INFECTION AND IMMUNITY, Sept. 1995, p. 3507–3513 Vol. 63, No. 9
0019-9567/95/$04.00+0

Monoclonal Antibody to a Conserved Epitope on Proteins Encoded by *Babesia bigemina* and Present on the Surface of Intact Infected Erythrocytes

SANKALE SHOMPOLE,[1,2] LANCE E. PERRYMAN,[2]† FRED R. RURANGIRWA,[1,2] TERRY F. MCELWAIN,[2] DOUGLAS P. JASMER,[2] ANTHONY J. MUSOKE,[3] CLIVE W. WELLS,[3] AND TRAVIS C. MCGUIRE[2]*

Biotechnology and Immunology Section, National Veterinary Research Laboratory, Kenya Agricultural Research Institute, Kabete,[1] and International Livestock Research Institute, Nairobi,[3] Kenya, and Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, Pullman, Washington 99164-7040[2]

Received 6 December 1994/Returned for modification 1 February 1995/Accepted 20 June 1995

To define *Babesia bigemina*-specific antigens on the surface of infected erythrocytes, monoclonal antibodies (MAbs) were identified by live-cell immunofluorescence. As determined by live-cell immunofluorescence, two MAbs made to the Mexico strain reacted with the Mexico strain and three Kenya strains, while three MAbs made to the Kenya-Ngong strain reacted with the Kenya strains but not the Mexico strain. Binding of MAb 44.18 (made to the Mexico strain) to a strain-common epitope was confirmed by immunoelectron microscopy and by surface-specific immunoprecipitation of [^{35}S]methionine-labeled proteins (200, 28, and 16 kDa in size), which also demonstrated that the MAb recognized an epitope on proteins encoded by *B. bigemina*. In immunoblots, the MAb bound to predominant antigens with sizes of 200 and 220 kDa in erythrocyte lysates infected with strains from Puerto Rico, St. Croix, Texcoco (Mexico), Kenya, and Mexico. Major antigens with sizes of 200 and 220 kDa were isolated from a MAb 44.18 affinity matrix. Calf serum antibodies to these isolated antigens bound to erythrocytes infected with either the Mexico or Kenya strains as determined by live-cell immunofluorescence, allowing the conclusion that at least one conserved surface epitope was recognized. Calf serum antibodies identified major labeled proteins with sizes of 200 and 72 kDa by surface-specific immunoprecipitation, and infected erythrocytes sensitized with these antibodies were phagocytized by cultured bovine peripheral blood monocytes. These results provide a rationale for evaluating antigens identified by MAb 44.18 individually and as components of subunit vaccines.

Four major species of the genus *Babesia*, which are intraerythrocytic protozoal parasites, cause disease in cattle. *Babesia bigemina* and *Babesia bovis* are the most important species in tropical and subtropical regions of the world, and both are transmitted by *Boophilus* spp. ticks (16). The relative importances of *B. bigemina* and *B. bovis* vary, depending on the distribution of tick vector species and other factors; however, in East Africa, disease caused by *B. bigemina* is most prevalent. The development of effective immunological control procedures including vaccines is constrained by a lack of knowledge of both the required target antigens and the required immunologic effector mechanisms (5). The induction of complete immunity to infection with *B. bigemina*, and other similar infections, may require several parasite antigens (7), including those on the surface of sporozoites (12), the surface and apical complex of extracellular merozoites (8, 17, 18, 29), and the surface of merozoite-infected erythrocytes (27).

B. bigemina-encoded proteins exposed on the surface of infected erythrocytes could be targets for protective immune responses because of their accessibility to antibody. Antibody binding to the surface antigens might lead to antibody-dependent complement lysis, antibody-dependent cellular cytotoxicity, and phagocytosis. In addition to the possible role of antibody as an effector mechanism, evidence for the role of T-lymphocyte responses in protective immune responses to similar infections is accumulating (25, 26). Since mature erythrocytes do not express major histocompatibility complex antigens on the surface, the mechanism of T-lymphocyte immunity to *Babesia* infections is not clear. Initial studies suggested that infection-specific antigens were present on the surface of fixed *B. bigemina*-infected erythrocytes (24). Our recent studies confirmed this suggestion by agglutination and live-cell immunofluorescence procedures by demonstrating that new antigens are exposed on the surface of intact *B. bigemina*-infected erythrocytes, (27). These new antigens are proteins encoded by *B. bigemina*, since a surface-specific immunoprecipitation assay detects [^{35}S]methionine-labeled proteins on infected erythrocytes (27). In addition, strain-common epitopes occur on antigens exposed on infected erythrocytes, since serum antibodies from calves infected with one *B. bigemina* strain bind to erythrocytes infected with other strains from distinct geographic regions (27). The occurrence of these antigens on the surface of infected erythrocytes may be accomplished by mechanisms suggested for other intraerythrocytic protozoa, including the deposition of antigens during invasion (6) or translocation of antigens to the surface of infected erythrocytes by the developing intracellular merozoites (20).

This paper presents the results of experiments to confirm the presence of conserved epitopes on the surface of intact *B. bigemina*-infected erythrocytes, to identify the *B. bigemina*-en-

* Corresponding author. Mailing address: Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, Pullman, WA 99164-7040. Phone: (509) 335-6030. Fax: (509) 335-6094.

† Present address: Department of Microbiology, Pathology, and Parasitology, College of Veterinary Medicine, North Carolina State University, Raleigh, NC 27606.

coded proteins exposed on the surface of infected erythrocytes, and to determine if calf serum antibodies to these proteins mediate phagocytosis by cultured monocytes. Monoclonal antibodies (MAbs) to surface-exposed epitopes on *B. bigemina*-infected erythrocytes were made, and they bound to the surface of erythrocytes infected with strains isolated from distinct geographic regions. MAb 44.18 was used to identify proteins containing the epitope and to isolate antigens from infected erythrocyte lysate. Calf serum antibodies to these isolated antigens mediated phagocytosis of infected erythrocytes by cultured bovine monocytes; this is a mechanism which may be important in the control of *B. bigemina* infections.

MATERIALS AND METHODS

***B. bigemina* strains.** The *B. bigemina* strains used for this work were maintained as cryopreserved stabilates (21), and these stabilates were used to infect calves and in vitro cultures (31). The Kenya strains (Kikuyu, Mariakani, and Ngong) were obtained from naturally infected cattle from farms in three different regions of Kenya with endemic *B. bigemina*. These strains were expanded in splenectomized calves and maintained at the National Veterinary Research Laboratory, Kabete, Kenya (27). The sources of the Mexico, Texcoco (Mexico), St. Croix, and Puerto Rico strains and the growth of the Mexico strain in microaerophilous stationary-phase cultures have been described previously (8).

Indirect immunofluorescence assay using intact *B. bigemina*-infected erythrocytes (live-cell immunofluorescence). Blood from calves infected with various strains or from cultures infected with the Mexico strain was collected with anticoagulant (1 mg of EDTA per ml of blood) and centrifuged to remove the plasma and buffy coat. Erythrocytes were washed three times with sterile phosphate-buffered saline (PBS) containing 0.01 M sodium phosphate and 0.14 M NaCl (pH 7.4). One hundred microliters of washed erythrocytes (1% solution in PBS) was reacted with 100 μl of PBS containing 20 μg of MAb per ml and 10 μg of ethidium bromide per ml for 30 min at room temperature. The erythrocytes were washed three times with PBS and reacted with 100 μl of fluorescein-conjugated rabbit antibody to mouse immunoglobulins (Organon Teknika, Durham, N.C.) diluted 1:100 in PBS. The mixture was incubated for 30 min at room temperature, washed three times with PBS, and examined by fluorescence microscopy. The specificity of the immunofluorescence was controlled by reacting MAbs with erythrocytes from an uninfected calf and by reacting isotype control MAbs with infected erythrocytes. To prove that the described reaction was restricted to epitopes exposed on the outer surface of infected erythrocytes, MAb 14.16 specific for p58 *B. bigemina* merozoite surface antigen (17) was used, and this MAb did not stain the merozoites in the infected erythrocytes prepared for live-cell immunofluorescence. However, in other reactions, MAb 14.16 bound merozoites in erythrocyte ghosts prepared by a procedure that disrupts the erythrocyte but results in live merozoites within the erythrocyte ghosts (17).

Production and purification of MAbs. Erythrocytes infected with *B. bigemina* strains from Kenya and Mexico were used to immunize mice for MAb production as described previously (17). Mice with serum antibodies which reacted with *B. bigemina*-infected erythrocytes as determined by live-cell immunofluorescence described in the preceding paragraph were used as spleen donors for hybridoma production. Supernatants from individual hybridomas were also screened on infected erythrocytes by live-cell immunofluorescence. In this way, hybridomas producing MAbs 44.18.34.12 (designated 44.18) and 44.52.6.12 (designated 44.56) to the Mexico strain and E6B5.5:3, E6B8.8:3, and E6C8.8:3 to the Kenya-Ngong strain were identified and cloned twice by limiting dilution. Ascitic fluid was made in pristane-primed BALB/c mice given hybridoma cells making MAb 44.18, and the MAb was purified by 50% ammonium sulfate precipitation followed by fractionation on DEAE-cellulose (18).

Immunoelectron microscopy. Packed Kenya-Ngong strain-infected erythrocytes from a calf with a 4.4% parasitemia were collected in 50 U of heparin per ml, diluted to about 5×10^6 erythrocytes per ml in Alsever's solution, and aliquoted into 1-ml fractions. The aliquots were washed three times in Alsever's solution and incubated with 0.9 mg or 0.09 mg of either MAb 44.18 or an isotype control MAb per ml. Another control containing no antibody was included. The erythrocyte suspensions were incubated for 30 min and then washed three times in Alsever's solution containing 1% bovine serum albumin. Washed erythrocytes were incubated with a 1:10 dilution of gold-conjugated goat anti-mouse immunoglobulin polyclonal serum (Biocell, Cardiff, United Kingdom) for 30 min, washed twice in Alsever's solution, and then fixed by adding equal volumes of 4% glutaraldehyde and 0.4% picric acid in 0.2 M sodium cacodylate buffer (pH 7.3). Fixation for 3 min at room temperature was followed by washing and incubation in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 h. Fixed erythrocytes were then washed and block stained with aqueous 2% uranyl acetate for 4 h and processed by graded acetone into epon-araldite resin mix. Ultrathin sections (thickness, 50 to 70 nm) were cut and mounted on copper grids. The sections were counterstained with Reynold's lead citrate and examined with an electron microscope (Zeiss EMICA, Oberkochenn, Germany).

Immunoaffinity chromatography. To isolate erythrocyte surface antigens identified by MAb 44.18, this MAb was coupled to cyanogen bromide-activated Sepharose 4B according to the manufacturer's recommendations (Pharmacia Fine Chemicals, Uppsala, Sweden). Two hundred milliliters of packed erythrocytes from a calf infected with the Kenya-Ngong strain of *B. bigemina* (10% infected erythrocytes) was solubilized in lysis buffer (50 mM Tris, pH 8.0, containing 5 mM EDTA, 5 mM iodoacetamide, 1 mM phenylmethylsulfonyl fluoride, 0.1 mM *Nα-p*-tosyl-L-lysine chloromethyl ketone, and 1% [vol/vol] Nonidet P-40). Lysate was passed through a column packed with the affinity matrix and washed, and the bound antigens were eluted with 0.1 M diethylamine and 0.5% (wt/vol) deoxycholate (pH 11.5) as previously described (18). Eluted proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and evaluated by being stained with Coomassie blue and transferred to nitrocellulose membranes for immunoblotting with MAb 44.18.

Production of monospecific serum. A calf (B2052) was immunized by intramuscular injection with 50 μg of affinity-purified antigen emulsified in Freund's complete adjuvant. Five booster immunizations consisting of 50 μg of antigen in Freund's incomplete adjuvant were done at 2-week intervals. Serum was obtained from the calf 10 days after the last immunization, and antibodies were evaluated by live-cell immunofluorescence and by immunoblotting of infected erythrocyte lysates.

PAGE and immunoblots. Blood stabilates from calves infected with *B. bigemina* strains from Mexico, Texcoco (Mexico), St. Croix, Puerto Rico, and Kenya were frozen and thawed three times and washed with cold PBS at 4°C until the supernatant was clear. The final pellet was disrupted in lysis buffer, and proteins from approximately 7×10^6 infected erythrocytes from each strain were separated by electrophoresis on 7.5 to 17.5% polyacrylamide gels with SDS (11). Separated antigens were electrophoretically transferred to a 0.45-nm-pore-size nitrocellulose membrane (Schleicher and Schuell, Keene, N.H.) (30). After the transfer, the nitrocellulose strips were incubated overnight at room temperature with MAb 44.18 (20 μg/ml) or calf B2052 serum antibodies to erythrocyte surface antigens diluted 1:160. Following the reaction, peroxidase-conjugated goat anti-mouse immunoglobulin antibodies diluted 1:4,000 (Cappel, Durham, N.C.) or GamMabind G-HRP (Genex Corp., Gaithersburg, Md.) (0.25 mg/ml) diluted 1:40,000 was used for the detection of mouse and bovine antibodies, respectively. Labeled conjugate binding was detected by an enhanced chemiluminescence method (Amersham Life Science Inc., Arlington Heights, Ill.). High-molecular-weight rainbow protein standards (Amersham) were also electrophoresed and transferred to nitrocellulose.

Surface-specific immunoprecipitation of metabolically labeled *B. bigemina*-infected erythrocyte proteins. Erythrocytes infected with the Mexico strain of *B. bigemina* were obtained from in vitro cultures (31), and those infected with the Kenya-Ngong strain were obtained from an infected splenectomized calf. These erythrocytes were labeled with [^{35}S]methionine (18), and since mature bovine erythrocytes are incapable of incorporating [^{35}S]methionine into nascent erythrocyte proteins (4), it was assumed that only *B. bigemina*-encoded proteins were labeled. To identify labeled *B. bigemina* antigens bound by MAb 44.18 and serum antibodies to erythrocyte surface antigens on the surface of live infected erythrocytes, 4×10^6 intact, infected erythrocytes were reacted with 10 μg of MAb 44.18 or 10 μl of serum antibodies to isolated surface antigens at 37°C for 1 h. Infected erythrocytes were washed three times with 10 ml of PBS to remove unbound antibodies, and then lysis buffer was added to the intact, infected erythrocyte pellet (27). After lysis, 100 μl of a 10% solution of protein G-Sepharose was added to remove labeled antigen-antibody complexes for analysis. The beads were washed, and the proteins were eluted and evaluated by SDS-PAGE as described previously (22). Standard immunoprecipitations were done by disrupting infected erythrocytes in lysis buffer before the immunoprecipitation reactions by using the same primary antibodies and procedures described above for the surface-specific immunoprecipitations. Approximately 5×10^6 cpm of labeled proteins was added to each immunoprecipitation reaction, and immunoprecipitates were separated by SDS-PAGE. Labeled proteins were visualized by fluorography (22).

Bovine peripheral blood monocyte cultures and antibody-dependent erythrophagocytosis assay. For each assay, 100 ml of peripheral blood (50 U of heparin per ml) was collected from a *Babesia*-free calf, and leukocytes were isolated by an erythrocyte lysis method (19). Monocytes were allowed to adhere to 13-mm^2 coverslips (Labtek, Naperville, Dl.) placed in 24-well plates (15), and 24 h later, the nonadherent cells were washed away. The monocyte cultures were maintained for 7 to 10 days before they were used in phagocytosis assays. *B. bigemina*-infected erythrocytes were reacted with calf B2052 serum taken before or after immunization, incubated for 1 h at 37°C, and added to the monocyte cultures. Phagocytosis of infected erythrocytes was evaluated microscopically; monocytes on 13-mm^2 coverslips were stained with Giemsa stain, and entire coverslips were examined for phagocytosed erythrocytes infected with *B. bigemina*. Four different coverslips were done for each experiment. The total number of monocytes on each coverslip was counted, and the number of monocytes with phagocytosed erythrocytes was also determined. The total number of monocytes with phagocytosed erythrocytes for each experiment (all four coverslips) was expressed as a percentage of the total number of monocytes. Five separate phagocytosis experiments were done, and the coverslips were evaluated by one person. Control experiments were done as described above, but preimmunization serum was used.

TABLE 1. Live-cell immunofluorescence assays to demonstrate MAb binding to the surface of *B. bigemina*-infected erythrocytes from different geographic regions

MAb	MAb isotype	Immunizing strain	Reaction by *B. bigemina* strain[a]			
			Kenya-Ngong	Kenya-Kikuyu	Kenya-Mariakani	Mexico
44.18	IgG_1	Mexico	+	+	+	+
44.52	IgG_1	Mexico	+	+	+	+
E6B5.5:3	$IgG2_b$	Kenya-Ngong	+	+	+	−
E6B8.8:3	$IgG2_b$	Kenya-Ngong	+	+	+	−
E6C8.8:3	$IgG2_b$	Kenya-Ngong	+	+	+	−

[a] +, visible reaction (green) on the surface of infected erythrocytes in live-cell immunofluorescence assays. Infected cells were identified by simultaneous staining with ethidium bromide (red merizoite staining). −, no visible reaction on the surface of infected erythrocytes.

RESULTS

Production of MAbs binding to the surface of intact *B. bigemina*-infected erythrocytes. Spleens from immunized mice with serum antibodies binding intact *B. bigemina*-infected erythrocytes as determined by live-cell immunofluorescence were used to make hybridomas. Hybridoma supernatants were also screened by live-cell immunofluorescence. Five hybridomas making antibodies which bound intact and infected, but not uninfected, erythrocytes were cloned twice and examined further. Two immunoglobulin (Ig) G1 MAbs, 44.18 and 44.52, were derived from a spleen donor immunized with the Mexico strain, and three IgG2b MAbs, E6B5.5:3, E6B8.8:3, and E6C8.8:3, were derived from a spleen donor immunized with the Kenya-Ngong strain.

As determined by live-cell immunofluorescence, MAbs 44.18 and 44.52 reacted with the surface of intact erythrocytes infected with the Mexico strain and three strains of *B. bigemina* from Kenya (Table 1). Figure 1A is a photograph of one of these reactions. MAbs E6B5.5:3, E6B8.8:3, and E6C8.8:3 reacted with a homologous strain (Kenya-Ngong) and with two other strains from Kenya as determined by live-cell immunofluorescence, but did not react with erythrocytes infected with the Mexico strain (Table 1). The appearance of the surface fluorescence on infected erythrocytes was uniform on some cells (Fig. 1A) and had a punctate pattern on other cells (data not shown). All erythrocytes with surface fluorescence also contained *B. bigemina* merozoites, as was determined by simultaneous staining with ethidium bromide (Fig. 1B). No immunofluorescence was observed when isotype control MAbs were reacted with infected (Fig. 1C) and uninfected erythrocytes (data not shown). MAb E6B5.5:3 made to the Kenya-Ngong strain and MAb 44.18 made to the Mexico strain reacted with different percentages of erythrocytes infected with the Kenya-Ngong strain of *B. bigemina* upon testing against various parasitemias (Table 2). However, the difference in the percentages of binding for the two antibodies with various parasitemias was similar to the difference of the same antibody with various parasitemias (Table 2). The mean percent binding in the four assays in Table 2 was 45% for MAb E6B5.5:3 and 48% for MAb 44.18.

Immunoelectron microscopy of *B. bigemina*-infected erythrocyte lysates. Figure 2 is an electron micrograph of a merozoite-containing erythrocyte with MAb 44.18 bound to the external surface. The MAb binding is visualized by the presence of gold-labeled goat anti-mouse immunoglobulin serum. Uninfected erythrocytes in the preparation did not have electron-dense gold particles bound to them (Fig. 2), and infected erythrocytes incubated first with an isotype control MAb did not have gold particles bound to them (data not shown). This result confirmed the surface location of the MAb 44.18 binding, because the erythrocytes were incubated with the MAb and gold-labeled goat anti-mouse immunoglobulin serum before fixation and embedding.

Immunoblots of *B. bigemina*-infected erythrocyte lysates. Immunoblots of infected erythrocyte lysates were done to identify antigens recognized by the five MAbs. All five MAbs bound to two major antigens (with sizes of approximately 200 and 220 kDa) from the Mexico and Kenya-Ngong strains (data not shown). MAb 44.18 was selected for further study because it bound antigens similar to those bound by the other MAbs in immunoblots and because it reacted with the Mexico strain and three Kenya strains as determined by live-cell immunofluorescence (Table 1). MAb 44.18 bound predominant antigens with sizes of approximately 200 and 220 kDa in immunoblots of

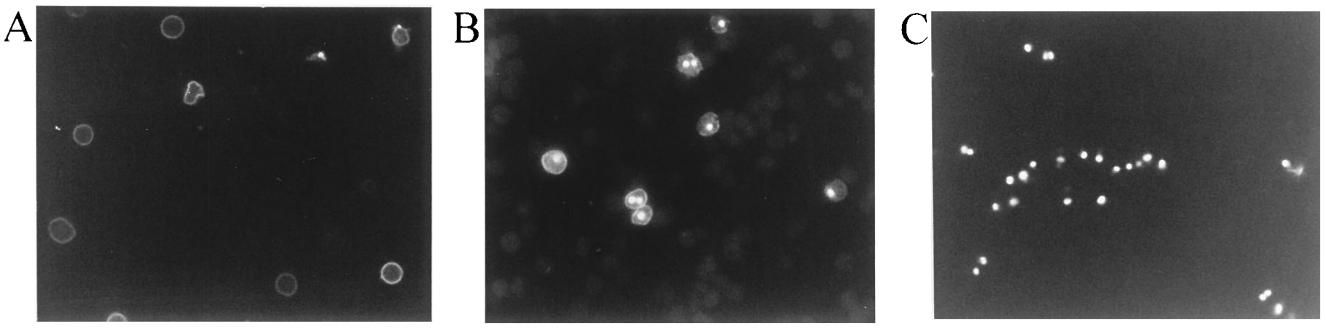

FIG. 1. MAb 44.18 binding to the surface of erythrocytes infected with *B. bigemina* as determined by live-cell immunofluorescence assays. (A) Fluorescence caused by MAb 44.18 binding to intact erythrocytes infected with the Kenya-Ngong strain. (B) A reaction similar to that in panel A, except that the intraerythrocytic merozoite nuclei were also stained with ethidium bromide. (C) Infected erythrocytes that reacted with IgG1 isotype control MAb and that were also stained with ethidium bromide. The merozoites in panel C were stained with ethidium bromide, but there was no staining of the infected erythrocyte surface. Magnification, ×600.

TABLE 2. Percent erythrocytes infected with the Kenya-Ngong strain at different levels of parasitemia

% Parasitemia[a]	% Infected erythrocytes[b]	
	MAb E6B5.5:3	MAb 44.18
0.5	64	40
0.8	30	47
1.5	40	44
4.4	44	59

[a] Blood was taken on postinfection days 7 (0.5% parasitemia), 8 (0.8%), 9 (1.5%), and 10 (4.4%).
[b] As determined by live-cell immunofluorescence with the indicated MAbs.

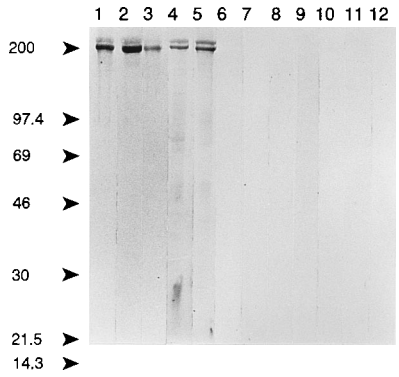

FIG. 3. Immunoblot of lysates from erythrocytes infected with five different strains of *B. bigemina* and probed with MAb 44.18. Strains were from Puerto Rico (lane 1), Mexico (lane 2), St. Croix (lane 3), Texcoco (Mexico) (lane 4), and Kenya-Ngong (lane 5). Uninfected erythrocyte lysate was used for lane 6. IgG1 isotype control MAb was reacted with the same strains in lanes 7 to 11, respectively, and uninfected erythrocyte lysate was used for lane 12. Molecular mass standards (in kilodaltons) are shown on the left.

lysates of erythrocytes infected with strains from Puerto Rico, Mexico, St. Croix, Texcoco (Mexico), and Kenya-Ngong (Fig. 3, lanes 1 to 5) but did not bind to uninfected erythrocyte antigens (Fig. 3, lane 6). The IgG1 isotype control MAb did not bind to infected (Fig. 3, lanes 7 to 11) or uninfected (Fig. 3, lane 12) erythrocyte antigens.

Surface-specific immunoprecipitation of metabolically labeled *B. bigemina* proteins. To further confirm the live-cell immunofluorescence results and to identify the parasite-encoded proteins recognized by MAb 44.18, a surface-specific immunoprecipitation method which involved first reacting the MAb with live infected erythrocytes was used. The cells were then washed and disrupted, and the MAb [^{35}S]methionine-labeled antigen complexes were isolated and analyzed by SDS-PAGE. MAb 44.18 immunoprecipitated [^{35}S]methionine-labeled antigens with sizes of approximately 200, 28, and 16 kDa from the Kenya-Ngong strain (Fig. 4, lane 5). Similar immunoprecipitation results were obtained with MAb 44.18 and [^{35}S]methionine-labeled antigens from the Mexico strain (data not shown). Major protein bands with sizes of approximately 200, 46, 31, 28, and 16 kDa were immunoprecipitated (Fig. 4, lane 1) when a standard immunoprecipitation procedure was performed. The procedure involved the lysis of the [^{35}S]methionine-labeled, Kenya-Ngong strain-infected erythrocytes before the reaction with MAb 44.18. Isotype control MAb did not immunoprecipitate similar proteins in either the standard or the surface-specific immunoprecipitation reactions (Fig. 4, lanes 2 and 6).

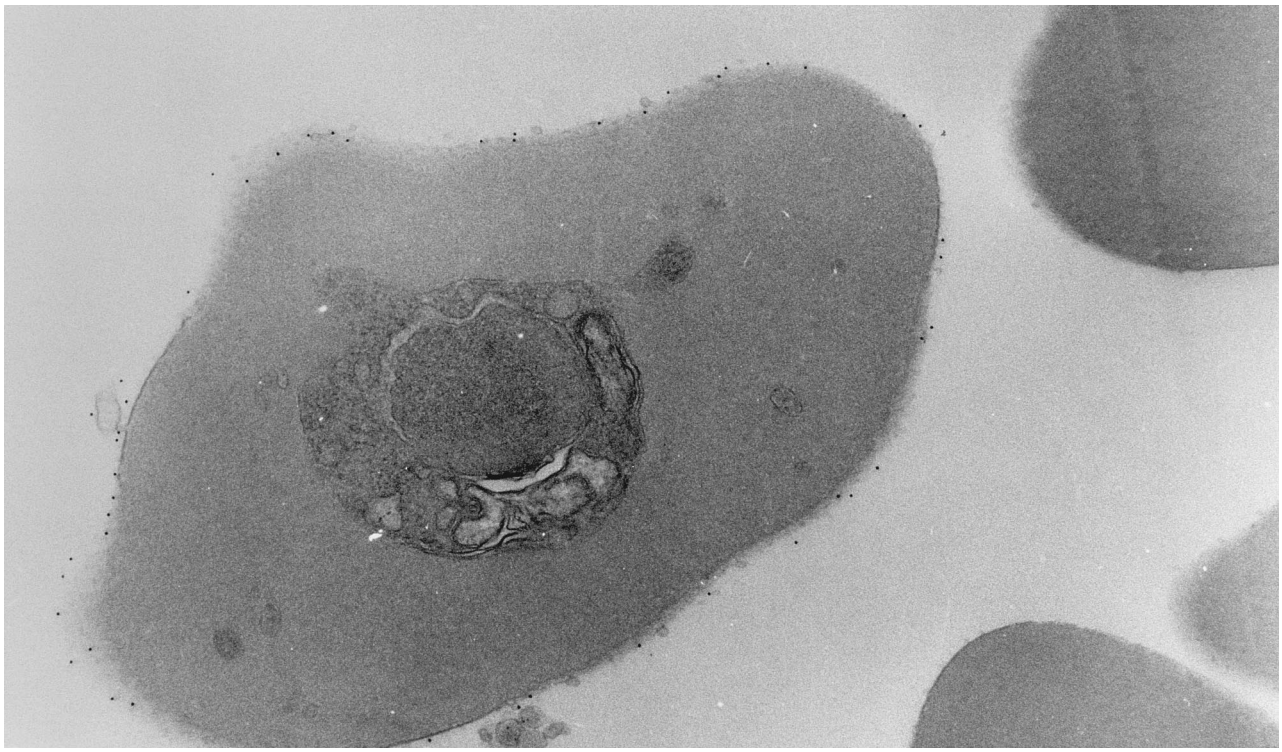

FIG. 2. Electron micrograph of MAb 44.18 binding to a *B. bigemina*-infected erythrocyte. The micrograph shows an infected erythrocyte. MAb 44.18 is visualized on the external surface by the binding of gold-labeled goat anti-mouse immunoglobulin serum to the external membrane. The uninfected erythrocytes do not have gold particles on their surfaces. The diameters of the gold particles are 10 nm. Magnification, ×41,000.

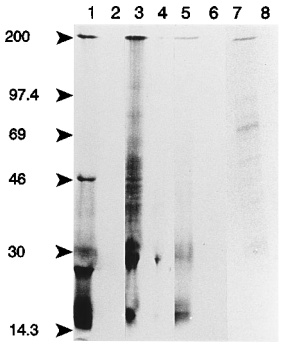

FIG. 4. Results of standard and surface-specific immunoprecipitations of [^{35}S]methionine-labeled, Kenya-Ngong strain-infected erythrocyte proteins. Lanes 1 to 4 are immunoprecipitates obtained by standard immunoprecipitation of labeled proteins that were reacted with antibodies after the lysis of labeled, infected erythrocytes. Lanes 5 to 8 are surface-specific immunoprecipitates obtained by the reaction of antibodies with labeled, infected erythrocytes before lysis. The primary antibodies were MAb 44.18 (lanes 1 and 5), IgG_1 isotype control MAb (lanes 2 and 6), calf B2052 serum antibodies to antigen isolated by MAb 44.18 affinity chromatography (lanes 3 and 7), and preimmunization calf serum (lanes 4 and 8). Molecular mass standards (in kilodaltons) are shown on the left.

Isolation of *B. bigemina* antigens recognized by MAb 44.18. MAb 44.18 was used in immunoaffinity chromatography to isolate antigens from lysates of erythrocytes infected with the Kenya-Ngong strain of *B. bigemina*. After they were washed, bound antigens were eluted with 0.1 M diethylamine and 0.5% (wt/vol) deoxycholate (pH 11.5) and dialyzed in PBS before separation by SDS-PAGE. Coomassie blue staining detected major bands with sizes of approximately 220 and 200 kDa and minor bands with sizes of 160, 86, 46, and 12 kDa (Fig. 5).

Antibody response to immunoaffinity-purified antigens. Calf B2052 serum antibodies to *B. bigemina* antigens eluted from a MAb 44.18 affinity matrix were evaluated. As determined by live-cell immunofluorescence, the serum antibodies (optimum dilution, 1:1,000) reacted with the surface of intact erythrocytes infected with three strains of *B. bigemina* from Kenya and one from Mexico. The appearance of the live-cell immunofluorescence was similar to that described for MAb 44.18 (Fig. 1C).

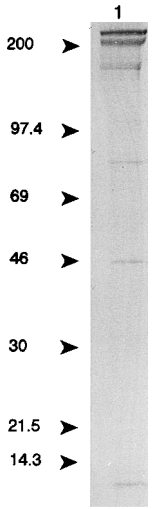

FIG. 5. Coomassie blue stain of antigen isolated from a MAb 44.18 affinity matrix and separated by SDS-PAGE. Lane 1 contains 56 μg of isolated antigen. Molecular mass standards (in kilodaltons) are shown on the left.

Preimmunization serum (1:1,000) did not react with the surface of infected erythrocytes, and the postimmunization serum (1:1,000) did not react with uninfected erythrocytes.

Immune calf serum antibodies immunoprecipitated major [^{35}S]methionine-labeled proteins with sizes of approximately 200 and 72 kDa and three other minor proteins in surface-specific reactions (Fig. 4, lane 7). Similar immunoprecipitation results were obtained for the Mexico strain (data not shown). Further, immunized calf serum antibodies bound a [^{35}S]methionine-labeled protein with a size of approximately 200 kDa in addition to other proteins, including those with sizes of 46, 28 and 16 kDa, as determined by a standard immunoprecipitation procedure (Fig. 4, lane 3).

Antibody-dependent erythrophagocytosis assay. Erythrophagocytosis assays were used to determine whether B2052 immune serum was capable of mediating effector mechanisms that may be involved in the resolution of *Babesia* infections. Cultured bovine monocytes readily phagocytosed *B. bigemina*-infected erythrocytes sensitized with B2052 immune serum antibodies to isolated erythrocyte surface antigens (Fig. 6A). The mean number of monocytes with erythrophagocytosis in five experiments was 23.9% (the standard deviation was 7.3%). Only a small percentage of monocytes (mean, 1.5%; standard deviation, 1.1%) phagocytized infected erythrocytes sensitized with a 1:2 dilution of preimmunization serum (Table 3). The mean percentage of erythrophagocytosis caused by immune serum was significantly different ($P < 0.0001$ by Student's t test) from that caused by preimmunization serum. Sensitized, uninfected erythrocytes were not phagocytized.

DISCUSSION

These results with MAbs documented that at least one strain-common epitope was exposed on the surface of *B. bigemina*-infected erythrocytes. As shown by live-cell immunofluorescence, two MAbs made to the Mexico strain bound to the surface of erythrocytes infected with the Mexico strain and three strains from Kenya. In contrast, three MAbs to the Kenya-Ngong strain bound to erythrocytes infected with three Kenya strains but not to erythrocytes infected with the Mexico strain. Not all infected erythrocytes were bound by MAbs. This observation was similar to the finding that not all live *B. bigemina*-infected erythrocytes were recognized by serum antibodies from infected calves (27) and was also similar to the reported failure of postinfection serum to bind to the exposed surface of all *B. bovis*-infected erythrocytes (2). Whether this was due to differential protein expression during merozoite development or, as in *B. bovis*, to an accumulation of antigen with time (2) is not known.

Evidence that MAb 44.18 bound to a surface-exposed epitope on intact *B. bigemina*-infected erythrocytes was derived from live-cell immunofluorescence and immunoelectron microscopy and from surface-specific immunoprecipitation assays. Erythrocytes that bound MAb 44.18 as shown by live-cell immunofluorescence and that were examined by phase microscopy were morphologically intact and contained hemoglobin. MAb 14.16, which reacts with a surface-exposed protein on viable merozoites (17), was unable to penetrate and bind merozoites inside erythrocytes during live-cell immunofluorescence, demonstrating that the erythrocyte membrane was impermeable to antibody. Live-cell immunofluorescence was confirmed by immunoelectron microscopy and surface-specific immunoprecipitation in which MAb 44.18 bound to intact, infected erythrocytes resulted in the immunoprecipitation of [^{35}S]methionine-labeled proteins. These proteins were considered to be encoded by *B. bigemina*, since mature bovine eryth-

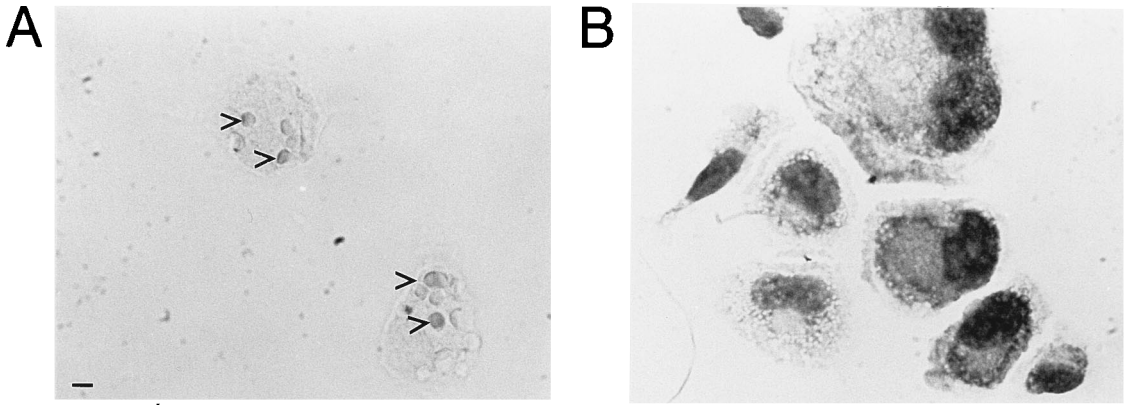

FIG. 6. Erythrophagocytosis of calf B2052 serum antibody-coated, Kenya-Ngong strain-infected erythrocytes by cultured bovine monocytes. (A) Phagocytized whole infected erythrocytes sensitized with calf B2052 serum antibody. Examples of phagocytized erythrocytes are indicated by arrowheads. Bar, 15 μm. (B) Cultured monocytes which failed to phagocytize infected erythrocytes sensitized with calf B2052 serum that was taken before immunization. Magnification, ×300.

rocytes do not incorporate [^{35}S]methionine into nascent erythrocyte proteins (4); however, definitive proof of parasite origin requires additional experiments. Similar surface-specific immunoprecipitation procedures have been used to identify parasite-synthesized antigens on the surface of *B. bovis* (3). It was concluded from results of these procedures that MAb 44.18 bound a conserved epitope on *B. bigemina*-encoded proteins exposed on the surface of intact, infected erythrocytes.

The conclusion that the epitope recognized by MAb 44.18 was conserved on strains from different geographic regions was derived primarily from live-cell immunofluorescence assays. This MAb bound intact erythrocytes infected with the Mexico strain (homologous) and erythrocytes infected with three Kenya strains (heterologous). Furthermore, MAb 44.18 reacted in immunoblots of infected erythrocyte lysates with two major *B. bigemina*-specific antigens with similar sizes from strains from Puerto Rico, Mexico, St. Croix, Texcoco (Mexico), and Kenya-Ngong. This latter observation argues that the epitope was conserved in these strains but does not demonstrate that it was exposed on the surface of infected erythrocytes, as was concluded for the Mexico and three Kenya strains.

MAb 44.18 bound predominant antigens with sizes of 220 and 200 kDa in immunoblots of erythrocyte lysates infected with five different strains of *B. bigemina*. Similarly, major proteins with sizes of 220 and 200 kDa were isolated from a MAb 44.18 affinity matrix. MAb 44.18 bound labeled proteins with sizes of 200, 28, and 16 kDa in surface-specific immunoprecipitation reactions and labeled proteins with sizes of 200, 46, 31, 28, and 16 kDa in standard immunoprecipitation reactions. A 200-kDa antigen was identified by all three procedures, although the 200-kDa protein identified by the immunoblots was not proven to be the same one identified by the immunoprecipitation reactions. The explanation for the absence of a 220-kDa protein in the immunoprecipitation reactions is unknown, as is that for the presence of smaller labeled proteins. These results could be explained by the identified proteins being derived from a 220-kDa precursor protein and that processing of all the labeled 220-kDa protein occurred before the immunoprecipitation reactions were done. There are a number of other possible explanations for these results, but the present data cannot differentiate among them. Cloning and expressing the *B. bigemina* genes encoding the proteins recognized by MAb 44.18 will be required to further characterize the relationships among these proteins. Two other recently described MAbs also bind isolate-common epitopes on *B. bigemina*-infected erythrocytes (32); however, a comparison of the recognized proteins has not been done.

TABLE 3. Monocyte phagocytosis of *B. bigemina*-infected erythrocytes sensitized with antibodies to antigens isolated from the MAb 44.18 affinity matrix

Expt no.	% Cultured monocytes phagocytizing erythrocytes[a]	
	Postimmunization	Preimmunization
1	13.5	1.3
2	20.0	3.3
3	27.4	0.9
4	32.5	0.6
5	26.2	1.6
Mean	23.9	1.5
Standard deviation	7.3	1.1

[a] At least 100 monocytes were examined microscopically in each experiment, the number with erythrophagocytosis were counted, and the percentage with erythrophagocytosis was calculated. Calf B2052 serum was used to sensitize the erythrocytes.

Changes occur in *B. bovis*-infected erythrocyte surface proteins (14), and surface-specific immunoprecipitation identifies parasite-synthesized antigens on infected erythrocytes (3). In a Mexico strain, 120- and 107-kDa proteins are immunoprecipitated, while 135-, 120-, and 107-kDa proteins are found on a United States strain (3). Similar proteins in a biologically cloned *B. bovis* strain are part of the antigenic variation of exposed erythrocyte surface antigens of this parasite (2). Most *B. bovis*-infected erythrocyte surface antigens are strain specific, although antibodies from calves infected with cloned lines cross-react with other cloned lines, suggesting that some epitopes may be conserved (2). In contrast, this and a previous study (27) demonstrate that epitopes on the surface of erythrocytes infected with *B. bigemina* are conserved. The role of these parasite-encoded proteins is unknown, but they likely contribute to endothelial cytoadherence of *B. bovis*-infected erythrocytes (1). The hemolysis resulting from the immune responses to these erythrocyte proteins could activate several systems involved in inflammation, including the kallikrein-kinin, coagulation, and complement systems, potentiating the disease caused by *Babesia* spp. (33).

Calf serum antibodies to *B. bigemina* antigens isolated with MAb 44.18 bound to two major labeled proteins (200 and 72

kDa) in surface-specific immunoprecipitations and recognized at least one conserved epitope on erythrocytes infected with the Mexico strain and three Kenya strains as determined by live-cell immunofluorescence. These antibodies also caused phagocytosis of infected erythrocytes by cultured bovine monocytes. A positive correlation between the opsonin titer and protective activity against *Babesia rodhaini* in rats occurs (23). Studies of *B. bovis* (9, 10, 15), *Babesia equi* (13), and *Babesia canis* (28) infections indicate that phagocytosis of infected erythrocytes by peripheral blood leukocytes is involved in the resolution of infections. These observations and the demonstration that MAb 44.18 recognized an epitope conserved among *B. bigemina* strains isolated from distant geographic regions provide a rationale for further characterization of the identified antigens and their evaluation as subunit vaccine components.

ACKNOWLEDGMENTS

This research was supported by U.S. Agency for International Development Cooperative Agreement DAN-4178-A-00-7056-00, by the Animal Health Research Center (Washington State University), and by Rockefeller Foundation African Dissertation Internship Award RF89034 (#139) and was done in collaboration with the Kenya Agricultural Research Institute.

We are grateful to Alberta Brassfield, Pat Mason, Debra Alperin, Steve Hines, Joseph Katende, Ntando Tebele, Angela Lesan, Anderson Wambugu, Joseph Buyu, Abu Oriko, and Catherine Nkonge for assistance.

REFERENCES

1. **Aikawa, M., J. Rabbege, S. Uni, M. Ristic, and L. H. Nutler.** 1985. Structural alteration of the membrane of erythrocytes infected with *Babesia bovis*. Am. J. Trop. Med. Hyg. **34:**45–49.
2. **Allred, D. R., R. M. Cinque, T. J. Lane, and K. P. Ahrens.** 1994. Antigenic variation of parasite-derived antigens on the surface of *Babesia bovis*-infected erythrocytes. Infect. Immun. **62:**91–98.
3. **Allred, D. R., S. A. Hines, and K. P. Ahrens.** 1993. Isolate specific parasite antigens of the *Babesia bovis* infected erythrocyte surface. Mol. Biochem. Parasitol. **60:**121–132.
4. **Barbet, A. F., L. W. Anderson, G. H. Palmer, and T. C. McGuire.** 1983. Comparison of proteins synthesized by two different isolates of *Anaplasma marginale*. Infect. Immun. **40:**1068–1074.
5. **Carson, C. A., and R. S. Phillips.** 1981. Immunologic response of the vertebrate host to *Babesia*, p. 441–444. *In* M. Ristic and J. P. Kreier (ed.), Babesiosis. Academic Press, New York.
6. **Copell, R. L., J. G. Culvernor, A. E. Bianco, P. E. Crewther, H. D. Stahl, G. H. Brown, R. F. Anders, and D. J. Kemp.** 1986. Variable antigen associated with the surface of erythrocytes infected with mature stages of *Plasmodium falciparum*. Mol. Biochem. Parasitol. **20:**265–277.
7. **Dalrymple, B. P.** 1993. Molecular variation and diversity in candidate vaccine antigens from *Babesia*. Acta Trop. **53:**227–238.
8. **Figueroa, J. V., G. M. Buening, D. A. Kinden, and T. J. Green.** 1990. Identification of common surface antigens among *Babesia bigemina* isolates using Mabs. Parasitology **100:**161–175.
9. **Goff, W. L., G. G. Wagner, and T. M. Craig.** 1982. The bovine immune response to tick derived *Babesia bovis* infection: serological studies of isolated immunoglobulins. Vet. Parasitol. **11:**109–120.
10. **Goff, W. L., G. G. Wagner, and T. M. Craig.** 1984. Increased activity of bovine ADCC effector cells during acute *Babesia bovis* infection. Vet. Parasitol. **16:**5–15.
11. **Hines, S. A., T. F. McElwain, G. M. Buening, and G. H. Palmer.** 1989. Molecular characterization of *Babesia bovis* merozoite surface proteins bearing epitopes immunodominant in protected cattle. Mol. Biochem. Parasitol. **37:**1–10.
12. **Hodgson, J. L., D. Stiller, D. P. Jasmer, G. M. Buening, G. G. Wagner, and T. C. McGuire.** 1992. *Babesia bigemina*: quantitation of infection in nymphal and adult *Boophilus microplus* using a DNA probe. Exp. Parasitol. **74:**117–126.
13. **Holbrook, A. A., A. J. Johnson, and P. A. Madden.** 1968. Equine piroplasmosis: intraerythrocytic development of *Babesia caballi* and *Babesia equi* (Laveran). Am. J. Vet. Res. **29:**297–303.
14. **Howard, R. J., B. J. Rodwell, P. M. Smith, L. L. Callow, and G. F. Mitchell.** 1980. Comparison of the surface proteins and glycoproteins on erythrocytes of calves before and during infection with *Babesia bovis*. J. Protozool. **27:** 241–247.
15. **Jacobson, R. H., F. Parrodi, I. G. Wright, C. J. Fitzgerald, and C. Dobson.** 1993. *Babesia bovis*: *in vitro* phagocytosis promoted by immune serum and by antibodies produced against protective antigens. Parasitol. Res. **79:**221–226.
16. **McCosker, P. J.** 1981. The global importance of babesiosis, p. 1–24. *In* M. Ristic and J. P. Kreier (ed.), Babesiosis. Academic Press, New York.
17. **McElwain, T. F., L. E. Perryman, W. C. Davis, and T. C. McGuire.** 1987. Antibodies define multiple proteins with epitopes exposed on the surface of live *Babesia bigemina* merozoites. J. Immunol. **138:**2298–2304.
18. **McElwain, T. F., L. E. Perryman, A. J. Musoke, and T. C. McGuire.** 1991. Molecular characterization and immunogenicity of neutralization sensitive *Babesia bigemina* merozoite surface proteins. Mol. Biochem. Parasitol. **47:** 213–222.
19. **McGuire, T. C., A. J. Musoke, and T. Kurtti.** 1979. Functional properties of bovine IgG1 and IgG2: interaction with complement, macrophages and skin. Immunology **38:**249–256.
20. **Murakami, K., and K. Tanabe.** 1985. An antigen of *Plasmodium yoelii* that translocates into the mouse erythrocyte membrane upon entry into the host cell. J. Cell Sci. **73:**311–320.
21. **Palmer, D. A., G. M. Buening, and C. A. Carson.** 1982. Cryopreservation of *Babesia bovis* for *in vitro* cultivation. Parasitology **84:**567–572.
22. **Palmer, G. H., and T. C. McGuire.** 1984. Immune serum against *Anaplasma marginale* initial bodies neutralizes infectivity for cattle. J. Immunol. **133:** 1010–1015.
23. **Parrodi, F., R. H. Jacobson, I. G. Wright, C. J. Fitzgerald, and C. Dobson.** 1991. The effect of immune serum and complement on the *in vitro* phagocytosis of *Babesia rodhaini*. Parasite Immunol. (Oxford) **13:**457–471.
24. **Ross, J. P. J., and K. F. Lohr.** 1968. Serologic diagnosis of *Babesia bigemina* infection in cattle by the indirect fluorescence antibody test. Res. Vet. Sci. **9:**557–562.
25. **Ruebush, M. J., and W. L. Hanson.** 1980. Thymus dependence of resistance to infection with *Babesia microti* of human origin in mice. Am. J. Trop. Med. Hyg. **29:**507–515.
26. **Schofield, L., J. Villaquiran, A. Ferreira, H. Schellekens, R. Nussenzweig, and V. Nussenzweig.** 1987. Gamma interferon, $CD8^+$ T cells and antibodies are required for immunity to malaria sporozoites. Science **330:**664–667.
27. **Shompole, S., T. F. McElwain, D. P. Jasmer, S. A. Hines, J. Katende, A. J. Musoke, F. R. Rurangirwa, and T. C. McGuire.** 1993. Identification of infected erythrocyte surface antigens with epitopes conserved among geographic strains of *Babesia bigemina*. Parasite Immunol. (Oxford) **16:**119–127.
28. **Simpson, C. F.** 1970. Electron microscopic comparison of *Babesia spp* and hepatic changes in ponies and mice. Am. J. Vet. Res. **31:**1763–1768.
29. **Suarez, C. E., T. F. McElwain, E. B. Stephens, V. S. Mishra, and G. H. Palmer.** 1991. Sequence conservation among merozoite apical complex proteins of *Babesia bovis*, *Babesia bigemina* and other apicomplexa. Mol. Biochem. Parasitol. **49:**329–332.
30. **Towbin, H., and H. Gordon.** 1984. Immunoblotting and dot immunoblotting: current status and outlook. J. Immunol. Methods **72:**313–340.
31. **Vega, C. A., G. M. Buening, T. J. Green, and C. A. Carson.** 1985. In vitro cultivation of *Babesia bigemina*. Am. J. Vet. Res. **46:**416–420.
32. **Vidotto, O., T. F. McElwain, R. Z. Machado, L. E. Perryman, C. E. Suarez, and G. H. Palmer.** *Babesia bigemina*: identification of B cell epitopes associated with parasitized erythrocytes. Exp. Parasitol., in press.
33. **Wright, I. G.** 1981. Biochemical characteristics of *Babesia* and physicochemical reactions in the host, p. 171–205. *In* M. Ristic and J. P. Kreier (ed.), Babesiosis. Academic Press, New York.