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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.

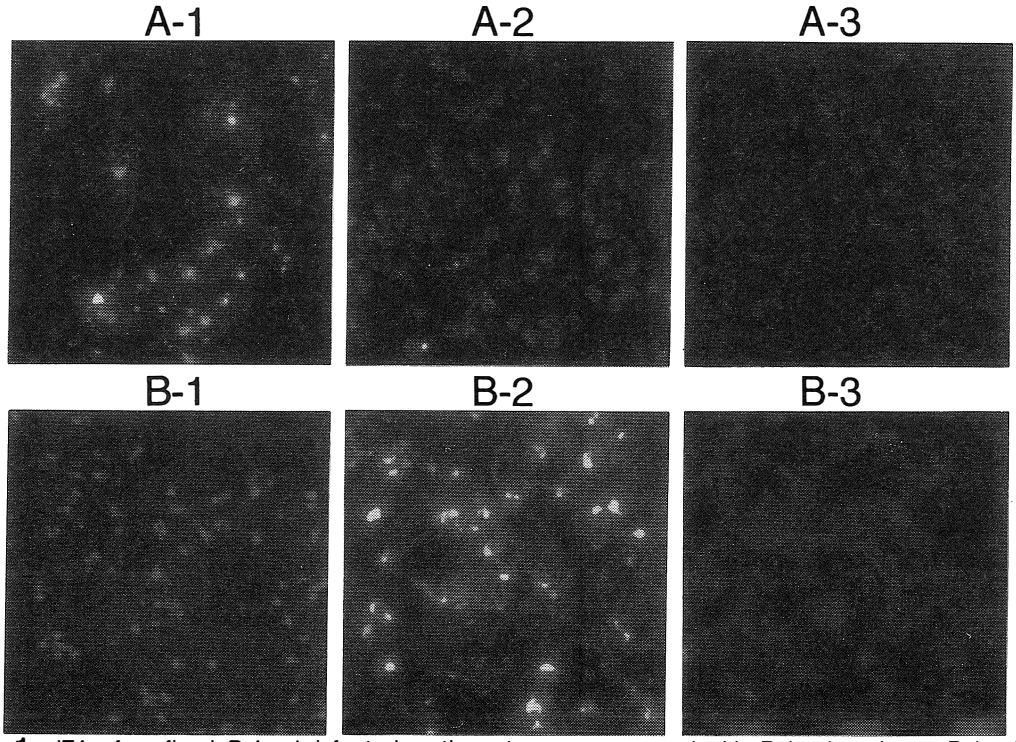

Fig. 1, IFA of un-fixed *Babesia*-infected erythrocyte smears reacted with *Babesia*-antisera. *Babesia microti* against *B. microti* (A-1) and *B. rodhaini* (A-2) antisera, or normal serum (A-3). *Babesia rodhaini* against *B. microti* (B-1) and *B. rodhaini* (B-2) antisera, or normal serum (B-3). x400

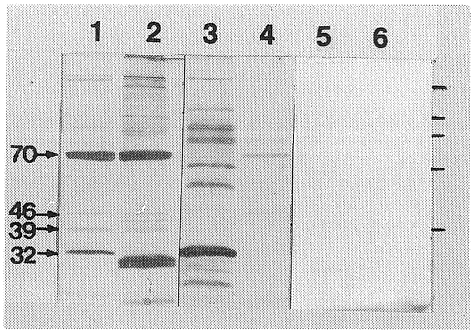

Fig. 2, Westernblot analysis of *Babesia* antigens. *Babesia rodhaini* (lane 1) and *B. microti* (lane 2) antigens detected using *B. microti anitserum. Babesia rodhaini* (lane 3) and *B. microti* (lane 4) antigens detected using *B. rodhaini* antiserum. *Babesia rodhaini* (lane 5) and *B. microti* (lane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

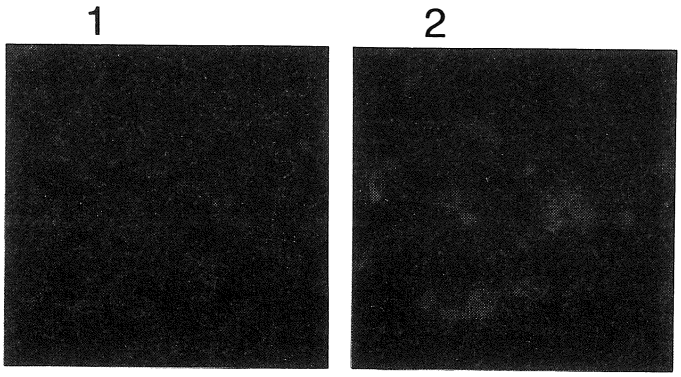

Fig. 3, IFA of un-fixed *Babesia* infected erythrocyte smears reacted with monoclonal anitbody #7. *Babesia microti* (1) and *B. rodhaini* (2). x400

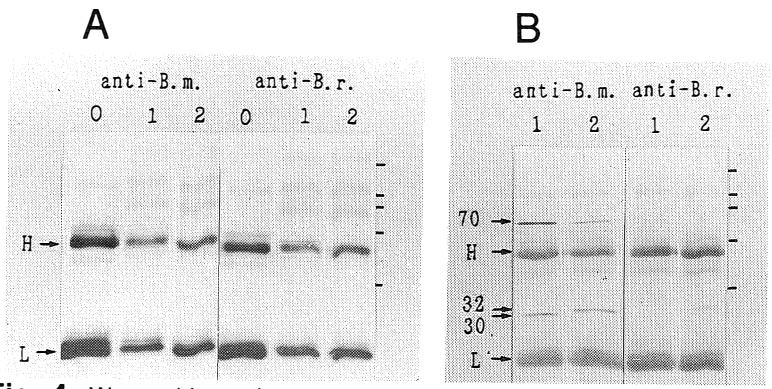

Fig. 4, Westernblot analysis of the immunoprecipitate materials from *Babesia* antigens with mAb #7 (A; lane 0, B; lane 1 and 2) or anti-*Toxoplasma gondii* mAb (A; lane 1 and 2). Normal erythrocyte (lane 0), *B. microti* (lane 1) and *B. rodhaini* (lane 2) antigens detected with *B. microti* (anti-B. 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.

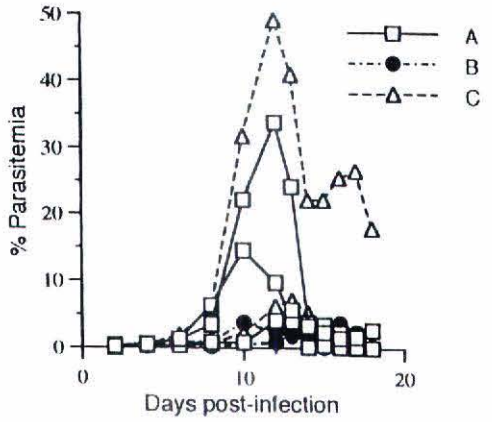

Fig. 5, progression of parasitemia in *B. microti*-exposed mice, given injections of mAb #7. (A): Injected 0.1 ml PBS i. v. from the day prior to exposure until the 14th day post-infection. (B): Injected 0.1 ml (100 μg) mAb #7 i. v. from the day prior to exposure until the 14th day post-infection. (C):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

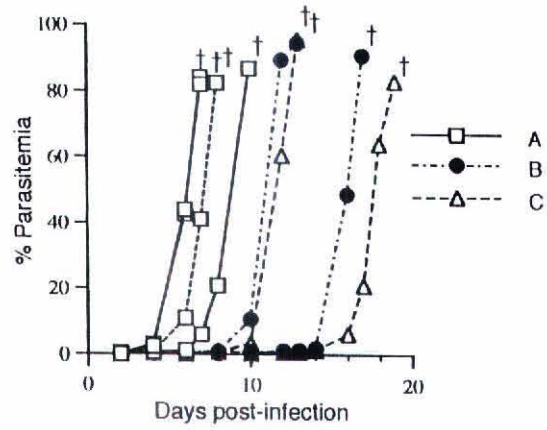

Fig. 6, progression of parasitemia in *B. rodhaini*-exposed mice, given injections of mAb #7. (A): Injected 0.1 ml PBS i. v. from the day prior to onset of death. (B): Injected 0.1 ml (100 μg) mAb #7 i. v. from the day prior to exposure until the 10th day post-infection. (C): Injected 0.1 ml (100 μg)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

Bautista, C. R. & Kreier, J. P. 1979. Effect of immune serum on the growth of *Babesia microti* in hamster erythrocytes in short-term culture. *Infect. Immun. 25*: 470-472.

Claveria, F. G., Omata, Y., Takehara, T., Saito, A., Maki, Y., Igarashi, I. & Suzuki, N. 1992. Anti-*Babesia rodhaini* Monoclonal Antibodies: Effect against *Babesia rodhaini* and Cross-reactivity with Human Strain of *Babesia microti*. *J. Protozool. Res. 2*: 112-122.

Cox, F. E. G. & Young, A. S. 1969. Acquired immunity to *Babesia microti* and *Babesia rodhaini* in mice. *Parasitology. 59*: 257-268.

Galfre, G., Howe, S. C., Milstein, C., Butcher, G. W. & Howard, J. C., 1977. Antibodies to major histocompatibility antigens produced by hybrid cell lines. *Nature. 266*: 550-552.

Kurtzhals, J., Andersen, B. J. & Christensen, N. f. 1988. Effects on in vitro growth of *Babesia microti* by cells and serum from *B. microti* and *Schstosoma mansoni* infected mice. *Acta. Vet. Scand. 29*: 357-362.

Meeusen, E., LLoyd, S. & Soulsby, E. J. L. 1984. *Babesia microti* in mice. Adoptive transfer of immunity with serum and cells. *Aust. J. Exp. Biol. Med. Sci. 62*: 551-566.

Roberts, J. A. 1968. Adoptive transfer of immunity to *Babesia rodhaini* by spleen cells from immune rats. *Aust. J. Exp. Biol. Med. Sci. 46*: 807-808.

Roberts, J. A. & Tracey-patte, P. D. 1975. *Babesia rodhaini*: A study of the effects of immune serum. *Int. J. Parasit. 5*: 577-581.

Simada, T., Shikano, S., Ono, K., Saito, A. & Suzuki, N. 1992. Changes of splenic lymphocyte subpopulation in mice inoculated with *Babesia microti* and *Babesia rodhaini*. *J. Vet. Med. Sci. 54*: 1071-1075.

Vernon, T. O. & Leonard, A. H. 1980. Immunoglobulin-Producing Hybrid Cell Lines. pp351-372. *IN*: Selected Methods in Cellular Immunology. Mishell, B. B. & Stanley, M. S. (Eds), W. H. Freeman & Company, New York.

Zivkovic, D., Seinen, W., Kuil, H., Albers-Van Bemmel, C. M. G. & Speksnijder, J. E. 1984. Immunity to *Babesia* in mice I. Adoptive transfer of immunity to *Babesia rodhaini* with immune spleen cells and the effect of irradiation on the protection of immune mice. *Vet. Immunol. Immunopathol. 5*: 343-357.