JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1991, p. 788–793
0095-1137/91/040788-06$02.00/0

Vol. 29, No. 4

Identification of *Anaplasma marginale* Long-Term Carrier Cattle by Detection of Serum Antibody to Isolated MSP-3

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Rapid and accurate detection of *Anaplasma marginale*-infected cattle would enhance anaplasmosis control procedures and evaluation of vaccines. Current tests based on detection of antibodies in serum are not widely used for several reasons, including the occurrence of either false-positive or false-negative results. We evaluated binding of antibodies in serum to a subunit antigen isolated from *A. marginale* initial bodies—major surface protein 3 (MSP-3). MSP-3 was detected in lysates of eight geographically different isolates of *A. marginale* and purified by affinity chromatography with monoclonal antibody AmG75C2. Antibodies from cattle infected with any of five geographically different isolates of *A. marginale* reacted in immunoblots with MSP-3. Sera from uninfected cattle and cattle infected with another rickettsial organism and two hemoprotozoal organisms failed to react with MSP-3. Six carrier cattle infected with the Florida isolate of *A. marginale* had antibody titers to MSP-3 ranging from 10^3 to 10^6 during a 5-year evaluation period. Since specific antibodies to isolated MSP-3 persist in high titers in long-term carrier cattle sera and MSP-3 is common among *A. marginale* isolates, it is recommended as a subunit antigen for an anaplasmosis test.

Anaplasma marginale, a rickettsia, causes bovine anaplasmosis, which occurs in most countries, including the United States (39). The organism grows in erythrocytes of infected cattle, resulting in anemia, weight loss, and death during some acute infections (44); infected and pregnant cattle may abort (7, 12) or give birth to infected calves (32). Anaplasmosis is transmitted biologically by ticks (38) that become infected by feeding on *A. marginale*-infected cattle, including carriers (47). Mechanical transmission occurs by other arthropods and additional mechanisms that transfer blood from infected to susceptible animals (8). In areas of endemic infection of cattle, this and other tick-borne diseases constrain efficient production of meat and milk and limit the introduction of susceptible cattle breeds with higher genetic potential (39). Cattle that survive the clinical disease associated with initial infection become carriers of *A. marginale* (11, 39, 43). Rickettsemia in carriers is usually below the limit reliably detectable by examination of stained blood smears (<0.1% infected erythrocytes) (20). Rickettsemia can be demonstrated, however, by inoculation of splenectomized calves with carrier blood (19) and by nucleic acid probe analysis (11, 13, 20).

Eventual control of *A. marginale* infection requires both development of an effective vaccine and identification of carrier cattle. There are two possible methods for routine carrier identification. One method is hybridization of infected cattle blood with a nucleic acid probe to detect *A. marginale* DNA. Hybridization of extracted DNA with an *A. marginale*-specific nucleic acid probe does not always detect known carriers (11), because of cyclic changes in rickettsemia levels (20). The polymerase chain reaction, however, might amplify *A. marginale* DNA to enable detection of all carrier cattle. The second method to identify carrier cattle is detection of *A. marginale*-specific antibody in serum. Carrier identification by antibody detection requires that infected cattle never rid themselves of *A. marginale* infection. Indefinite persistence of *A. marginale* organisms in infected cattle has been documented (11, 39), yet conclusive data allowing generalization to life-long persistence in all infections are lacking. Proving that life-long persistence occurs in *A. marginale*-infected cattle would be facilitated by using both a nucleic acid probe procedure that would detect all carrier cattle and a sensitive and specific test for antibody.

Serologic tests for anaplasmosis are not widely used, primarily because the error rate is too high. A part of the error with current tests is false-positive reactions caused by erythrocyte contamination of the *A. marginale* antigen used in the tests (2, 10) and the presence of antibodies to erythrocytes in some cattle sera. The frequency of antibodies to erythrocytes is markedly increased in sera from cattle vaccinated with blood-based vaccines, such as those for *Babesia* sp. (4, 10). The specificity of the current tests, including complement fixation (24, 31), card agglutination (2), capillary tube agglutination (40), enzyme-linked immunosorbent assay (4, 10, 45, 46), indirect immunofluorescence (14–16, 30), immunoblots (1), and radioimmunoassay (42), could be improved by using purified *A. marginale* antigen. Another part of the error is false-negative reactions occurring with some tests (26) either because of low sensitivity or because some nonprimary binding tests, such as complement fixation, do not detect all immunoglobulin isotypes (27). Therefore, an improved test should use a defined *A. marginale* antigen that is conserved among isolates and has a primary binding format capable of detecting all immunoglobulin isotypes.

To develop a subunit diagnostic test that solves the problems of existing tests and meets the characteristics defined in the preceding paragraph, we identified immunodominant proteins of *A. marginale* by immunoprecipitation with sera from infected cattle (36). One candidate antigen had an apparent molecular mass of 86 kDa and induced high antibody titers during infection of cattle with different *A.*

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marginale isolates (36). Here we describe the isolation and characterization of the 86-kDa protein and evaluate it as the basis for a diagnostic test for bovine anaplasmosis. We have renamed this protein major surface protein 3 (MSP-3), in keeping with current *Anaplasma* protein designations (33).

MATERIALS AND METHODS

Monoclonal antibody production and characterization. Five BALB/c mice were immunized with MSP-3 purified from *A. marginale* proteins separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Initial bodies were purified for electrophoresis from cattle erythrocytes infected with the Florida isolate of *A. marginale* (41). After electrophoresis, a gel slice was taken from an area calculated from the migration of prestained molecular weight markers to contain MSP-3. The purity of the eluted MSP-3 was evaluated on silver-stained gels after SDS-PAGE (35).

Sera from immunized mice were evaluated by immunoprecipitation of *A. marginale* proteins metabolically labeled with [^{35}S]methionine, followed by SDS-PAGE and fluorography (3, 35). Immunoprecipitation was chosen for evaluation because the protein identified by immune mouse sera could be compared on the same gel with MSP-3 identified by diluted immune cattle sera as previously described (36). The mouse having the strongest serum immunoprecipitation reaction with MSP-3 was selected as a spleen cell donor for fusion with X63.Ag8.653 tumor cells to make hybridomas (28, 29).

Supernatants from hybridomas were evaluated in pools containing 10 supernatants each. The evaluation procedure was also immunoprecipitation of metabolically labeled *A. marginale* proteins, followed by SDS-PAGE and fluorography. Besides identification of pools containing antibody to MSP-3, it was assumed that monoclonal antibody binding labeled MSP-3 during the immunoprecipitation reaction would function well when attached to a solid matrix for affinity chromatography. Individual supernatants from pools reacting with MSP-3 were also tested by immunoprecipitation. An individual supernatant which contained mouse antibodies to MSP-3 was identified. The hybridoma producing this supernatant was cloned twice by limiting dilution, and the immunoglobulin G1 monoclonal antibody produced was designated AmG75C2. Immunoprecipitation of AmG75C2 with ^{125}I-labeled initial body surface proteins was done as previously described (37).

Affinity chromatography. Ascitic fluid was obtained from pristane-treated BALB/c mice given hybridoma cells producing monoclonal antibody AmG75C2. The monoclonal antibodies were isolated from ascitic fluid by ammonium sulfate precipitation and DEAE-cellulose chromatography (29). Isolated antibody was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia LKB Biotechnology, Piscataway, N.J.) for affinity purification of MSP-3 (35).

Purified initial bodies from the Florida isolate of *A. marginale* were lysed with 1% Nonidet P-40 as previously described and passed through the affinity column containing monoclonal antibody AmG75C2 (35). Unbound proteins were removed by washing. The dissociating agent used to remove MSP-3 was determined by sequential elutions with 2 and 4 M potassium thiocyanate, 0.02 M diethylamine (pH 11.5), and 0.25 M glycine hydrochloride (pH 2.5). Diethylamine was the most efficient eluant and was used in subsequent isolation of MSP-3. Presence of MSP-3 in the eluates was determined by SDS-PAGE, followed by silver staining, and by Western immunoblotting with 10 μg of AmG75C2 per ml as the primary antibody, rabbit anti-mouse immunoglobulin as the secondary antibody, and [^{125}I]protein A for detection of bound complexes (18). After washing to remove unbound [^{125}I]protein A, the reaction was visualized by autoradiography.

Antiserum to MSP-3 was made by injecting three rabbits with 20 μg each of isolated MSP-3 emulsified in Freund complete adjuvant. This was followed by two biweekly injections with 20 μg each of MSP-3 emulsified in Freund incomplete adjuvant and two biweekly injections with 40 μg each of MSP-3 emulsified in Freund incomplete adjuvant. The rabbit antiserum was evaluated in immunoblots and dot blots by using [^{125}I]protein A to detect bound antibody.

Long-term *A. marginale* carrier cattle. Serum was collected over a 5-year period from six Holstein steers, each inoculated at 3 months of age with 10 ml of blood stabilate having 48% erythrocytes infected with the Florida isolate of *A. marginale*. These calves became rickettsemic within 30 days after inoculation and had peak percentages of infected erythrocytes ranging from 23 to 42%. Rickettsemias were undetectable by 6 months postinfection by blood smear examination; however, blood from each animal was inoculated into individual splenectomized calves at 2 and 3 years postinfection (11). Blood from all six cattle induced anaplasmosis in recipient splenectomized calves at both time points. As reported, DNA extracts of erythrocytes from these six cattle reacted with an *A. marginale* RNA probe prepared from the msp1β gene (11).

Cattle infected with *A. marginale* isolates. Five 6-month-old Holstein steers were infected with blood stabilate containing 10^9 *A. marginale* Washington O isolate-infected erythrocytes (34). Calves became rickettsemic within 28 to 38 days postinfection and had peak infected-erythrocyte concentrations ranging from 4 to 8%. Sera were taken at 44 days postinfection for reaction against isolated MSP-3 in immunoblots. Sera from four Holstein calves taken 68 to 82 and 281 days after exposure to ticks infected with the South Idaho isolate were obtained from Steve Landry, Agricultural Research Service, U.S. Department of Agriculture, Moscow, Idaho. Sera from four calves infected with the Virginia isolate for 61 to 118 days have already been described (36). Serum samples were taken from two North Texas isolate-infected calves at 103 and 131 days postinfection (36).

Normal cattle and cattle with other infections. Sera from 36 normal adult cattle from northern Canada (5) were obtained from Lorne Babiuk, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, Saskatchewan, Canada. Eight sera were obtained from normal Holstein calves located at a dairy in western Oregon. Sera from five calves infected with *Babesia bovis* for 122 days were previously characterized (17). Sera from three Holstein calves infected with *B. bigemina* for 325 days were obtained from Lance Perryman in our department. Sera from four Holstein calves infected for 22 to 99 days with a rickettsia isolated from an aborted bovine fetus (9) that cross-reacts serologically with *Cowdria ruminantium* were obtained from Tim Crawford in our department.

***A. marginale* isolates.** Eight *A. marginale* isolates were used in this study. The origins of six isolates, Washington C, Washington O, South Idaho, Florida, Virginia, and North Texas, have already been described or referenced (28). The Missouri isolate was from Gerald Buening, Department of Microbiology, College of Veterinary Medicine, University of Missouri, Columbia. The Mississippi isolate was from Richard Hidalgo, School of Veterinary Medicine, Louisiana State University, Baton Rouge.

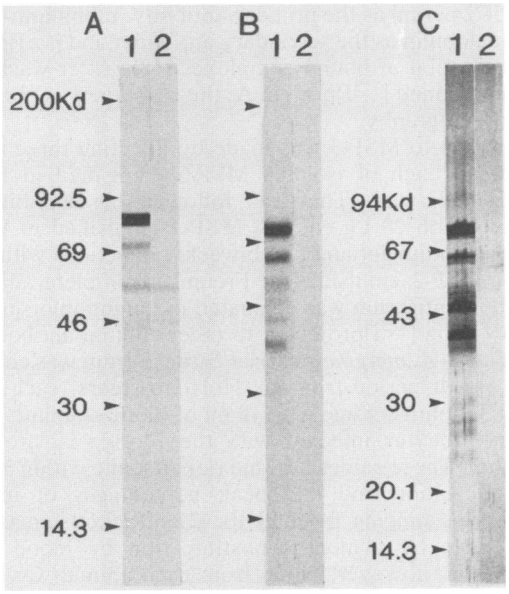

FIG. 1. Characterization of monoclonal antibody AmG75C2 reactivity. (A) Immunoprecipitation of [^{35}S]methionine-labeled *A. marginale* proteins with AmG75C2 (lane 1) and an isotype control monoclonal antibody (lane 2), followed by SDS-PAGE and fluorography. (B) Immunoblot of MSP-3 isolated by affinity chromatography reacted with AmG75C2 (lane 1) and with a control eluate from the same column (lane 2). (C) Silver stain following SDS-PAGE of isolated MSP-3 (lane 1) and a control eluate from the same column (lane 2). Kd, Kilodaltons.

Immunoblots with cattle sera. The procedure for Western immunoblots used [^{125}I]protein G to detect cattle antibodies reacting with 0.5 to 2 μg of isolated MSP-3 transferred to nitrocellulose (17). Each titer was the reciprocal of the last serum dilution reacting with MSP-3, the largest protein isolated from AmG75C2 affinity columns.

RESULTS

Sera and monoclonal antibodies derived from mice immunized with MSP-3 isolated from SDS-PAGE were evaluated by immunoprecipitation of metabolically labeled *A. marginale* proteins. Monoclonal antibody AmG75C2, derived from a twice-cloned hybridoma, immunoprecipitated labeled proteins having apparent molecular masses of 83 and 70 kDa (Fig. 1A). To show that the larger protein bound by AmG75C2 was similar to the one previously described as Am86 (35), metabolically labeled proteins immunoprecipitated with AmG75C2 and diluted calf serum were compared. The larger protein immunoprecipitated by AmG75C2 had the same apparent molecular weight as the immunodominant protein immunoprecipitated by diluted serum from an *A. marginale*-infected calf and previously identified as Am86 (36). Furthermore, immunoprecipitation of ^{125}I-labeled initial body surface proteins (37) with AmG75C2 identified a single labeled protein of the same apparent molecular weight previously identified as Am86 with rabbit antiserum to initial bodies (37). In accordance with other *A. marginale* surface proteins (33), the largest one identified by AmG75C2 was designated MSP-3.

Proteins isolated from a monoclonal antibody AmG75C2 affinity column were evaluated of SDS-PAGE, followed by immunoblotting (Fig. 1B), and by silver staining (Fig. 1C).

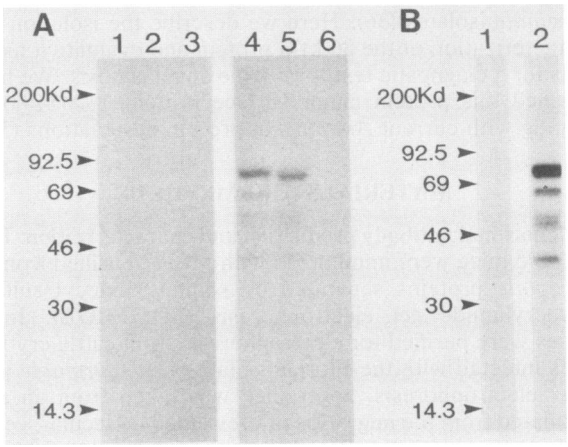

FIG. 2. Immunoblot of isolated MSP-3 and initial body lysate. (A) Lanes: 1 to 3, reaction with an isotype control monoclonal antibody; 4 to 6, reaction with AmG75C2; 1 and 4, isolated MSP-3; 2 and 5, lysate of 1.3×10^9 Florida isolate initial bodies; 3 and 6, lysate of 3×10^7 uninfected erythrocytes. (B) Lanes: 1 and 2, isolated MSP-3; 1, reaction with uninfected-calf serum (1:1,000); 2, reaction with infected-calf serum (1:1,000). Kd, Kilodaltons.

AmG75C2 reacted with six proteins of 80, 65, 55, 50, 44, and 40 kDa (Fig. 1B) in an immunoblot, and silver staining revealed a similar pattern (Fig. 1C). Monoclonal antibody AmG75C2 also reacted in an immunoblot with similar proteins in affinity column eluate and whole initial body lysate (Fig. 2A), except that the largest protein identified migrated to slightly different positions in the eluate and the lysate. AmG75C2 did not react in an immunoblot with erythrocyte lysate proteins (Fig. 2A). Antibodies from the serum of an *A. marginale*-infected calf reacted in an immunoblot with all of the proteins identified by AmG75C2 (Fig. 2B). Immunoblot reactions of AmG75C2 with multiple polypeptides in whole initial body lysate and similar proteins eluted from an AmG75C2 affinity column (Fig. 2A) indicated that these polypeptides shared the same or a similar epitope.

The presence of MSP-3 epitopes in different isolates of *A. marginale* was assessed by reacting a rabbit antiserum against affinity-purified MSP-3 with lysates from eight geographically different isolates. The specificity of the rabbit antiserum was evaluated by immunoblotting isolated MSP-3 and initial body lysate. With a 1:500 dilution of antiserum, at least five proteins with apparent molecular weights similar to those of the proteins bound by AmG75C2 (Fig. 1A) were bound (Fig. 3A). Results in Fig. 3C demonstrated that rabbit anti-MSP-3 serum reacted in a dot blot assay with initial body lysates of eight geographically different isolates but not with *B. bovis* merozoite lysate or uninfected-erythrocyte lysate.

To verify the specificity of the reaction of antibodies in serum from infected cattle with MSP-3 in an immunoblot, sera from normal cattle were tested. Sera from 36 adult cattle from Saskatoon, Saskatchewan, Canada, were negative at a dilution of 1:100, the only dilution tested (Table 1). Further, eight sera from 2-month-old calves were negative (Fig. 4), as were preinfection sera from 15 other calves (Table 1). Sera from five calves infected with *B. bovis*, five infected with *B. bigemina*, and four infected with a new rickettsia isolated from an aborted bovine fetus (9) all failed to react with MSP-3 in immunoblots.

Sera from nonsplenectomized calves infected with stabi-

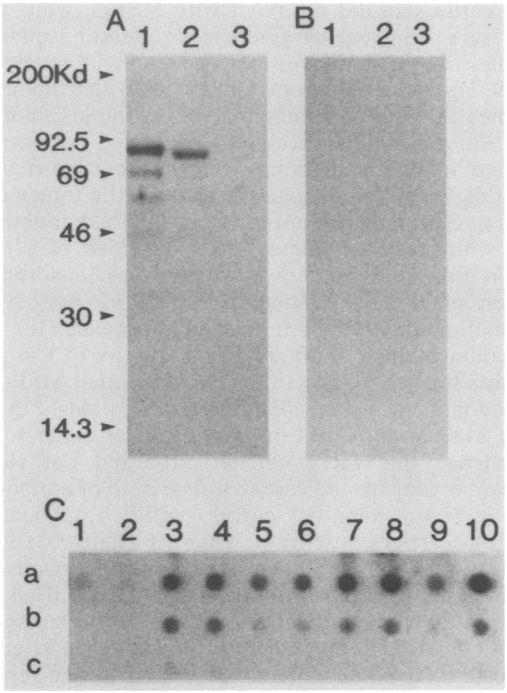

FIG. 3. Reactivity of rabbit anti-MSP-3 serum with isolated MSP-3 and initial bodies from eight geographically different isolates. Panels A and B show immunoblots. Lanes: 1 isolated MSP-3; 2, Florida isolate initial body lysate; 3, uninfected-erythrocyte lysate. The immunoblot in panel A was reacted with rabbit anti-MSP-3 serum (1:500), while that in panel B was reacted with normal rabbit serum (1:500). Panel C shows a dot blot. Columns: 3 to 10, Virginia, Mississippi, Missouri, Washington O, North Texas, Washington C, Idaho, and Florida initial body lysates, respectively. Rows: a to c, 2.5×10^8, 2.5×10^7, and 2.5×10^6 organisms, respectively. Columns: 1, rows a to c, 10-fold dilutions of uninfected-erythrocyte lysate, beginning with 5×10^7 cells; 2, rows a to c, *B. bovis* merozoite lysate in 10-fold dilutions, beginning with 3.3×10^7 organisms. The dot blot in panel C was reacted with rabbit anti-MSP-3 serum (1:500). Kd, Kilodaltons.

lates of five geographically different isolates of *A. marginale* were tested to determine whether these isolates induced antibodies that reacted with MSP-3 isolated from the Florida isolate. Calves infected with the Washington O, South Idaho, Virginia, and North Texas isolates became parasitemic after infection as determined by microscopic examination of stained blood smears. At 44 to 281 days postinfection, sera from all of these calves reacted with isolated MSP-3 in an immunoblot (Table 1).

Affinity-purified MSP-3 was evaluated by immunoblot with pre- and postinfection sera from six long-term carrier cattle infected with the Florida isolate of *A. marginale*. Preinfection sera were unreactive at a dilution of 1:100 (Table 2). Antibody titers in postinfection sera were determined by reacting 10-fold dilutions of serum in an immunoblot (Fig. 5). The antibody titers exceeded 1,000 at 2 months and 1, 3, and 5 years after infection, with the maximum titer being 10^6 (Table 2). As reported, blood from each of these six cattle was infectious for splenectomized calves at both 2 and 3 years postinfection (11). These earlier transmission data demonstrated that these cattle were *A. marginale* carriers, and the data in Table 2 demonstrated that they maintained high antibody titers to MSP-3.

TABLE 1. Immunoblot reactivities of cattle sera with MSP-3 after infection with various *A. marginale* isolates

Exposure to *A. marginale*	No. positive[a]/ no. tested
None	0/44
Washington O isolate	
Preinfection	0/5
44 days postinfection	5/5
South Idaho isolate	
Preinfection	0/4
68–82 days postinfection	4/4
281 days postinfection	4/4
Virginia isolate	
Preinfection	0/4
60 days postinfection	4/4
North Texas isolate	
Preinfection	0/2
103 and 131 days postinfection	2/2
Florida isolate	
Preinfection	0/6
60 days postinfection	6/6
1, 3, and 5 yr postinfection	6/6

[a] Positivity was defined as a visible reaction with MSP-3 at a 1:100 dilution of serum.

DISCUSSION

Development of a useful subunit diagnostic test for *A. marginale* infection requires a sensitivity that will detect all infected cattle. The test also must identify cattle infected with various geographically different isolates of *A. marginale* because some isolates have differences in structure as indicated by morphologic (21, 22), biochemical, (3) and immunologic (6, 14, 15, 23, 25, 28) studies. Our previous work identified an *A. marginale* protein as a candidate for a subunit diagnostic test (36). This protein (previously Am86, now MSP-3) was detected by immunoprecipitation of serum with [^{35}S]methionine-labeled *A. marginale* initial body proteins (36). To evaluate MSP-3 as the basis of a subunit

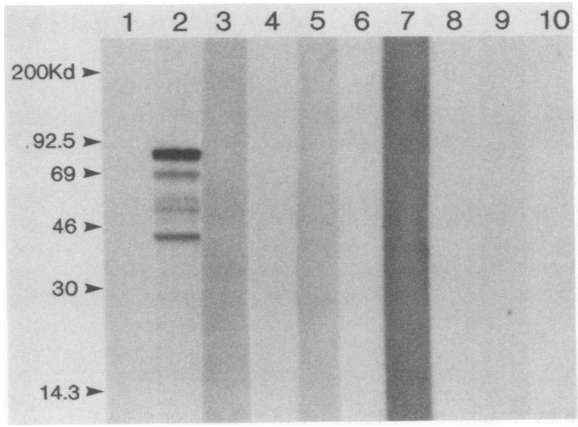

FIG. 4. Immunoblots of MSP-3 with uninfected-calf sera. Lanes: 1 to 10, isolated MSP-3; 1, reaction with uninfected-calf serum as a negative control (1:1,000); 2, reaction with infected-calf serum as a positive control (1:1,000); 3 to 10, reaction with sera from eight uninfected calves at a 1:100 dilution. Kd, Kilodaltons.

TABLE 2. Immunoblot antibody titers[a] to MSP-3 in sera from long-term carrier cattle infected with the Florida isolate of *A. marginale*

Animal no.[b]	Titer at the following time after infection[c]:			
	2 mo	1 yr	3 yr	5 yr
B110	10^3	10^4	10^4	10^4
B122	10^4	10^5	10^3	10^3
B170	10^6	10^5	10^4	10^3
B178	10^6	10^5	10^3	10^3
B182	10^6	10^6	10^4	10^4
B185	10^4	10^4	10^3	10^4

[a] Each titer is the reciprocal of the last serum dilution causing a reaction with MSP-3.

[b] All six cattle were shown to be carriers by disease transfer to splenectomized recipient calves at 2 and 3 years after infection (11). They were positive by DNA hybridization at 5 years postinfection (20).

[c] There was no visible reaction at a 1:100 dilution of serum at time zero postinfection.

diagnostic test, we purified MSP-3 and determined whether a test using the subunit could detect antibodies in sera from *A. marginale*-infected cattle, including long-term carriers.

A monoclonal antibody, AmG75C2, that was specific for *A. marginale* and bound MSP-3 was made. Specificity of AmG75C2 for *A. marginale* proteins was demonstrated by immunoprecipitation of protein metabolically radiolabeled in a system that results in labeling of only *A. marginale* proteins (3). Specificity was verified by failure of AmG75C2 to react with erythrocyte and leukocyte proteins by indirect immunofluorescence (data not shown), with erythrocyte lysate in immunoblots, and with *B. bovis* merozoite and erythrocyte lysates in dot blots. Binding of AmG75C2 with MSP-3 was demonstrated in immunoprecipitation reactions of metabolically and surface-labeled proteins of *A. marginale*. Additional proteins having apparent molecular masses below 80 kDa were present in initial body lysate and had the same epitope recognized on MSP-3 by AmG75C2 or a similar epitope. These additional proteins were assumed to be proteolytic digestion products of MSP-3, although other explanations are plausible.

Immunoblot reaction of MSP-3 with cattle sera was specific for *A. marginale* infection. Preinfection calf sera and normal cattle sera did not react with MSP-3, while postinfection sera from calves given five different isolates had antibodies to MSP-3. Calves experimentally infected with *B. bovis*, *B. bigemina*, and a new rickettsia (9) also did not have antibodies in their sera that reacted in immunoblots with MSP-3. *Babesia*-infected calves were tested because babesiosis and anaplasmosis often occur in the same regions of the world. With regard to carriers, six cattle infected for 2 months to 5 years with the Florida isolate had antibodies to MSP-3 with titers exceeding 1,000. Detection of all carrier cattle in this study contrasts with a previous report of detection of 79% by complement fixation, 84% by card agglutination, and 97% by indirect fluorescence (16).

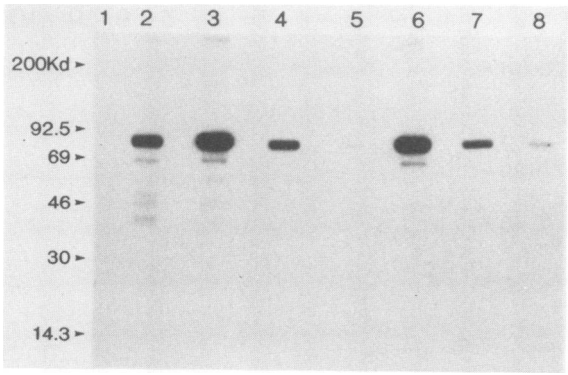

FIG. 5. Determination of antibody titers in sera from carrier cattle by immunoblots. Lanes: 1 to 8, isolated MSP-3; 1, reaction with uninfected-calf serum as a negative control (1:1,000); 2, reaction with infected-calf serum as a positive control (1:1,000); 3 to 5, reaction with 10-fold dilutions, beginning with 1:10,000, of B-170 serum taken 2 months after infection; 6 to 8, same as 3 to 5 except that B-178 serum was titered. Kd, Kilodaltons.

Detection of high antibody titers (10^3 to 10^6) in carrier cattle sera further supports the conclusion that MSP-3 is an immunodominant *A. marginale* protein (36). Also, the presence of MSP-3 antibodies in known carrier cattle sera and the evidence that these carrier cattle did not clear *A. marginale* organisms indicate that detection of antibodies to MSP-3 in serum identifies carrier cattle. Amplification of DNA from carrier cattle by polymerase chain reaction and hybridization with an *A. marginale*-specific nucleic acid probe may allow detection of all carriers (11). If all DNA-positive cattle have antibodies to MSP-3, either an antibody test or a nucleic acid probe test would identify carriers. The test choice then would depend on ease, cost, and reproducibility.

Data presented in this paper demonstrate that MSP-3 is suitable for development of a subunit diagnostic test for anaplasmosis. The current format of immunoblotting to detect antibodies to MSP-3 and diagnose anaplasmosis is suitable for laboratory-based applications. However, a more readily available source of antigen and an easier test format are needed before widespread application of an MSP-3 subunit diagnostic test. We hypothesize that a synthetic immunodominant peptide based on the DNA sequence of the gene coding for MSP-3 can be identified. Once this peptide is identified, a reproducible synthetic peptide can be made and used in sensitive primary binding tests for antibody in serum. It is anticipated that the gene for MSP-3 can be identified by screening protein expression libraries of *A. marginale* DNA with monoclonal antibody to MSP-3.

ACKNOWLEDGMENTS

This work was supported by grants from the following agencies: the U.S. Department of Agriculture (58-91H2-7-84), the USDA Bi-National (US-Israel) Agricultural Research and Development Fund (US-1561-88), the U.S. Agency for International Development (DAN-1328-G-SS-4093-00), and the Washington Technology Center (09-1064).

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