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Anil-*Babesia rodhaini* Monoclonal Antibodies: Effect against *Babesia rodhaini* and Cross-reactivity with Human Starin of *Babesia microti*

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ABSTRACT

Anti-*Babesia rodhaini* monoclonal antibodies (mAb), namely: 1-E7, 2-H2 and 3-B8, significantly suppressed the development of high parasitemia in BALB/c mice infected with *B. rodhaini* and all mAb-treated mice survived the infection. While, only monoclonal antibody 3-B8 showed some inhibitory effect against *Babesia microti*, with mice showing high parasitemia of 18.04 ± 2.69 %, at 9 days post-exposure. Westernblot analysis of *B. rodhaini* and *B. microti* parasite extract reacted with anti-*B. rodhaini* monoclonal antibodies showed cross-reactive bands of molecular weights 62 and 55 kilodaltons. Comparison of the antigenic components of *B. rodhaini* and *B. microti* using polyspecific sera revealed several shared or common parasite antigens of molecular weights 62, 55, 45-47, 41, 30-31 and 26-28 kilodaltons.

INTRODUCTION

Several studies have documented protection against *Babesia* spp. either by passive transfer of immune serum (Kurtzuhals et al., 1988; Ishimine et al., 1979; Bautista and Kreier, 1979; Abdahlla et al., 1978; Rogers 1974; Philips 1969; Mahoney 1967), or by adaptive transfer of splenocytes and lymph node cells from immunized mice (Meeusen et al., 1984a, 1984b; Reubush and Hanson 1980), or by passive transfer of immune spleen cells and injection of non-viable antigenic materials to recipient mice (Meeusen et al., 1985; Smith and Ristic 1981). Although, some workers are convinced of the role of nonspecific factors particularly in cases related to cross-protection induced by heterologous agents (Zivkovic et al., 1983/1984; Clark et al., 1976, 1977; Cox and Turner 1970), voluminous data from previous studies have provided strong evidence to suggest the principal involvement of antibodies (Ab) in host protection against babesiosis.

Until recently, in most vaccine-related research such as that of Tetzlaff et al. (1990) on *Babesia microti*

using polyspecific sera, and those of Figueroa et al. (1989), Wright et al. (1985, 1983), Smith et al. (1979) on bovine babesiosis, greater emphasis has been directed towards the immunochemical analysis of *Babesia* antigens (Ag) and their capacity to induce protective immunity. In this paper, we report our findings using monoclonal antibodies against two protective *B. rodhaini* Ag and their cross-reactivity with the human isolate of *B. microti*.

MATERIALS AND METHODS

Experimental animals and parasites: Six to eight wks old BALB/c mice were used in all experiments and were reared and kept in our animal facility. *Babesia rodhaini* and *Babesia microti* were maintained in BALB/c mice and both parasite species were syringe-passaged through intraperitoneal (ip) inoculation of infected blood in heparin and phosphate-buffered saline (PBS). *B. microti* was generously supplied by Dr. Heydorn of the Institute for Parasitology and Tropical Veterinary Medicine, The Free University of Berlin. This *B. microti* was originally isolated from a human case of babesiosis and was subsequently adapted to mice.

Hybridoma and monoclonal antibody screening: BALB/c mice were primed by ip inoculation of $1x10^4$ *B. rodhaini* parasitized red blood cells (PRBC). One wk after exposure, infected mice were given three intramuscular (IM) injections of 100 µl Ganaseg (5mg/ml, Squibb & Sons Inc., Argentina) per injection, spaced out in one wk. Mice that survived the treatment regimen were inoculated with a booster dose of $1x10^4$ PRBC. Cell hybridization protocol follows essentially that described by Oi and Herzenberg (1979). Three days after the boost, immune spleen cells were fused with P3Ns1-1, a BALB/c myeloma cell line, using 50 % polyethylene glycol 1500 in 75 mM Hepes (Boehringer Mannheim GmbH, Germany). Cell fusion suspension in 2 % hypoxanthine aminopterin thymidine (HAT) + 10 % fetal calf serum (FCS) + Cellgrosser culture medium (Meguro Laboratories, Osaka, Japan) was plated into five 96-wells plates, and was kept in a CO_2-supplied incubator at 37 ^{O}C. Wells that showed healthy growth of cell colonies were checked for antibody (Ab) production using indirect fluorescence antibody test (IFAT). Positive colonies were subcultured and were screened for clones that produced Ab specific against the parasite and/or against PRBC membrane. Hybridomas that produced relevant Ab were expanded in 24-wells trays, and were maintained in Freund's incomplete adjuvant (FIA) and/or pristane primed BALB/c mice. Ascitic fluid obtained from mice showing ascitic tumors was examined for mAb with IFAT. Of the 13 relevant clones inoculated into mice, only three clones got established, namely; mAb 1-E7, 2-H2 and 3-B8. Monoclonal Ab were precipitated from ascitic fluid diluted with PBS (v/v 50:50) using 50 % ammonium sulfate, and were dialized in three changes of PBS at 4 ^{O}C. Protein concentration was determined with the double wavelength beam spectrophotometer (Hitachi, Tokyo, Japan). Precipitated mAb were stored at -70 ^{O}C until use. Isotyping of mAb was done using the mouse mAb isotyping kit (Amersham, International pLc, UK).

Indirect fluorescence antibody test (IFAT):

A. Fixed parasites and PRBC: Infected blood diluted in PBS was applied on wells and fixed in methanol, and then was incubated with titrated (4^1-4^{12}) hybridoma culture-supernatant, ascitic fluid, precipitated mAb or polyspecific Ab (normal and *Babesia* immune mouse serum) at 37 ^{O}C for 30 min. After three washings of PBS and once with distilled water, the wells were air-dried and then incubated with fluorescein isothiocyanate (FITC) conjugated anti-mouse IgG (Tago Inc., Berlingame, CA) for 30 min. The slides were washed three times with PBS and mounted in 90 % glycerol-PBS.

B. Live parasites and PRBC: Infected blood obtained through cardiac puncture using heparinized syringe was transferred into microcentrifuge tubes, washed once with citrate buffer (pH 7.0), and three times with PBS by centrifugation at 5,000 rpm, 10 min/wash. To the tubes, mAb (1:10) or polyspecific Ab (1:100) in PBS were added and incubated for 30 min in a 37 ^{O}C waterbath with constant gentle shaking. After three washings with PBS, the cell pellets were incubated with FITC conjugated anti-mouse IgG, as described above. To remove excess FITC conjugated anti-mouse IgG, the cells were washed three times with PBS, resuspended in 90 % glycerol-PBS, and then mounted on glass slides. Both fixed and unfixed preparations were examined with a Nikon fluorescence microscope.

***Sodium dodecyl sulfate (SDS)- polyacrylamide gel electrophoresis (PAGE)*:** Parasite Ag mixed with an equal amount of sample buffer (2.5M Tris-HCl, pH 6.8, 4.6% SDS, 10% glycerol, 10% 2 mercaptoethanol, 0.05% bromophenol-blue) was heated over boiling water for 5 min and kept in a 37 ^{O}C water bath with shaking for another 5 min. The sample was ultrasonicated at 70W, 3 x 10 s each time and centrifuged for 5 min at 15,000 rpm. Per well, 10-15 µl of the soluble parasite extract obtained was loaded on a standard 10% acrylamide gel. Fractionated proteins were immunoblotted and/or stained with Coomassie blue. Low molecular weight markers used were purchased from BIO-RAD Laboratories, Richmond, CA.

Immunoblotting: Electrophoretic transblotting of fractionated proteins or Ag into nitrocellulose paper (0.22 µm pore size, GVHP, Nippon Millipore Kogyo, Yonezawa, Japan) followed essentially that of Towbin et al. (1979). Transblotted nitrocellulose sheets were stained with 5% amidoblack black, destained with 10 % acetate and washed three times with PBS-0.05 % Tween 20 (PBS-T20), 10 min/wash. To remove nonspecific reactants, the membrane sheets were washed with 10 % methanol for 10 min, followed by three washings in PBS-T20, and reacted with the different test Ab (normal and immune mouse serum) and mAb. Polyspecific Ab and mAb were diluted 1:100 in 5 % skim milk (Difco Co., Michigan, USA), respectively. Reaction time with mAb lasted 2 - 6 h, about half the time at room temperature (RT) with constant gentle shaking, and the remaining time at 4 ^{O}C. The membranes were washed three times with PBS-T20, and incubated with 1.5 % peroxidase-conjugated anti-mouse IgG (BIO-RAD Laboratories, Richmond, CA), in 5 % skim milk, for 1 h at RT, with constant gentle shaking. After 3 washings in PBS-T20, the membranes were reacted with freshly prepared substrate consisting of 0.02 % 3,3-diaminobenzidine-4HCl and 15 µl H_2O_2 in 0.1 M Tris-HCl (pH 7.4).

Babesia rodhaini and Babesia microti parasite extract preparation: Blood was drawn out from infected mice through cardiac puncture using heparinized syringe with PBS and Alsever solution. Parasite Ag were processed following the procedure described by Sugimoto et al. (1991) with some modifications, Blood was overlaid on Ficoll-Conray solution (1:1 ratio) and centrifuged at 1,440 rpm for 20 min. The cell pellets were washed three times in 10 mM Tris-HCl (pH 7.5) - 150 mM NaCl buffer at 2,200 rpm, 10 min/wash. Cells were hemolyzed using 27 units hemolysin (Asao et al., 1984; Kozaki et al., 1987) per ml of Tris- NaCl buffer, generously provided by Dr. Sugimoto of the National Institute of Animal Health, Tsukuba, Japan. Hemolysin-treated PRBC were incubated for 2 h in a 37 ^{O}C waterbath with occasional shaking. The level of hemolysis was checked after 1 h of incubation using a hemocytometer. To obtain maximum hemolysis, incubation was allowed to continue longer depending on the level of parasitemia obtained from blood, with the addition of 13 - 17 hemolysin units/ml. The lysate was kept in ice-cold water for about 30 min, and ethylenediamine tetraacetic acid (EDTA) solution (pH 7.5) was added to make 5 mM final lysate concentration. Five ml of lysate/tube was overlaid on a 10 ml 60 % and 40 % Percoll discontinuous density gradient (Pharmacia LKB Biotechnology, Uppsala, Sweden) prepared with Tris-(HCl)-NaCl-EDTA buffer (pH 7.4), and centrifuged at 20,000 rpm for 20 min. Parasites obtained from the visible interphase band between 60 % and 40 % Percoll solution were washed with Tris-NaCl-EDTA buffer 3 times at 10,000 rpm, first wash for 10 min and the last two washings for 5 min/wash. To the parasite suspension, an equal amount of lysis buffer consisting of 50 mM Tris-HCl (pH 8.0), 0.5 % Triton X-100, Noninet P-40 and 1 mM PMSF was added and kept at -70 ^{O}C until use.

Purification of mAb: Monoclonal Ab were purified from ascitic fluid by ion-exchange chromatography using DEAE To.yo Pearl 650M (Toso, Tokyo, Japan) in 50 mM Tris-HCl buffer (pH 7.5) with gradient concentration of 0-400 mM NaCl. The eluted mAb in the fractions were detected with IFAT and the protein concentration was determined using a double wavelength beam spectrophotometer.

Immunoaffinity purification of Ag : Immunoabsorbent column was prepared using 30 mg of purified mAb covalently bound to 300 mg TSK gel Tresyl-5PW (Toso, Tokyo, Japan) in 1 M phosphate buffer (PB), pH 7.5, at 4 ^{O}C for 16 h with gentle shaking. The remaining binding sites were blocked by incubation with 0.02 M Tris-HCl buffer (pH 8.0) for 1 h at RT, and then washed with 0.1 M PB (pH 7.4). Ultrasonicated parasite extract that was passed through the affinity column was extensively washed with 0.1 M PB (pH 7.4), and the bound Ag were eluted with 0.1 M citrate-HCl buffer (pH 1.6). Eluted fractions were neutralized immediately with 1 M Tris solution and were concentrated by ultra filtration, and then stored at -70 ^{O}C.

Passive transfer of immunity : Tubes containing 300 µl each of the precipitated mAb 1-E7 (1.0 mg), 2-H2 (2.7 mg) or 3-B8 (3.84 mg) were incubated with $3x10^{4}$- *B. rodhaini* PRBC suspended in 1.2 ml of

PBS and 10.8 ml of 20 % FCS-Eagle's minimum essential medium (MEM) for 1 h in a CO_2-supplied incubator at 37^{O}C. The cell suspensions were centrifuged for 20 min at 2,000 rpm, and the supernatant was aspirated leaving 1 ml of the content to which 2 ml of PBS was added. In the experimental groups (3 mice per group), each mouse was ip-inoculated with 1 ml of mAb-treated PRBC. Control groups, likewise, consisted of 3 mice per group, each mouse was ip-injected with incubated PRBC without mAb (control group A). For control B, each mouse was injected with the same dose of PRBC which were neither incubated nor treated with mAb. Each mouse in the experimental groups was ip-injected 0.2 ml mAb suspension containing mAb liters of 0.67 mg, 1.8 mg and 2.56 mg for 1-E7, 2-H2 and 3-B8, respectively. Preparation of *B. microti* inoculum was prepared similar to that described for *B. rodhaini*. Each mouse was inoculated with $18x10^6$ PRBC. Experimental groups mAb 1-E7, 2-H2 and 3-B8 were injected daily mAb liters of 1.26 mg, 2.48 mg and 3.32 mg, respectively. Thin blood smears were prepared at 2 - 3 day interval. Percent parasitemia was calculated by counting the number of PRBC out of 500-1,000 (depending on the level of parasitemia) total red blood cells covering various microscopic fields.

RESULTS AND DISCUSSION

Monoclonal antibodies 1-E7, 2-H2 and 3-B8 were of the isotype IgG2b. With fixed parasite material, we observed strong fluorescence on PRBC membrane and against both extracellulay- and intracellulary-located parasites. With live material fluorescence was likewise, noted on PRBC membrane, and against extracellular parasites only.

Mice exposed to *B. rodhaini* parasite incubated with precipitated mAb preparation and administered daily injections of mAb exhibited significantly lower percent parasitemia ($p< 0.05$) starting the 5th day post-exposure (PE), compared to the control groups (Fig. 1A). At 19 day PE, percent parasitemia of mAb-treated groups were 15.0 ± 0.86% (1-E7), 12.95 ± 4.9% (2-H2) and 13.32 ± 7.15% (3-B8) respectively. After the last injection, however, parasitemia increased in all mAb-treated groups, and at 38 day PE, registered peak percent parasitemia of 65.0 ± 17.2% (1-E7), 69.6 ± 16.5% (2-H2) and 46.0 ± 8.1% (3-B8). Afterwards parasitemia decreased in all experimental groups and ranged from 6-21 % at 43 - 48 day PE, and all mAb-treated mice survived the infection. While all control mice died between 27 and 30 day PE with peak percent parasitemia ranging from 87.2 - 93.7 %.

Fig. 1B shows the effect of anti- *B. rodhaini* mAb on *B. microti* infection. The first 5 day after exposure showed no significant difference between mAb-treated and control groups. At 7 day PE, however, percent parasitemia for mAb 1-E7- and 3-B8- treated mice were 3.92 ± 3.23 % and 3.69 ± 2.0%, respectively. Those of mAb 2-H2- treated mice and the control groups A and B were slightly higher at 13.7 ± 3.45%, 19.34 ± 10.65% and 12.96 ± 9.47%, respectively. Days following the 7th day PE, parasitemia in all

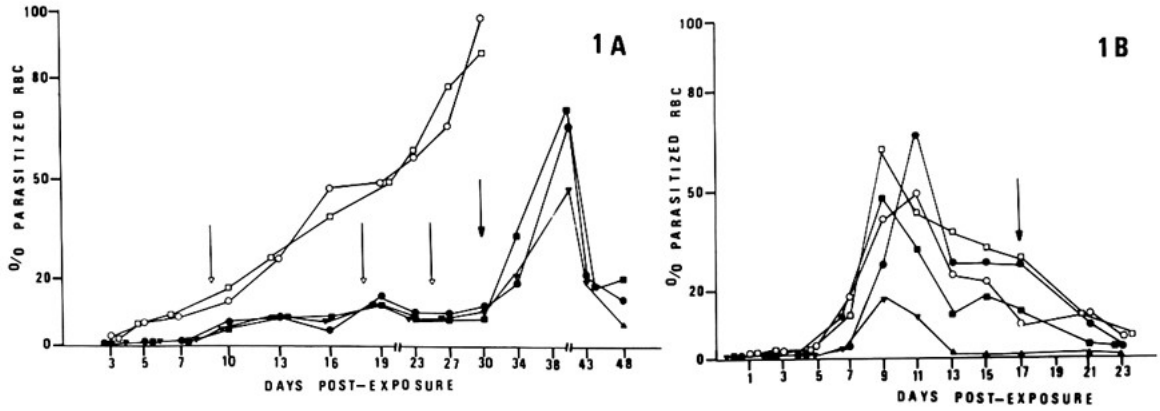

Figure 1. Passive transfer of immunity using anti-*Babesia rodhaini* mAb against *B. rodhaini* parasite (1A) and against *Babesia microti* parasite (1B). Per experimental group consisted of three mice, each mouse was exposed to mAb-incubated parasitized red blood cells (PRBC) and was injected daily with mAb 1-E7 (●), mAb 2-H2 (■), or mAb 3-B8 (▲). Inoculation dose: *B.rodhaini* PRBC 1 x 10^4 ; *B. microti* PRBC 18 x 10^6. Control group A (○) exposed to the same inoculum dose of incubated, non-mAb-treated PRBC. Control group B (□) exposed to the same inoculum dose of non-incubated, non-mAb-treated PRBC. Last day of mAb injection (↓); no mAb administration (⇩). Each points represents the mean value per three mice.

groups increased significantly, except for mAb 3-B8-treated mice which showed peak parasitemia of 18.01 ± 2.69 % at 9 day PE, and was kept at levels less than 0.5 % from the 13th to the 23rd day PE. In contrast, peak parasitemia of mAb 1-E7- and 2-H2- treated groups were 66.47 ± 15.42%, 48.78 ± 8.95%, and those of controls A and B were 48.97 ± 27.5% and 61.69 ± 28.24%, respectively. All control and experimental mice survived the infection.

Results of westernblot analysis showed cross reactive Ag between *B. rodhaini* and *B. microti*. As shown in Fig. 2 (lanes B), parasites extracts of *B. microti* and *B. rodhaini* reacted with *B. rodhaini* hyperimmune serum revealed common bands of M.W 66, 62, 55, 45 - 47 and 30 - 31 kilodaltons (kDa). Reaction with *B. microti* polyspecific Ab showed similar bands as indicated above, in addition to two other bands of M. W 26 - 28 kDa. Another parasite Ag of M. W 72 kDa was apparent in the *B. rodhaini* lanes which was detected with both *B. rodhaini* and *B. microti* immune sera.

Likewise, westernblot analysis (Fig. 2) showed mAb 1-E7 and 2-H2 reactive with the 62 and 55 kDa *B. microti* parasite components. With mAb 3-B8, we noted a weak band of M.W 62 kDa. With *B. microti* parasite extract, similar bands were detected by all three mAb. A fine band M.W 72 kDa was noted. This band seemed to be a non-specific reactant detected by certain Ab presumably present in crude mAb preparation obtained from ascitic fluid, since no such band was detected when immunoaffinity purified Ag was reacted with purified mAb.

Repeated westernblot analysis consistently yielded weak 62 and 55 kDa bands. We tried to verify our findings by reacting purified mAb with parasite Ag obtained through immunoaffinity chromatography. As shown in Fig. 3, parasite Ag collected during the first run and second run of immunopurified *B. rodhaini*

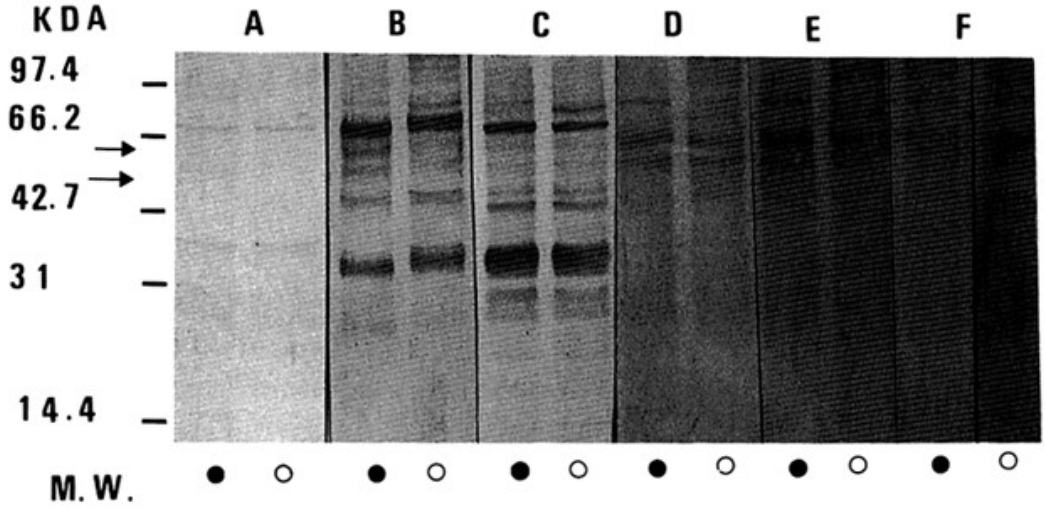

Figure 2. Westernblot analysis of *B. rodhaini* (○) and *B. microti* (●) parasite extract reacted with normal serum (A), *B. rodhaini* hyperimmune serum (B), *B. microti* immune serum (C), mAb 1-E7 (D), mAb 2-H2 (E), mAb 3-B8 (F). Kilodaltons (kDa) : Molecular weight (M.W). Arrows indicate the 62 and 55 kDa parasite antigens reactive with mAb.

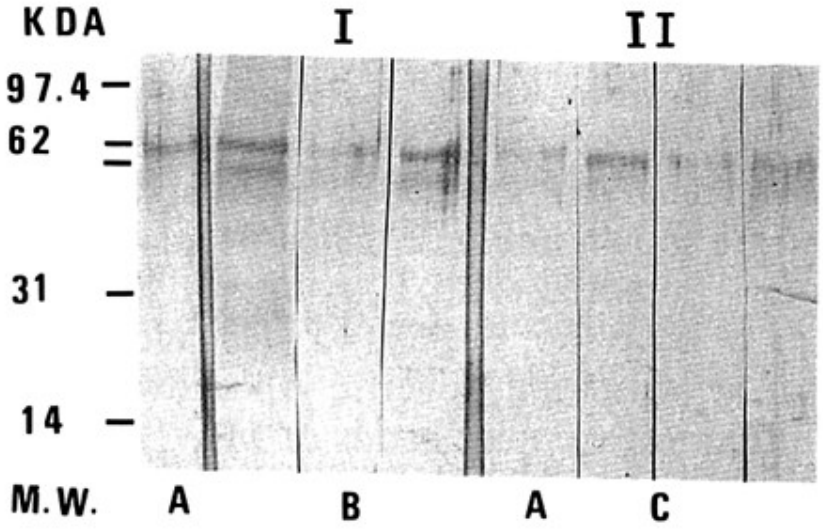

Figure 3. Westernblot analysis using immunoaffinity-purified *B. rodhaini* antigens. Crude parasite extract (lane A). Purified antigens of the first cycle (Group I, lane B) and second cycle (Group II, lane C) reacted with mAb 1-E7 and mAb 2-H2, respectively.

extract using a mixture ofmAb 1-E7 and 2-H2, both yielded two distinct bands of M.W 62 and 55 kDa. when reacted with mAb 1-E7 and 2-H2.

Although, the establishment of *B. rodhaini* infection in mice injected with mAb was not totally suppressed, the significantly lower percent parasitemia suggests some protective or inhibitory effect of mAb against the parasite. The daily injection of mAb seemed to have augmented and helped sustained the level of these specific protective Ab against the parasite, in addition to all other protective Ab in circulation. This may effect a slowing down in the penetration rate of the parasite inside red blood cells as

gleaned from an increased parasitemia, a few days after the last injection, and on certain day of mAb (Fig. 1a). Abdalla et al. (1978), noted that *B. rodhaini* immune serum delayed the onset of parasitemia, but it neither prevented the development of injection nor protected the mice from death, even with further supplementation of immune serum during infection. We observed a similar pattern of the progress of infection, however, all mAb-treated mice outlived the infection (Fig. 1a). While, we administered a daily injection of mAb to mice, Abdalla et al. (1978) supplemented immune serum two to three times only after exposure, throughout the duration of the experiment. The survival of mAb-treated mice infected with the usually fetal *B. rodhaini* noted by us is interesting. Thoongsuwan and Cox (1973) had recovered three antigenic strains of *B. rodhaini* from the virulent parent strain by means of in vitro treatment with immune globulin. They alluded the loss of virulence of the recovered strains to loss of antigenicity (i.e. either loss or decreased immunogenicity), rather than to selection of a variant with antigen(s) that differed from that of the parent strain. Timms et al. (1990), likewise, attributed the appearance and loss of *Babesia bovis* virulence to the mixture of subpopulations of varied virulence consisting a parent strain. They, too, maintained that the mechanisms of differential gene expression and phenotypic selection of subpopulations have to operate for virulence in *B. bovis* to be altered. In the present study, whether the loss of virulence in mAb-treated mice resulted from the loss or diminished immunogenicity as a consequence of mAb-treatment (Thoongsuwan and Cox 1973) or from the combined mechanisms of differential gene expression and phenotypic selection (Timms et al., 1990), and/or from continuous passage of the parasite in mice for a long time (Howard et al., 1980) is an interesting question that warrants further investigation.

Against *B. microti*, only mAb-3-B8 showed some protective activity to mice, although, less significantly compared to its effect against *B. rodhaini*. The absence of inhibitory effect of anti-*B. rodhaini* mAb-1-E7 and 2-H2 against *B. microti*, seemingly suggests a difference in the antigenic determinants or epitopes of both the 62 and 55 kDa between these species. Thus, these mAb may be alluded to, as non-protective against *B. microti*. Likewise, our data suggest common epitope for the M.W 62 kDa Ag recognized by mAb-3-B8 in both species.

Westernblot analysis of both *B. rodhaini* and *B. microti* parasite extracts revealed shared Ag. The 66, 62, 55 and 45 - 47 kDa Ag may share similarities with the immunochemically determined surface Ag of *Babesia bigemina* (Figueroa et al. 1989), and to the 60 kDa protein obtained from *B. rodhaini* infected blood (Howard et al., 1980). Tetzlaff et al. (1990) had isolated and characterized a gene of M.W 55 kDa which he hypothesized to be likely associated with a virulent strain of *B. microti*. Interestingly, we noted, a 55 kDa parasite component in *B. rodhaini* and *B. microti*, which reacted with both mAb 1-E7 and 2-H2, and with immune sera.

At present, we have started purifying the 62 and 55 kDa *B. rodhaini* Ag. Antigens will be used for

further experimentations related to the findings reported in this paper.

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