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Characterization of Epitopes on an 18 kDa Piroplasm Surface Protein of *Babesia equi*

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ABSTRACT

Monoclonal antibodies were produced against *Babesia equi* piroplasms. Three MoAbs reacting with an 18 kDa surface membrane protein (p18) of *B. equi* in immunoblot analysis, were used to characterize the epitopes on p18. All the three MoAbs recognized the same epitope on p18 as indicated by competitive ELISA. Negative results in two-site ELISA suggest absence of repetitive epitopes on p18. Triton X-114 phase partitioning confirmed that 18 kDa antigen is an integral membrane protein of *B. equi* piroplasms. As these MoAbs identified a single protein and showed no crossreaction with *B. caballi* or equine erythrocyte proteins, these can be a candidate to be used in the differential diagnosis of mixed equine piroplasma infections.

INTRODUCTION

Equine babesiosis is one of the major problems in horse trade especially due to the danger of disease transmission during the movement of race horses from country to country. There is a need to improve the existing serological assays used to detect the equine piroplasmosis as no single reliable test is available. Most researchers propose that a combination of at least two different assays should be used to increase the diagnostic reliability of the serodiagnosis of *Babesia* infections (Tenter and Friedhoff 1986; Weiland 1986; Weiland and Reiter 1988).

Efforts have been made to demonstrate parasite-specific antigens by the generation of monoclonal antibodies (MoAbs). A competitive-inhibition enzyme-linked immunosorbent assay (ELISA) using a MoAb recognizing a geographically conserved epitope has been described (Knowles et al. 1991a and b). None of the antigens diagnostic for European isolates of *B. equi* were recognized by sera from field-infected horses from Brazil (Bose and Hentrich 1994). Previously, we identified six immunodominant *B. equi* piroplasm proteins. Out of these, Mr 18, 30 and 32 were found to be membrane bound proteins (Ali et al. 1993). It is necessary to determine whether these proteins are involved in reactions in various serological tests for equine babesiosis. In this experiment we produced MoAbs against *B. equi* piroplasms and further characterized 18 kDa surface protein using these MoAbs.

MATERIALS AND METHODS

Parasite and antigen preparations

Non-splenectomized horses were inoculated with *Babesia equi*-infected blood that had been obtained from National Veterinary Services Laboratories (United States Department of Agriculture, Iowa, USA). Piroplasms of *B. equi* were purified from infected horse erythrocytes as described before (Ali et al. 1993). For preparation of enzyme-linked immunosorbent assay antigen, purified piroplasms were treated with 2% Nonidet P-40 in phosphate-buffered saline (PBS, pH 7.2) at 4 °C for 3 hrs. After centrifugation at 2,000*g* for 10 min, the supernatant was used for ELISA according to the method of Shimizu et al. (1988).

South African *B. equi* antigen used in the Westernblot analysis was kindly supplied by Dr. E. Zweygarth, Onderstepoort Veterinary Institute, Onderstepoort, South Africa. Dr. V. T. Zablotsky of All Russia Institute of Experimental Veterinary Medicine (VIEV), Moscow, Russia has kindly provided the Russian *B. equi* antigen used in this experiment.

Preparation of monoclonal antibodies

Six weeks old BALB/c mice were subcutaneously immunized with 100 µg protein/0.1ml of *B. equi* piroplasms emulsified with the same volume of Freund's complete adjuvant. They were boosted with the same amount of the antigen two weeks after the first injection. The spleen was removed three days after the second booster. Spleen cells (1×10^8 cells) were fused with 1×10^7 myeloma cells (P3Ul) in the presence of ploye thylene glycol (PEG 1500, Boehringer Mannheim, GmbH, Germany). Hybrid cells were selected by HAT medium (hypoxanthine aminopterine-thymidine). Hybridoma supernatants were screened by immunoblotting. The antibody-producing hybridomas were cloned

twice by the limiting dilution method in a 96 well plastic microplate.

To determine the class, subclass and light chain type of the MoAb's, ammonium sulphate precipitated and protein G affinity purified MoAb's were examined by a MoAb isotyping kit (Amersham International plc. UK).

Purification and biotinylaffon of MoAbs

MoAbs were purified from hybridomas grown in serum-free medium and from ascitic fluids by precipitation with 50% ammonium sulphate and protein G affinity chromatography using MAb Trap G II kit (Pharmacia LKB, Sweden). For biotinylation of MoAbs, sulfosuccinimidobiotin (Rockford, Illinois, USA) was used to covalently bind biotin to purified MoAbs. One ml of the purified MoAbs (1 mg/ml) was mixed at a ratio of 250 µg of ester/mg of antibody and incubated at room temperature for 4 hrs. Incubation reaction was stopped by the addition of 20 µl of 1 M NH_4 Cl per 250 µg of ester. The mixture was dialyzed against PBS and 0.1 % thimersal was added as preservative. Labelled MoAbs were stored at 4 °C till use. The specificity and optimal concentration of the biotinylated MoAbs was determined by a standard ELISA, as described below, using *B. equi* piroplasm antigen and avidin-conjugated peroxidase (Zymed Laboratories, CA, USA).

Phase partition by Triton X-114

Piroplasm proteins were subjected to Triton X-114 phase partition according to the method of Bordier (1981). Purified piroplasms were treated with Tris-buffer saline (TBS) containing 1% (v/v) Triton X-114 and 1 mM phenylmethylsulfonyl fluoride (PMSF) on ice for 30 min. The lysate was centrifuged for 1 hr at 100,000*g* and the pellet was discarded. The supernatant was carefully layered over an equal volume of a 6% (w/v) sucrose cushion in TBS containing 1 mM PMSF. After incubation at 30 °C for 15 min, the sample was centrifuged at 1,000*g* for 15 min at room temperature. The top aqueous phase and bottom detergent phase were collected. Each fraction was precipitated with 4 volumes of acetone and centrifuged at 6,000*g* for 10 min at 4 °C. The pellets were stored at -80 °C.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis and immunoblotting

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblot were carried out as described (Ali et al. 1993). Briefly, one-dimensional SDS-PAGE was performed using a 12.5% polyacrylamide gel according to the method described by Laemmli (1970). Samples were solubilized in Tris-HCl buffer (0.0625 M, pH 6.8) containing 2% (w/v) SDS and 5% (w/v) 2-mercaptoethanol (Laemmli 1970) at 100 °C for 2 min. After separation by SDS-PAGE, the piroplasm proteins were electrophoretically transferred to polyvinylidene difluoride (PVDF) membrane sheets (Immobilon

transfer membranes; Millipore, USA) according to the immunoblotting technique of Dunn (1986). The sheets were blocked for 1 hr at room temperature in 0.01 M PBS (pH 7.2), 0.05% (v/v) Tween-20 (PBST) containing 5% skim milk (SM). These were washed three times with PBST and incubated for 1 hr at room temperature with MoAbs with 1% SM. The sheets were washed and incubated for 1 hr at room temperature with goat anti-mouse IgG peroxidase conjugate (Jackson Inc., USA) diluted 1:1,000 in PBST with 1% SM. After washing, the sheets were treated with a freshly prepared substrate solution containing 1.3 mM diaminobenzidine tetrahydrochloride (DAB; Nakarai Chemicals, Ltd, Kyoto, Japan), 1.3 mM $CoCl_3$ and 0.02% (v/v) hydrogen peroxide in PBST.

Enzyme linked immunosorbent assay

ELISA was performed according to the method described previously (Shimizu et al. 1988) with a minor modification. Briefly, optimal concentrations of antigen, antibody and enzyme conjugate were determined by checker board titration. Serial two fold dilutions of 36 μg per ml piroplasm proteins/0.1 ml in 0.05 M carbonate/bicarbonate buffer (pH 9.6) per well, were tested through ELISA using serial two-fold dilutions of enzyme conjugate (from 1:500 to 1:2,000), standard positive and negative (Ali et al. 1993) horse sera (from 1:20 to 1:1,280) and unlabelled MoAbs (from 1.25 μg/ml to 50 μg/ml). For color development, 0.1 ml of substrate (0.2 mM azino-di-[3-ethylbenzthiazoline] sulfonic acid) was used. The plates were read at 405 nm in a spectrophotometer (Corona Electric, Tokyo, Japan). The end point of ELISA was estimated from the dose-response curve and the antibody titer was expressed as the reciprocal of the highest dilution of MoAbs showing an absorbance over 0.1.

Competitive binding assay between MoAbs

The assay was performed according to the method described previously (Zavala et al. 1983). The wells of microplate were coated with 0.1 ml of *B. equi* piroplasm lysate (protein concentration 18 μg/ml) in 0.05 M carbonate-bicarbonate buffer (pH 9.6) and kept ovemight at 4 °C. Non-specific sites were blocked with 0.5% SM in PBST at room temperature for 1 hr.

As an antibody competitor, 0.1 ml of serial 2-fold dilutions of unlabelled purified monoclonal antibodies (0-20 μg) were added. After incubation at room temperature for 1 hr, the plates were washed with PBST and 0.1 ml of biotin-labelled monoclonal antibodies (20 μg IgG/ml) were added. Following incubation at room temperature for 1 hr and washing with PBST, 0.1 ml of avidin-peroxidase (Zymed Laboratories, CA, USA) diluted 1:1,000 in 0.2% SM-PBST was added and the plates were incubated at room temperature for 1 hr. The wells without *B. equi* antigen followed by biotin-labelled antibody were used as

negative controls. For color development, 0.1 ml of substrate (0.2 mM azino-di-[3-ethylbenzthiazoline] sulphonic acid) was used. The absorbance of the wells was read at 405 nm in a spectrophotometer (Corona Electric, Tokyo, Japan).

Two site-ELISA

Two-site ELISA was performed by the method described previously by Zavala et al. (1983) and Burkot et al. (1984). The microplate was coated with 0.1 ml of purified unlabelled MoAb C2, C5 and D3 (20 μg/ml) and left overnight at 4 °C. After washing with PBST, non-specific binding sites were blocked as described above. Plates were washed and 0.1 ml of serial 2-fold dilutions of 18 μg/ml solubilized merozoite antigen were added. After incubation at room temperature for 1 hr, 0.1 ml of biotin-labelled homologous MoAb (20 μg/ml) was added. Plates were incubated at room temperature for 1 hr, and processed as described above. Antibody-coated wells incubated without antigen followed by biotin-labelled antibody were used as negative controls.

RESULTS

Characterization of the monoclonal antibodies

Immunoblotting was carried out for the determination of the specificity of MoAbs and for the identification of the molecules recognized by these MoAbs. A total of 54 clones secreting antibodies against *B. equi* piroplasms were obtained in 5 fusion experiments. Supernatants of three (i.e. C2, C5 and D3) out of 54 hybridomas, that reacted with the 18 kDa protein (p18) of *B. equi* in immunoblotting, were selected for further studies. None of the three MoAbs reacted either with *B. caballi* or equine erythrocyte proteines in Westernblot analysis (Fig. 1: Lanes 3 to 5). These monoclonal antibodies also recognized an 18 kDa protein in the Westernblot of a *B. equi* strain collected from Russia (Fig. 2: Lanes 1 to 3). Other dominant proteins of the Russian strain were Mr 40.5, 28 and 16.5 kDa. However, these MoAbs did not recognize p18 in Westernblot of *B. equi* strain collected from South Africa (Fig. 2: Lane 4). Determination of immunoglobulin isotype showed that all of the three monoclonal antibodies were IgG1 (K).

Triton X-114 phase partition of piroplasm antigen

The Triton X-114 phase partition method was used to determine whether 18 kDa antigen is an integral membrane protein. When the extracted materials were electroblotted and reacted with MoAbs, all of which recognized a 18 kDa molecule of the *B. equi* piroplasm, this molecule was partitioned exclusively into the detergent phase (Fig.1: Lane 2). This immunoblot was probed with MoAb C5 and was representative of all the three MoAbs used in this study.

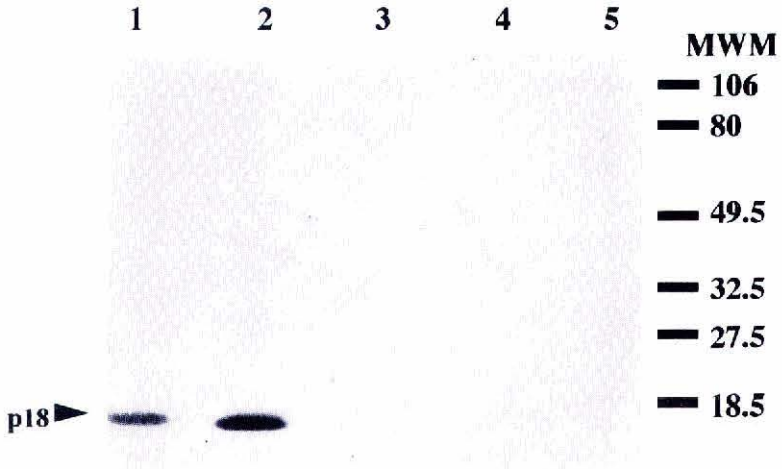

Fig. 1 Recognition of 18 kDa *Babesia equi* piroplasm surface protein by MoAbs. *B. equi* and *B. caballi* piroplasm proteins were subjected to Triton X-114 phase partitioning, separated by electrophoresis on 12.5 % SDS-polyacrylamide and transferred to PVDF membrane sheet. The blot was probed with MoAb C5 and is representative of all the three MoAbs used in this study. Lanes: 1. *B. equi* whole piroplasm antigen; 2. *B. equi* detergent phase; 3. *B. caballi* whole piroplasm antigen; 4. *B. caballi* detergent phase; 5. Equine erythrocyte proteins. Position of 18 kDa protein is shown on the left (MW Marker x10^3).

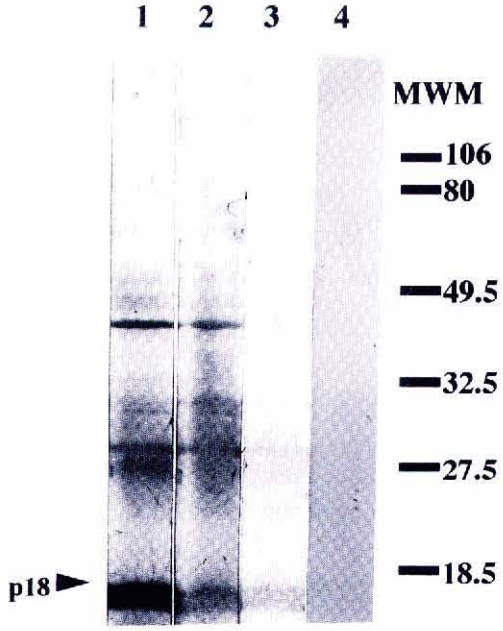

Fig. 2 Reaction of MoAbs with *B. equi* whole piroplasm antigens obtained from South Africa and Russia. Piroplasm proteins were separated by electrophoresis on 12.5% SDS-polyacrylamide gel and were transferred to PVDF membrane sheet. Russian *B. equi* antigen probed with MoAbs C2(Lane 1), C5(Lane 2) and D3(1ane3); Lane 4. South African antigen probed with C2. Position of 18 kDa protein is shown on the left (MW Marker x10^3).

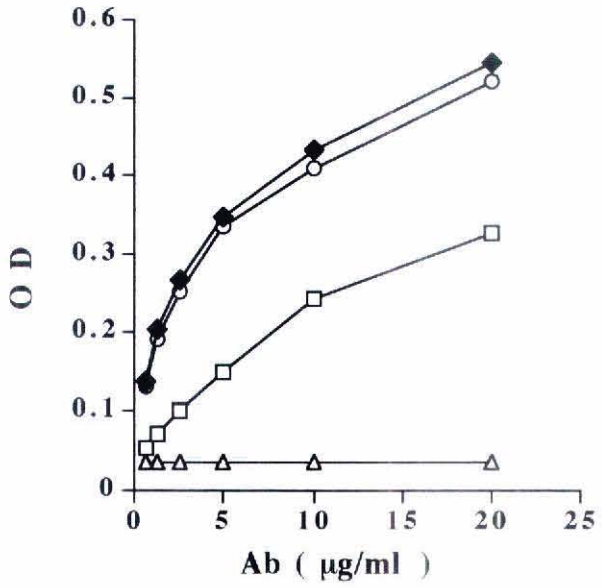

Fig. 3 Determination of sensitivity of biotin-labelled MoAbs. Serial 2-fold dilutions of biotin-labelled MoAbs (20 µg/ml) were incubated in microplate wells coated with solubilized *B. equi* piroplasm antigen. Antibodies bound to the antigen-absorbed well were detected by ELISA. Wells without antigen were used as negative controls. All values are the mean of triplicated samples. —□— C2; —◆— C5; —○— D3; —Δ— control (without antigen)

Sensitivity of biotin-labelled MoAbs

Checker board titration showed that the 0.1 ml of *B. equi* piroplasm antigen (18 µg/ml) per well could produce a optimum reaction in ELISA. Therefore, this amount of the antigen was used for the determination of reactivity of the biotinlabelled MoAbs. From the results (shown in Fig. 3), 20 µg/ml biotin-labeled MoAb was used in the following test, since this amount of each MoAb could give the optimum reaction. The specificity of the biotin-labelled MoAbs was demonstrated by the lack of reactivity in negative control wells.

Competitive ELISA between MoAbs

Competitive ELISA was performed to determine whether any of the MoAbs recognized the same epitopes on the 18 kDa protein. MoAbs at different concentration (0-20 µg) were used in competition with the heterologous biotin-labelled MoAb (20 µg/ml). Results were expressed as percentage of inhibition of binding of each MoAb by the others. As shown in Fig. 4, each MoAb inhibited binding of homologous as well as heterologous biotinylated MoAb. MoAbs C5 and D3 produced a reduction of 83.3% and 84.6% in the binding of

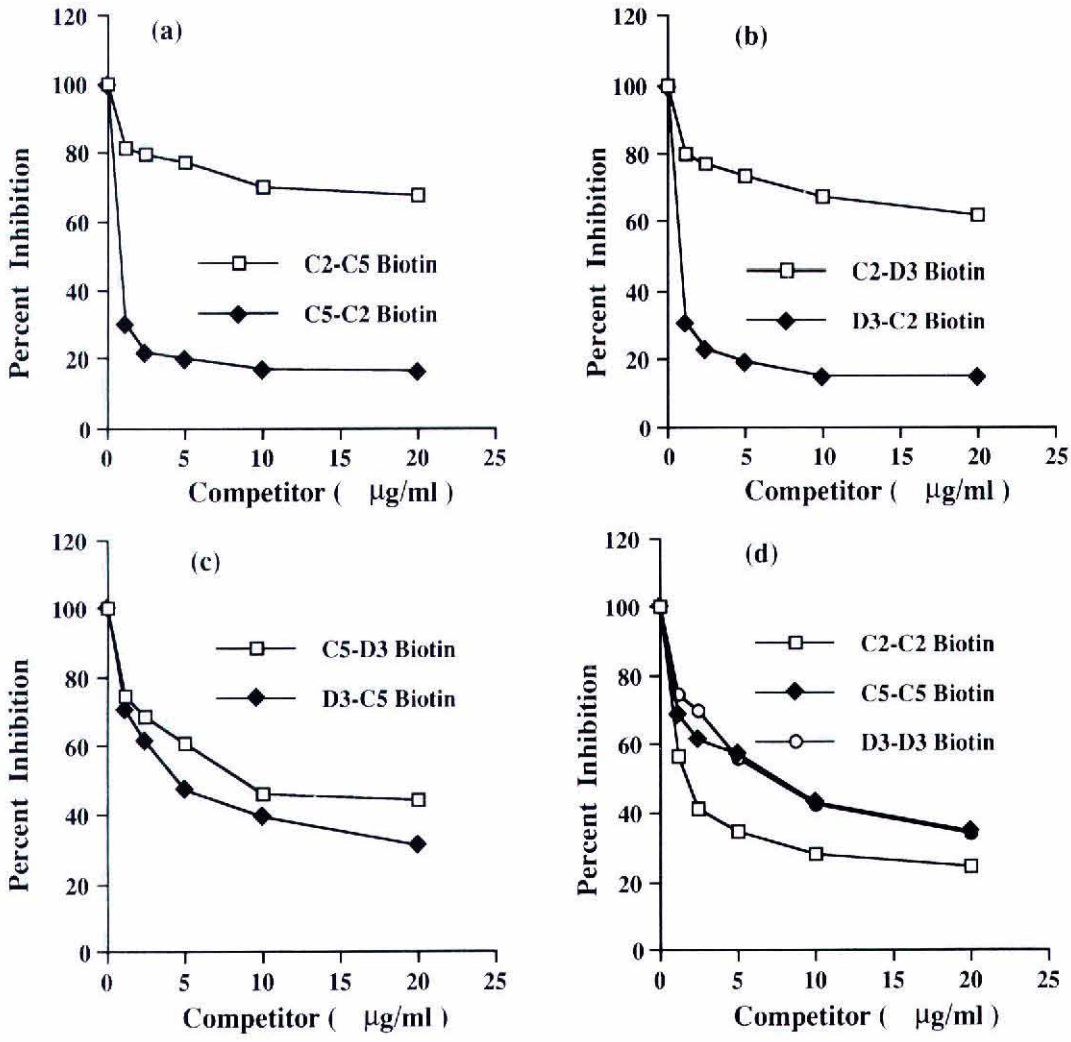

Fig. 4 Competitive ELISA assay between MoAbs. Unlabeled MoAbs as competitors (0-20 μg) were incubated in microplate wells coated with 0.1 ml of the piroplasms antigen (18 μg/ml). After washing, the wells were incubated with biotin-labeled MoAbs (20 μg/ml). (a) MoAb C2 to compete with biotinlabelled MoAb C5 and their reciprocal assay. (b) MoAb C2 to compete with biotin-labeled MoAb D3 and their reciprocal assay. (c) MoAb C5 to compete with biotin-labeled D3 and their reciprocal assay. (d) Homologous unlabeled MoAb as a competitor of biotin-labeled MoAb. All values given are the mean of triplicated samples.

biotin labelled C2 at 20 μg/ml concentration. The reciprocal figures were 32.6% and 37.8% for biotynilated C5 and D3, respectively. The percentage of inhibition of biotin labelled D3 by C5 was 55.7% at 20 μg/ml level. The figure in reciprocal assay was 69.0%. The inhibitory effects in competition binding of homologous antibodies were from 65 to 75%.

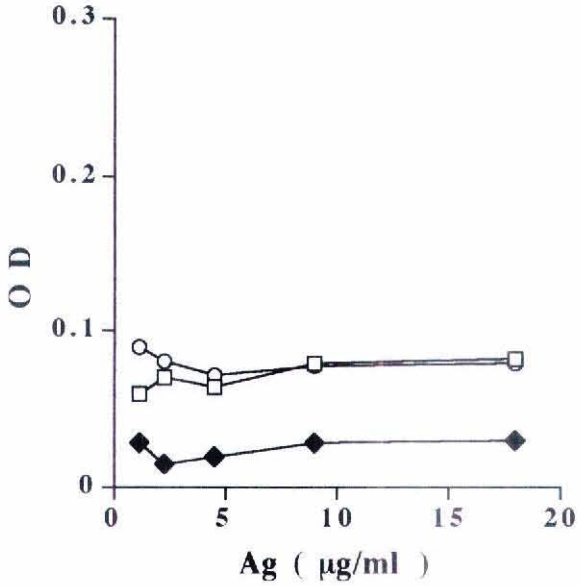

Fig. 5 Single-antibody two-site ELISA for detecting repeated epitope within the 18 kDa protein of *B. equi* merozoite. Piroplasm solubilized antigens were incubated in microplate wells coated with one of the MoAbs (C2, C5, D3) specific to 18 kDa protein. After washing, homologous biotin-labeled MoAb was used to detect the antigen captured on the microplate surface. All values given are the mean of triplicated samples. —□— C2;—◆— C5;—○— D3

Two-site ELISA

Two-site ELISA was performed to determine the presence of repetitive epitopes in the 18 kDa protein of *B. equi*. MoAbs immobilized on microplate wells were incubated with *B. equi* merozoite lysate and then homologous biotin-labelled MoAb was added to the plate. As shown in Figure 5, biotin-labelled MoAbs C2,C5 and D3 did not show any positive reactions.

DISCUSSION

Exposed epitopes on the sporozoite and merozoite membranes or on infected erythrocyte membranes are likely to be the targets for a protective immune response in *Babesia* infections (McElwain et al. 1987). The contamination of

babesial proteins with large amounts of host cell products is a problem for the isolation of pure babesial antigens. The identification of such antigens is required if their efficacy as immunogen has to be investigated. Generation of MoAbs that bind to merozoite surface proteins is a way which leads to the identification of protective immunogen.

The aims of the present study were to develop monoclonal antibodies (MoAbs) against the previously identified immunodominant proteins of *B. equi* (Ali et al. 1993) and to use these MoAbs in different serological assays and cDNA screening experiments. As a first step, a panel of MoAbs was developed against *B. equi* piroplasms. Three MoAbs reacting with an 18 kDa surface membrane protein (p18) of *B. equi* in immunoblot analysis were used to characterize the epitopes on p18.

The results of competitive ELISA indicate that MoAbs recognized the same epitope on the p18 molecule as each MoAb inhibited the binding of homologous as well as heterologous biotinylated MoAb. Comparatively higher reduction in binding of biotin-labelled C2 in competition with C5 and D3 in inhibition assay may be due to the differences in their affinity for the epitope on the p18.

In this experiment, Triton X-114 phase partitioning of *B. equi* piroplasms proteins confirmed that 18 kDa antigen is an integral membrane protein of *B. equi* piroplasms. None of the three MoAbs mentioned in this study reacted either with *B. caballi* or with equine erythrocyte proteins. These MoAbs recognized the p18 in the Russian strain of *B. equi* but not in the strain obtained from South Africa. This suggests that there are antigenic differences between the *B. equi* strains isolated from different parts of the world. Bose and Hentrich (1994), demonstrated that none of the antigens diagnostic for European isolates of *B. equi* were recognized by sera from field-infected horses from Brazil. Single protein identification and absence of crossreaction with *B. caballi* or equine erythrocyte proteins recommend the use of these MoAbs in differential diagnosis of mixed equine piroplasmosis infections.

REFERENCES

Ali, S., Sugimoto, C., Matsuda, M., Sugiura, T., Kanemaru, T., Onuma, M. & Kamada, M. 1993. Protein characterization of *Babesia equi* piroplasms isolated from infected horse erythrocytes. *Parasitol. Res. 79:* 639-643.

Bordier, C. 1981. Phase separation of integral membrane proteins in Triton X-114 solution. *J. Biol. Chem. 256:* 1604-1607.

Bose, R. & Hentrich, B. 1994. Identification of antigens diagnostic for European isolates of *Babesia equi* by two-dimensional electrophoresis and Western blotting. *Parasitol. Res. 80:* 182-185.

Burkot, T.R., Zavala, F., Gwadz, R.W., Collins, F.H., Nussenzweig, R.S. & Robert D.R. 1984. Identification of malaria-infected mosquitos by a two-site enzyme linked immunosorbent assay. *Am. J. Trop. Med. Hyg. 33:* 227-231.

Dunn, S.D. 1986. Effects of the modification of transfer buffer composition and denaturation of proteins in gels on the recognition of proteins of Western blots by monoclonal antibodies. *Anal. Biochem. 157:* 144-153.

Knowles, D.P., Perryman, L.E., Goff, W.L., Miller, C.D., Harrington, R.D. & Gorham, J.R. 1991a. A monoclonal antibody defines a geographically conserved surface protein epitope of *Babesia equi* merozoites. *Infect. Immun. 59:* 2412-2417.

Knowles, D.P., Perryman, L.E., Kappmeyer, L.S. & Hennager, S.G. 1991b. Detection of equine antibody to *Babesia equi* merozoite proteins by a monoclonal antibody-based competitive inhibition enzyme-linked immunosorbent assay. *J. Clin. Microbiol. 29:* 2056-2058.

Laemmli, U.K. 1970. Cleavage of structural protein during the assembly of the head of bacteriophage T4. *Natur 227:* 680-685.

McElwain, T.F., Perryman, L.E., Davis, W.C. & McGuire, T.C. 1987. Antibodies define multiple proteins with epitopes exposed on the surface of live *Babesia bigemina* merozoites. *J. Immunol. 138:* 2298-2304.

Shimizu, S., Suzuki, K., Nakamura, K., Kadota, K., Fujisaki, K., Ito, S. & Minami, T. 1988. Isolation of *Theileria sergenti* piroplasms from infected erythrocytes and development of an enzyme-linked immunosorbent assay for serodiagnosis of *T. sergenti* infection. *Res.Vet. Sci. 45:* 206-212.

Tenter, A.M. & Friedhoff, K.F. 1986. Serodiagnosis of experimental and natural *Babesia equi* and *B. caballi* infections. *Vet. Parasitol. 20:* 49-61.

Weiland, G. 1986. Species-specific serodiagnosis of equine piroplasma infections by means of complement fixation test (CFT), immuno-fluorescence (IIF), and enzyme-linked immunosorbent assay (ELISA). *Vet. Parasitol. 20:* 43-48.

Weiland, G. & Reiter, I. 1988. Methods for the measurement of serological response to *Babesia*. pp. 143-162. In: Babesiosis of Domestic animals and Man. Ristic, M.(Ed), CRC Press Inc., Boca Raton, Florida.

Zavala, F., Cochrane, A.H., Nardin, E.H., Nussenzweig, R.S. & Nusenzweig, V. 1983. Circumsporozoite proteins of malaria parasites contain a single immunodominant region with two or more identical epitopes. *J. Exp. Med. 157:* 1947-1957.