti-CD4 or anti-CD8 mAb for three successive days prior to the infection, and with additional same dose every three days post-inoculation. Control mice were inoculated with 0.5 mg normal rat IgG (Caltag Laboratories, South San Francisco, CA). Mice were injected with 1.0 mg of anti-IFN-γ and anti-IL-4 mAb for four consecutive days PI, and then every three days till the end of experiment.

Preparation of spleen cells: Infected and control mice were killed under anesthesia, and their spleen were removed aseptically, minced with scissors, and squeezed between two frosted glass slides.The cell suspension was filtered through a sterile stainless mess to remove tissue fragments. Contaminated erythrocytes were lyzed with 0.83% ammonium chloride, washed with Hank's balanced salt solution (HBSS) and resuspended to a concentration of $5x10^6$ cells/ml in RPMI 1640 medium (Gibco, Grand Island, NY).

Flow cytometric analysis of T cell subsets: Spleen cells suspended in RPMI 1640 containing 2.0% fetal bovine serum (FBS) were incubated with FITC-conjugated anti-CD4 or anti-CD8 mAb for 30 min on ice. After washing with PBS, cell samples were analyzed on a flow microfluorometer (FACScan, Becton Dickinson, Mountain View, CA).

Preparation of Babesia-lysate antigen (BLA): Blood from mice with about 80% parasitemia was obtained through cardiac puncture using heparinized syringe and washed three times. Pelleted PRBC were frozen with liquid nitrogen and thawed, this step repeated three times. The thawed sample was centrifuged at 144,000g for 30 min at 4 C, and the supernatant obtained was designated as BLA.

Detection of IFN-γ and IL-4 activity in spleen cell culture: $5x10^6$/ml spleen cells from infected and control mice were prepared and cultured in RPMI 1640 containing 5.0 % FBS, 100 units penicillin/ml and 100 mg streptomycin/ml, in the presence of BLA.Culture supernatant was harvested 72 hr post-incubation and stored at -80C until use. Concentrations of IFN-γ and IL-4 were measured using ELISA kits (Holland Biotechnology, Netherlands; Endogen Inc., Boston, MA).

RESULTS AND DISCUSSION

BALB/c nu/+ mice infected with *B. microti* developed acute infection registering 41.75% peak parasitemia on day 10, subsequently followed with remission, while nude mice failed to resolve infection (Fig. 1). Although parasitemia in nude mice decreased to 38.2% on day 12, a high level of infection was sustained ranging between 45-60% until the 40th day PI. SCID mice developed rapid acute infection with peak parasitemia of 83.2% and failed to control primary infection as the nude mice did, with immunocompetent mice showing spontaneous remission (Fig. 2). These results confirm earlier observation of the failure of nude mice to resolve *B. microti* and *Plasmodium* infection (Clark and Allison 1974). SCID mice having no functional T and B cells failed to resolve primary infection registering similar levels of parasitemia as nude mice. Cavacini et al. (1990) demonstrated that B-cell deficient mice could control *B. microti* as well as *P. chabaudi* infection. These data put together apparently indicate that T-cell mediated mechanism(s) may indeed play a vital role in the resolution of *B. microti* in mice.

Treatment of BALB/c mice with either anti-CD4 or anti-CD8 mAb resulted in more than 92% depletion of its corresponding subpopulation. Treatment with normal rat IgG had no effect on $CD4^+$ or $CD8^+$ subpopulation, showing essentially similar results with those of untreated mice (data not shown). CD8-depleted mice showed a similar pattern of parasitemia until day 8 PI, but cleared primary infection (Fig. 3), similar to that observed in normal rat IgG-treated or untreated control groups. Although involvement of $CD8^+$ T cells have been suggested to contribute to the development of protective immunity against *P. chabaudi* (Podoba and Stevenson 1991), our present findings indicate that $CD8^+$ T cells seem to have no effect on the course of *B. microti*. In contrast, depletion of $CD4^+$ T cells in BALB/c mice resulted in a course of infection similar to that of nude mice (Fig. 3). CD4-depleted mice had a peak parasitemia of 69.5% on day 10 PI, and they failed to clear the infection exhibiting between 34-47% parasitemia. These results demonstrate the importance of $CD4^+$ T cells in the clearance of infection with *B. microti*. A requirement for $CD4^+$ T cells in protective immunity has been demonstrated in *P. berghei* (Waki et al 1992), *P. chabaudi* (Cavacini et al 1986; Süss et al 1988) and *P. yoelli* (Jayawadena et al 1982; Vinetz et al 1990).

In *P. chabaudi*, the presence of $CD4^+$ cells is necessary at early phase of acute infection to control parasitemia (Langhorne et al 1990). In order to examine whether $CD4^+$ T cells are required throughout a primary infection to control the rise of infection, treatment of mice with anti-CD4 mAb was started three days prior, and seven and 14 days after *B. microti* inoculation. As shown in Figure 4, the administration of anti-CD4 mAb prior to the infection resulted to the development of of high parasitemia just like in nude mice, but depletion of $CD4^+$ cells at 7 and 14 days PI did not affect their ability to control of parasitemia. These results also suggest that $CD4^+$ cells are necessary at early phase of acute infection, e.i. before the peak parasitemia.

The role of $CD4^+$ cells that contribute to the resolution of acute phase of *B. microti* is not known. In *P. chabaudi* infection, two subsets of $CD4^+$ cells have been suggested to be responsible at different stage of primary infection, showing Th1 cells predominating until after peak parasitemia, and Th2 cells predo-

minating after the decrease of parasitemia (Langhorne et al 1989). To determine the role of $CD4^+$ cell subsets in *B. microti* infection, lymphokine production in spleen cells was assayed during the first 10 days PI. Production of IFN-γ and IL-4 in the supernatant of spleen cell culture in the presence of specific *Babesia* antigen obtained from anti-CD4, anti-CD8-treated, or untreated mice was determined. High concentration of IFN-γ was detected in cultures on day 4 from untreated and anti-CD8-treated mice, but production of IFN-γ of spleen cells from CD4-depleted mice was completely inhibited (Fig. 5). IL-4 was not detectable in all groups during the first 10 days, except that a very low concentration of IL-4 was noted in untreated and anti-CD8 treated mice only on day 8 PI, (data not shown). These data demonstrated that IFN-γ is apparently produced in high amounts by $CD4^+$ cells during early phase of *B. microti* infection and suggest that Th1 cells are apparently involved in the recovery of acute infection with *B. microti* and collaborate previous reports that DTH response occurs in parallel with resistance against *B. microti* (Ruebush et al 1986).

To examine the role of IFN-γ produced by CD4+ T cells in the resolution of *B. microti*, mice were treated with anti- IFN-γ or anti-IL-4 mAb. As shown in Fig. 6, treatment of mice with anti-IL-4 mAb resulted in the resolution of infection, similar to that of normal rat-IgG treated or untreated groups. Mice treated with anti IFN-γ mAb, however, showed higher peak parasitemia (65.0%) compared to untreated mice (46.0%) and to anti-IL-4 mAb treated group (50%), and was sustained at greater than 10% parasitemia until the 26th day PI. However, the effect of anti- IFN-γ mAb was less marked among mice that received lower dose of anti-CD4 mAb. If treatment of mice with anti-IFN-γ mAb was started on the 7th day PI, mice showed lower parasitemia than those mice that received the treatment on day 0 (Fig. 7). The role of IFN-γ on the blood stage of malaria infection has been reported (Waki et al 1992; Clark et al 1987; Shear et al 1989; Meding et al. 1990) and has been suggested to activate macrophages. In the present study, treatment of mice with anti- IFN-γ alone showed higher parasitemia relative to the control group, however, obstructive effect of anti- IFN-γ on the recovery of infection was not significant compared to anti-CD4. It may be supposed that one type of antibody is insufficient to deplete all IFN-γ activity in vivo, thus the need to prepare other types of mAb against IFN-γ . Also, it seems that IFN-γ alone cannot replace the capacity of CD4+ cells in clearing acute primary infection, and that additional factors such as IL-2, tumor necrosis factor as suggested in malarial infection (Traverne et al 1990; Traverne et al 1987) may be necessary in the resolution of *B. microti*.

Based on our results, we conclude that $CD4^+$ cells play an essential role in the resolution of *B. microti* acute primary infection and that IFN-γ produced by $CD4^+$ cells, at least in part, is responsible for the control of early stage of acute *B. microti* infection in mice.

ACKNOWLEDGEMENT

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Figure legends

Figure 1. Course of *B. microti* infection in BALB/c nu/+ (□) and nu/nu (●) mice. Parasitemia was determined in groups of five mice every two days.

Figure 2. Course of *B. microti* infection in SCID (■) and CB-17 (○) mice. Parasitemia was determined in groups of three mice every two days.

Figure 3. Course of *B. microti* infection in mAb-untreated BALB/c mice (■) or mice treated with normal rat IgG (○), anti-CD4 (●) and anti-CD8 (▲) mAb. Parasitemia was determined in groups of three mice every two days.

Figure 4. Course of *B. microti* infection in mAb-untreated BALB/c mice (○), or mice trated with anti-CD4 mAb three days prior parasite inoculation (●), and on day 7 (■), and day 14 (△) post inoculation.

Figure 5. IFN-γ production of spleen cells obtained from untreated BALB/c mice (■), or from mice treated with anti-CD 4 (▨), and with anti-CD 8 mAb (▩).

Figure 6. Course of *B. microti* infection in untreated BALB/c mice (●), in mice treated with anti-IFN-γ (■) and anti-IL-4 (△) mAb.

Figure 7. Course of *B. microti* infection in untreated BALB/c mice (○), in mice injected with anti-IFN-γ mAb beginning on the day of inoculation (■), and on day 8 (△) post inoculation.

Figure 1

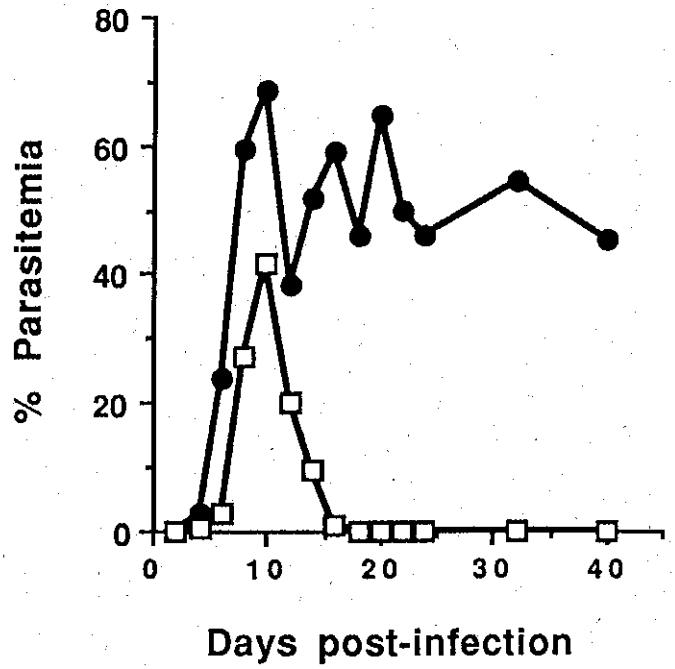


Figure 2

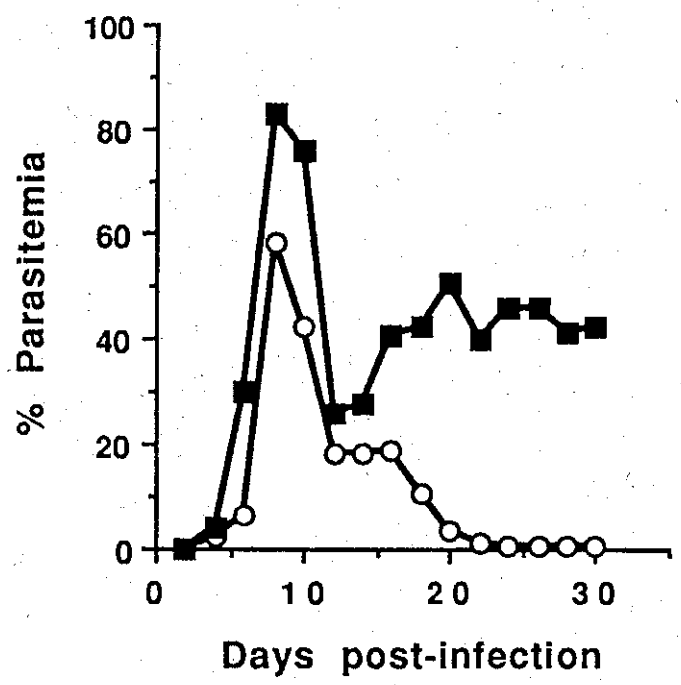


Figure 3

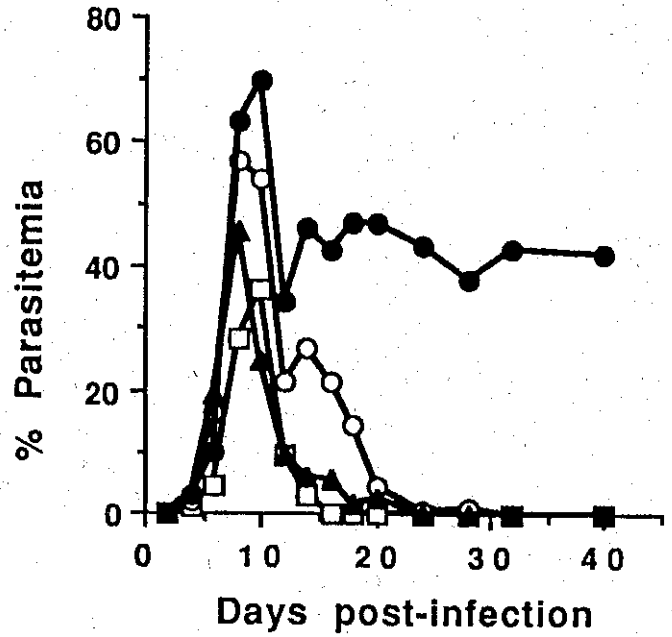


Figure 4

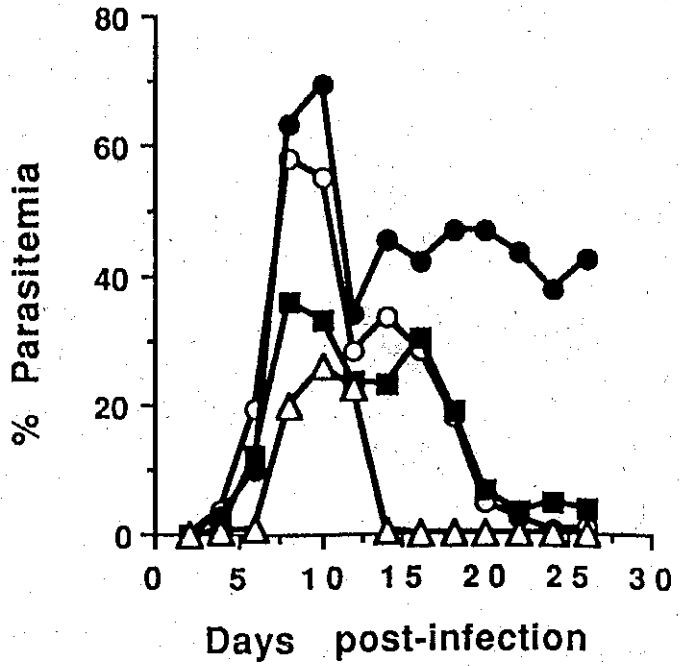


Figure 5

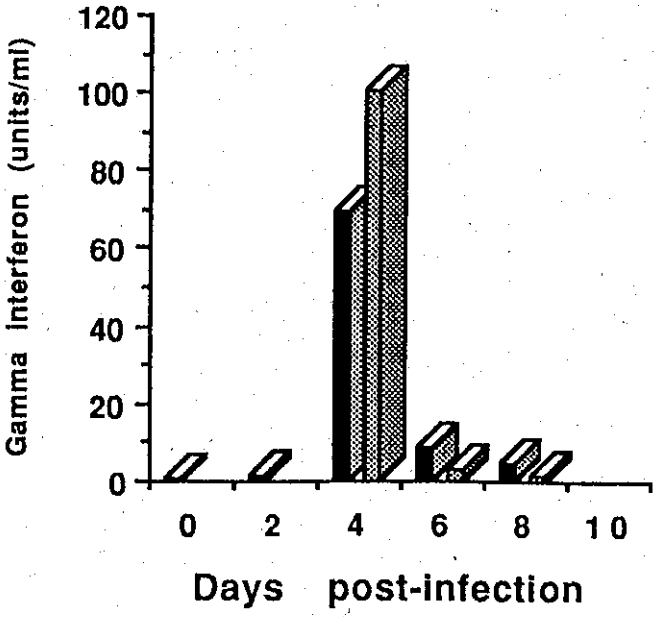


Figure 6

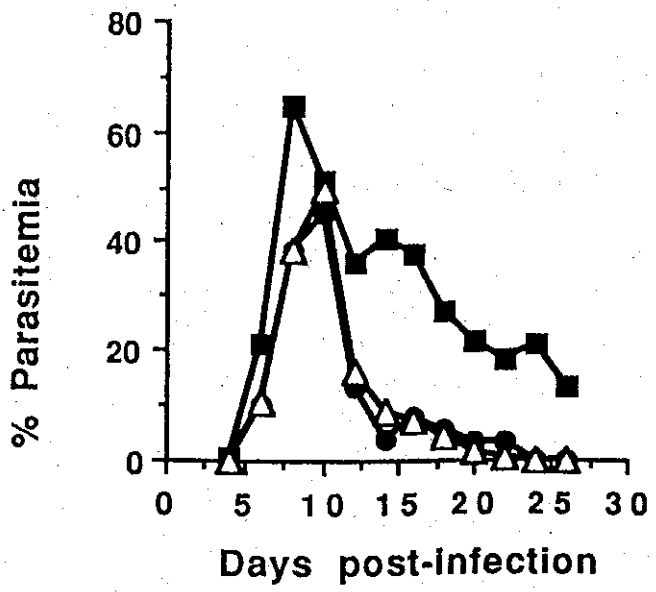
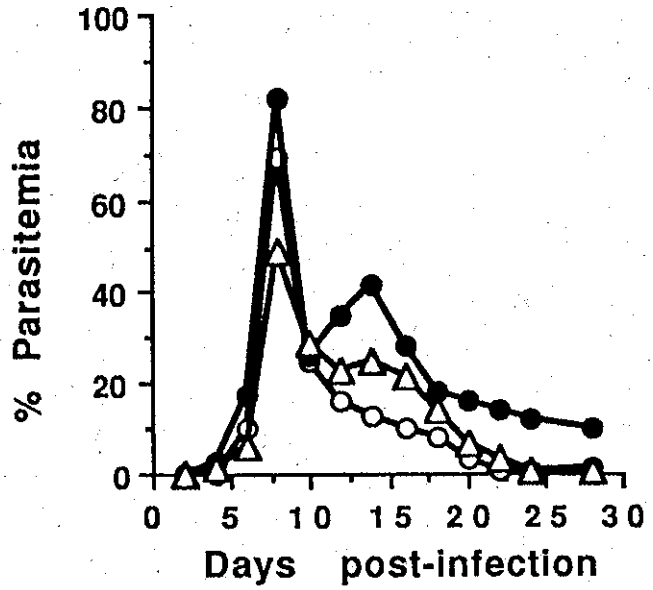


Figure 7


100
80
60
40
20
0
% Parasitemia
0
5
10
15
20
25
30
Days post-infection