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Evaluation of *Anaplasma marginale* Major Surface Protein 3 (MSP3) as a Diagnostic Test Antigen

A. RICK ALLEMAN* AND ANTHONY F. BARBET

Department of Pathobiology, College of Veterinary Medicine, University of Florida, Gainesville, Florida 32611-0880

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An immunodominant surface protein, major surface protein 3 (MSP3), has been proposed as an antigen suitable for use in the diagnosis of bovine anaplasmosis. We further characterized MSP3 to examine its potential as a test antigen for the serological diagnosis of carrier cattle. The specificity of this antigen in detecting infected cattle as well as the conservation of MSP3 between strains of *Anaplasma marginale* was evaluated by using immunoblots of *A. marginale* proteins separated by one- and two-dimensional polyacrylamide gel electrophoreses. Immune sera from animals infected with *Anaplasma ovis*, *Ehrlichia risticii*, and *Ehrlichia ewingii* reacted with the MSP3 antigen of *A. marginale*. One-dimensional gel electrophoresis of *A. marginale* proteins demonstrated size polymorphism of MSP3 between different geographic isolates. Two-dimensional gel electrophoresis revealed at least three different antigens migrating at the 86-kDa molecular size, and sera from animals infected with different strains of *A. marginale* reacted with different 86-kDa antigens. These results indicate that MSP3 may share cross-reactive epitopes with antigens found in *A. ovis* and some *Ehrlichia* spp. In addition, MSP3 is not conserved between different isolates of *A. marginale*, and at least in the isolate from Florida, what was previously identified as MSP3 is actually a group of three or more 86-kDa antigens with different isoelectric points. The cross-reactivity of MSP3 with some *Ehrlichia* spp., the variability of MSP3 between isolates, and the multiple 86-kDa antigens recognized by various sera suggest that MSP3 is not a suitable candidate for use as a recombinant test antigen.

Anaplasma marginale is an arthropod-borne, rickettsial hemoparasite which invades the erythrocytes of cattle, causing a clinical disease known as anaplasmosis. This disease has a global distribution which includes the United States. Worldwide economic losses are difficult to calculate, but losses in the United States alone are estimated to be over $100 million annually (14, 24). In certain areas of the United States, the incidence of infected cattle may be as high as 37% (23, 24).

Acute anaplasmosis is usually seen in cattle over 1 year of age and is characterized by a severe hemolytic anemia, resulting in weight loss, abortion, decreased milk production, and often death in infected animals over 3 years of age (47). In the acute stages, the disease is easily diagnosed by finding organisms on routine blood smear evaluation. However, animals that survive the infection will remain carriers and maintain a low level of parasitemia which cannot be detected microscopically (36, 49). These carrier cattle serve as a perpetual source of infection for susceptible cattle (42). Cyclic rickettsemia has been detected and quantitated in carrier cattle by nucleic acid probe hybridization (9, 16). Although the number of infected erythrocytes is at times very low (<0.000025%), ticks are able to acquire infection from these cattle at infectivity rates of up to 80% (10). Replication of *A. marginale* in the tick vector allows easy transmission of the disease from only a few infected ticks. This firmly establishes the important role that persistently infected cattle play in the transmission of the disease. In order to reduce the economic losses associated with anaplasmosis, control efforts must include an effective way of identifying and decreasing transmission from carrier cattle.

* Corresponding author. Mailing address: Department of Pathobiology, College of Veterinary Medicine, University of Florida, Gainesville, FL 32611-0880. Phone: (904) 392-4700, extension 5858. Fax: (904) 392-9704. Electronic mail address: ARALL@GNV.IFAS.UFL.EDU.

Several types of serological tests have been described for the diagnosis of infection with *A. marginale*, including complement fixation, capillary tube agglutination, card agglutination, indirect immunofluorescence, radioimmunoassay, and enzyme-linked immunosorbent assays (2, 3, 5, 8, 13, 20, 27, 28, 39, 48). These tests lack acceptable specificity and/or sensitivity, particularly in the detection of carrier cattle (2, 3, 5, 8, 11, 13, 22, 23, 43). This is primarily because the antigens used in these tests are a crude mixture of *A. marginale* and erythrocyte material (17, 18). Because of the importance of carrier cattle in disease transmission, an effective diagnostic test with recombinant *A. marginale* proteins would be an important tool in reducing disease transmission and economic losses.

An 86-kDa surface protein of *A. marginale* (major surface protein 3 [MSP3]) may have potential for use as a diagnostic test antigen (25). This protein is one of the most immunodominant proteins of *A. marginale*, and high levels of antibody to MSP3 were detected in cattle sera as early as 30 days postinfection and in carrier cattle infected for 5 years or longer (25, 30). Affinity-purified MSP3 was used experimentally as a test antigen and showed excellent sensitivity and specificity in detecting carrier animals (25). However, the cross-reactivity of sera from animals infected with related organisms was not firmly established. In addition, little is known about the conservation of this protein between various strains of *A. marginale* or whether sera from animals infected with isolates from various geographic locations might differ in their reactivities to MSP3.

We separated *A. marginale* proteins by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and two-dimensional (2-D) gel electrophoresis and immunoblotted these proteins using antisera from animals infected with *A. marginale* or other related organisms to further characterize MSP3. This was done to determine if a recombinant form of

the protein would be suitable for use as a diagnostic test antigen to detect carrier cattle.

MATERIALS AND METHODS

***A. marginale* strains.** Florida (FL), Virginia (VA), South Idaho (SI), and Washington O (WA) isolates of *A. marginale* stored as stabilates (37) were used to infect splenectomized calves. Infected, whole blood was collected in EDTA from calves during periods of peak parasitemias (FL, 70%; VA, 36%; SI, 42%; WA, 40%) and was centrifuged at 10,000 × *g* for 15 min, and the serum and buffy coat were removed. Packed erythrocytes were washed three times in phosphate-buffered saline (PBS), resuspended to a packed cell volume of 50%, and stored at −70°C.

Initial body preparation. *A. marginale* initial bodies were isolated from infected erythrocytes as described previously (33). Isolated, intact initial bodies were visualized by Wright-Giemsa staining. The pellets of initial bodies were resuspended in equal volumes of PBS for use in SDS-PAGE. The initial bodies used in 2-D gel electrophoresis were resuspended in equal volumes of lysis buffer containing 9.5 M urea, 2% Nonidet P-40, 1.6% ampholyte 5/7 (Bio-Lyte 5/7; Bio-Rad Laboratories, Richmond, Calif.), 0.4% ampholyte 3/10 (Bio-Lyte 3/10; Bio-Rad Laboratories), and 5.0% β-mercaptoethanol. Protein concentrations were determined spectrophotometrically by the Micro BCA Protein Assay (Pierce, Rockford, Ill.). Initial body preparations were stored in small aliquots at −70°C. *Anaplasma centrale* initial bodies were also prepared as described above.

***Babesia bovis* and *Babesia bigemina* antigen preparation.** *B. bovis* and *B. bigemina* antigens were prepared from organisms maintained in microaerophilic stationary-phase culture as described previously (21). Briefly, infected erythrocytes were centrifuged at 10,000 × *g* and the supernatant was removed. Packed cells were resuspended to 20 times the volume in 10 mM sodium phosphate. The solution was centrifuged and the supernatant was removed. The pellets were then resuspended in an equal volume of 10 mM sodium phosphate.

Antisera used for immunoblots. Six Holstein calves were infected with blood stabilate containing an FL isolate of *A. marginale*. Two other Holstein calves were each infected with either a VA or an SI isolate. Antisera from all calves were collected at 50 and 70 days postinfection. Antiserum from a cow experimentally infected with a WA isolate, rabbit-anti-MSP3 polyclonal sera, an anti-MSP3 monoclonal antibody (MAb), and an anti-MSP2 MAb were supplied to us courtesy of Travis McGuire and Guy Palmer at Washington State University. The reactivities of the MAbs (25, 26, 31, 32) and the rabbit-anti-MSP3 polyclonal serum (25) have been described previously. Sera obtained from calves prior to infection with *A. marginale* and an antitrypanosome MAb were used as negative controls in immunoblot experiments.

A. centrale and *A. ovis* antisera with indirect fluorescent-antibody titers of 1:4,000 were supplied courtesy of Susan Oberle (The Salk Institute, San Diego, Calif.). Hyperimmune sera from cattle infected experimentally with *B. bovis* and *B. bigemina* were supplied by David Allred (University of Florida, Gainesville). Sera from cattle experimentally infected with *Cowdria ruminantium* were supplied by Michael Bowie (University of Florida, Gainesville). Equine sera from animals infected with *Ehrlichia equi* and *Ehrlichia risticii* had indirect fluorescent-antibody titers of 1/1,600 and were obtained from Ibulaimu Kakoma (University of Illinois, Urbana). Serum from a dog with canine ehrlichiosis was obtained from Rose Raskin (University of Florida, Gainesville). This dog was infected with *E. ewingii*, but serum from this animal had an indirect fluorescent-antibody titer of 1/160 for *Ehrlichia canis* and 1/64 for *Ehrlichia chaffeensis.*

SDS-PAGE. Initial body preparations containing 6.0 to 10.0 μg of protein were solubilized in one-half their volume of a 3× sample buffer containing 0.1 M Tris (pH 6.8), 5% (wt/vol) SDS, 50% glycerol, 7.5% β-mercaptoethanol, and 0.00125% bromophenol blue and were heat denatured at 100°C for 3 min. Proteins were separated on 7.5 to 17.5% (wt/vol) gradient polyacrylamide-SDS gels. Gels were fixed in 25 mM Tris–191.8 mM glycine–20% methanol and were electrophoretically transferred to nitrocellulose (Hybond ECL; Amersham International plc, Buckinghamshire, England).

2-D gel electrophoresis. Isoelectric focusing gels were prepared according to the manufacturer's instructions (Bio-Rad) (6) with the following modifications. An acrylamide–*N*,*N*′-methylenebisacrylamide solution containing 9.5 M urea, 2.0% (vol/vol) Nonidet P-40, 4.1% acrylamide–bis (30.8% T, 2.6% C), 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate and 5.8% (vol/vol) ampholyte 5/7 was polymerized in glass tubing (3 by 140 mm). Initial body preparations were incubated for 2 h at room temperature in four times their volume of the previously described lysis buffer and five times their volume of sample buffer containing 9.5 M urea, 2.0% Triton X-100, 5% β-mercaptoethanol, 1.6% ampholyte 5/7, and 0.4% ampholyte 3/10. The solution was then centrifuged at 100,000 × *g* for 2 h at 25°C. A volume of the supernatant containing 20 μg of protein was loaded onto each tube gel, and each tube was overlaid with 50 μl of overlay buffer containing 9.5 M urea, 0.8% ampholyte 5/7, 0.2% ampholyte 3/10, and 0.0025% bromophenol blue. Tube gels were electrophoresed at 400 V for 16 h and 800 V for 2 h with a model V16 vertical gel electrophoresis system (Bethesda Research Laboratories, Gaithersburg, Md.). Tube gels were extruded from the glass tubes and were equilibrated for 5 min in buffer containing 0.0625 M Tris-HCl (pH 6.8), 10.0% glycerol, 2.0% (wt/vol) SDS, 5.0% β-mercaptoethanol, and 0.00125% bromophenol blue. The focused proteins were then electrophoresed on 7.5 to 17.5% (wt/vol) gradient polyacrylamide-SDS gels and were treated and transferred to nitrocellulose membranes as described above.

Immunoblots with antisera. Nitrocellulose membranes containing the transferred proteins were blocked with 5% (wt/vol) milk in PBS with 0.25% polyoxyethylene-sorbitan monolaurate (Tween 20) to inhibit the nonspecific binding of primary and secondary antibodies. The membranes were washed with 1% (wt/vol) milk in PBS with 0.25% Tween 20 and were probed with antisera from animals infected with one of the following organisms; *A. marginale*, *A. centrale*, *A. ovis*, *B. bovis*, *B. bigemina*, *C. ruminantium*, *E. equi*, *E. risticii*, or *E. ewingii*. Normal sera from the respective uninfected species were used as negative controls. Serum dilutions of 1/100 or greater were used. Rabbit anti-MSP3 polyclonal sera was used at a dilution of 1/5,000. As a negative control, normal rabbit serum was used at the same dilution. Anti-MSP3, anti-MSP2, and negative control anti-trypanosome MAbs were used at concentrations of 5 μg/ml. The membranes were again washed with 1% (wt/vol) milk in PBS with 0.25% Tween 20 and were probed with either species-specific anti-immunoglobulin G–horseradish peroxidase (HRP)-conjugated antibody at a dilution of 1/2,000 (Sigma Immuno Chemicals, St. Louis, Mo.) or HRP-conjugated protein G at a dilution of 1/15,000 (Sigma Immuno Chemicals). The membranes were processed for enhanced chemiluminescence (ECL) with detection reagents containing luminol as a substrate (ECL Western blotting [immunoblotting] detection reagents; Amersham International plc). The membranes were exposed to Hyperfilm-MP (Amersham International plc) to visualize the bound antibody.

RESULTS

Specificity experiments. SDS-PAGE-separated *A. marginale* initial body proteins were transferred to nitrocellulose and were probed with antisera from animals infected with related rickettsial agents or protozoal hemoparasites. This was done to determine if animals infected with these organisms contain antibodies which cross-react with the 86-kDa protein (MSP3) of *A. marginale*. As a negative control, normal sera from various species were reacted with *A. marginale* proteins. When available, protein preparations from related organisms were used in a homologous reaction with respective antisera to serve as a positive control.

Sera from a sheep infected with *A. ovis* (Fig. 1a), a horse infected with *E. risticii* (Fig. 1c), and a dog infected with *E. ewingii* (Fig. 1c) showed strong reactivity with MSP3 of *A. marginale*. These sera as well as sera from animals infected with *E. equi* and *C. ruminantium* also showed reactivity against other *A. marginale* antigens. Sera from animals infected with *A. ovis*, *C. ruminantium*, *E. ewingii*, and *E. equi* showed reactivity against a 36-kDa antigen, possibly MSP2 (35) (Fig. 1a, b, and c). Sera from animals infected with *C. ruminantium* (Fig. 1b) and *E. equi* (Fig. 1c) reacted with an antigen with a molecular size slightly smaller than that of MSP3. Sera from animals infected with *A. centrale* (Fig. 1a) or either *Babesia* spp. (Fig. 1b) failed to react with any *A. marginale* antigens, although strong reactivity was demonstrated in reactions involving homologous preparations.

Conservation of MSP3 between isolates of *A. marginale* from different geographic locations. The conservation of MSP3 between isolates of *A. marginale* from different geographic locations was evaluated by SDS-PAGE and immunoblots with initial body preparations of isolates of *A. marginale* from FL, VA, SI, and WA. The preparations were separated on 7.5 to 17.5% (wt/vol) gradient polyacrylamide gels as described above. They were then transferred to nitrocellulose and were reacted with various dilutions of antiserum from an animal experimentally infected with an FL isolate and a MAb to MSP3, AMG75C2 (25), for the definitive identification of the MSP3 antigen. An optimal dilution of this antiserum was established for demonstration of the immunodominant MSP3 protein (data not shown). Preinfection bovine sera and a MAb to a trypanosome surface protein were used as negative controls and showed no reactivity to MSP3.

Initial body preparations from these same isolates were then probed with antiserum at a single dilution established previ-

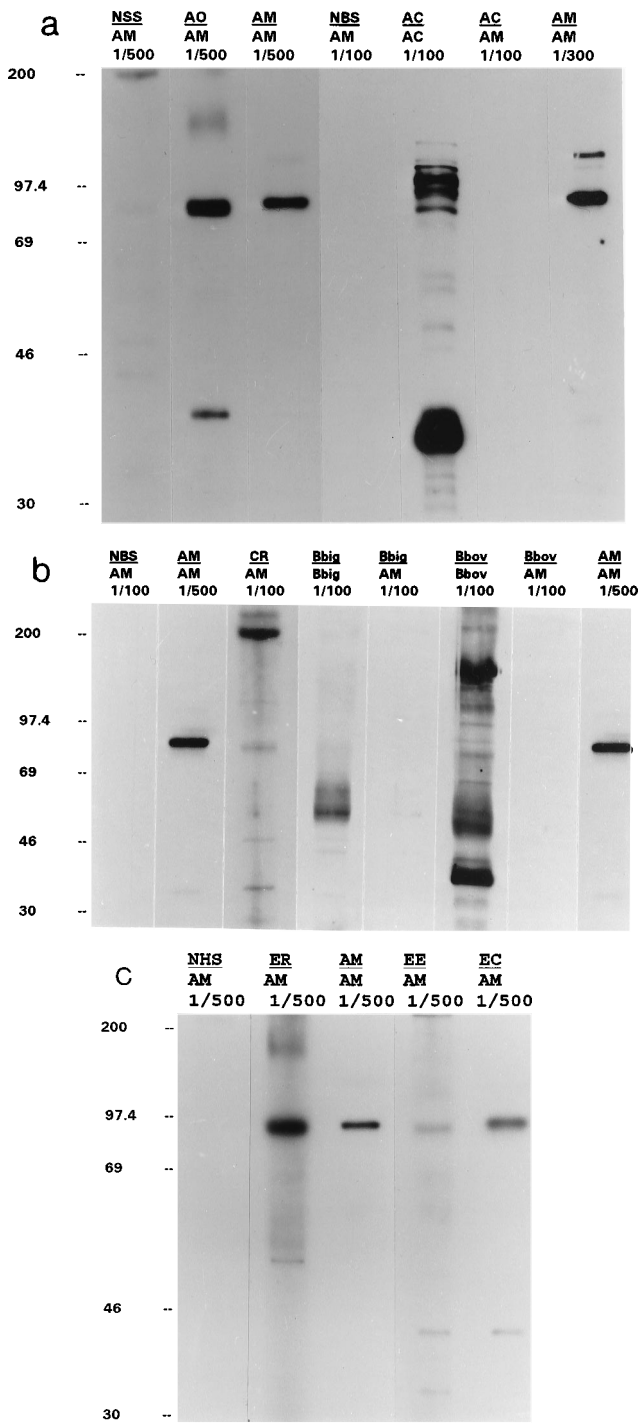

FIG. 1. Specificity experiments with immunoblots. (a) *A. marginale* (AM) or *A. centrale* (AC) initial body preparations reacted with normal sera from noninfected sheep (NSS), noninfected cattle (NBS), or antisera from animals infected with *A. ovis* (AO), *A. centrale* (AC), or *A. marginale* (AM). (b) *A. marginale* (AM), *B. bovis* (Bbov), or *B. bigemina* (Bbig) initial body preparations reacted with normal serum from a noninfected cow (NBS) or antisera from animals infected with *A. marginale* (AM), *C. ruminantium* (CR), *B. bigemina* (Bbig), or *B. bovis* (Bbov). (c) *A. marginale* (AM) initial body preparations reacted with normal serum from a noninfected horse (NHS) or antisera from animals infected with *A. marginale* (AM), *E. risticii* (ER), *E. equi* (EE), or *E. ewingii* (EC). The labels above each lane indicate the serum sample used (top), the initial body preparation used (center), and the dilution of the serum (bottom). Molecular size standards (in kilodaltons) are given on the left.

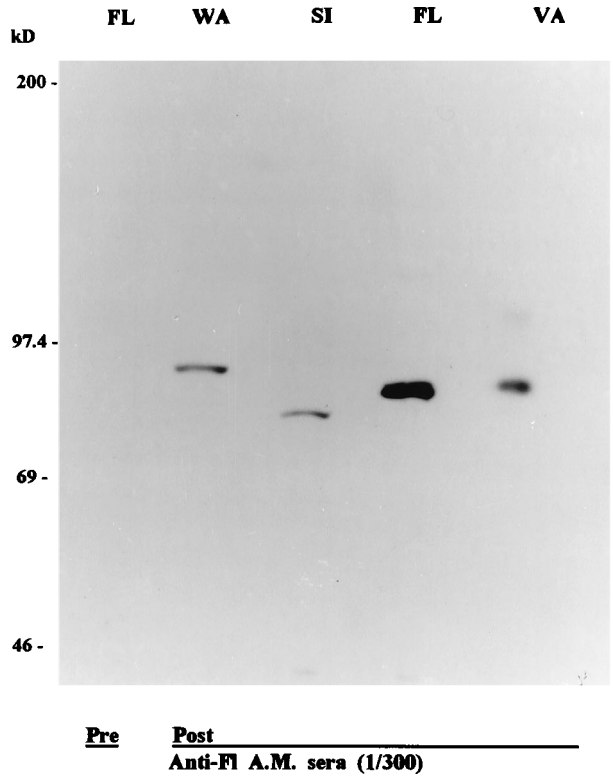

FIG. 2. Size polymorphism of MSP3 by using immunoblots of initial body preparations from FL, WA, SI, and VA isolates of *A. marginale* (AM) and normal serum from a noninfected cow (lane FL [Pre]) or antisera from a cow infected with an FL isolate of AM (Post; lanes WA, SI, FL, and VA). Serum was diluted to 1/300. Molecular size standards (in kilodaltons) are given on the left.

ously (Fig. 2). The side-by-side locations of the various isolates demonstrate variations in the sizes of the MSP3 proteins. The 86-kDa MSP3 antigen is seen in the FL isolate (Fig. 2). An antigen of similar size is seen when this same antiserum is reacted with proteins from a VA isolate. However, in reactions with the SI isolate, an antigen with a slightly smaller molecular mass is seen, and the WA isolate has an antigen with a molecular mass greater than 86 kDa.

Immune response to MSP3. Realizing that MSP3 is not conserved between isolates from different geographic locations, we then investigated the possibility that animals infected with different isolates may contain immune sera that vary in their reactivities to MSP3 from a single isolate. This is an important consideration in attempting to develop a diagnostic test antigen derived from a single isolate. Initial body preparations from an FL isolate were separated by 2-D gel electrophoresis as described above in order to determine if comigration of antigens with similar molecular sizes occurs. Electrophoretically separated proteins were transferred to nitrocellulose and were probed with antisera from cattle infected with an FL, VA, SI, or WA isolate of *A. marginale* as well as an anti-MSP3 MAb (AMG75C2), rabbit-anti-MSP3 polyclonal sera (25), and an anti-MSP2 MAb (ANF19E2). Preinfection bovine sera, normal rabbit sera, and a MAb to a trypanosome surface protein were used as negative controls and showed no reactivity to MSP3 (data not shown).

In an homologous reaction with anti-FL serum, two major areas of reactivity with a molecular mass of 86 kDa were seen, one with an apparent pI of 6.5 and the other with a pI of approximately 6.2 (Fig. 3). There was slight reactivity with an

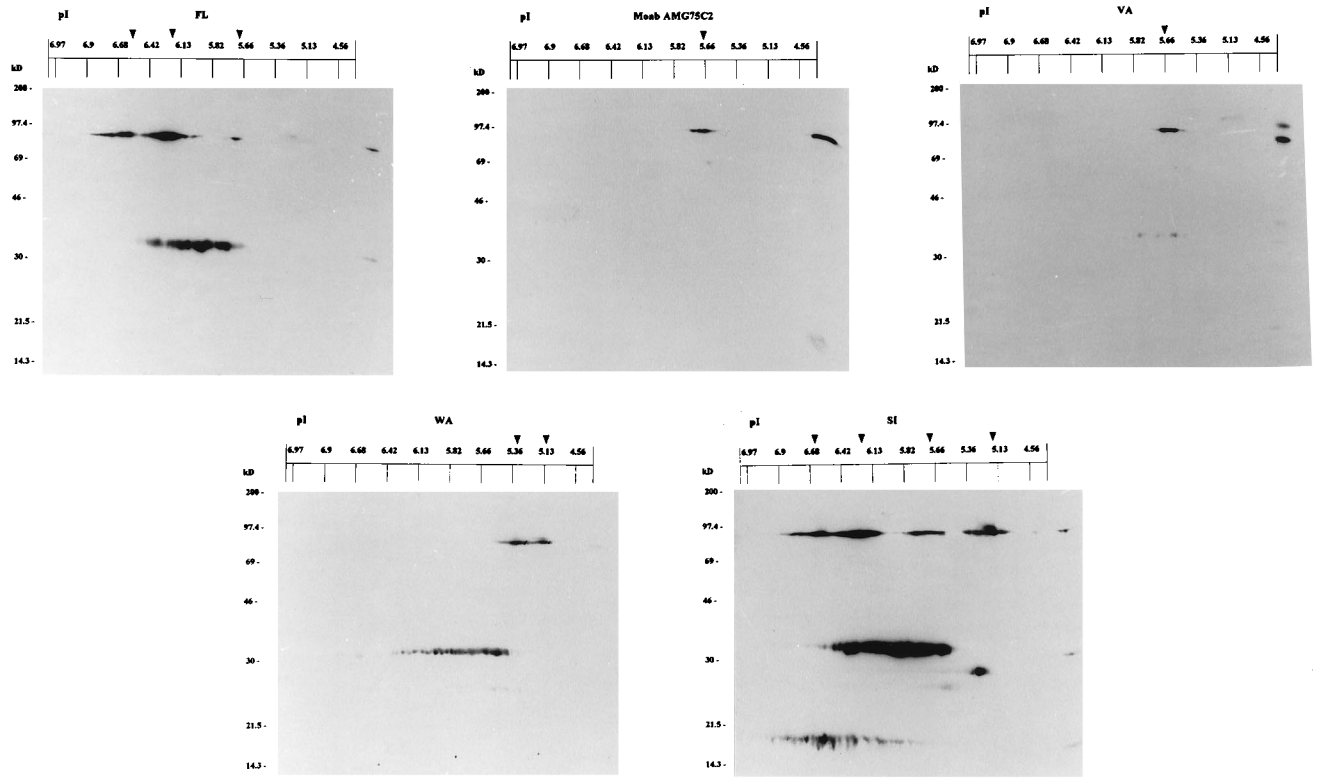

FIG. 3. Immunoblots with an initial body preparation of an FL isolate of *A. marginale* separated by 2-D gel electrophoresis. The letters centered above each immunoblot indicate the antibody used in the reaction. FL, antiserum from a cow infected with an FL isolate of *A. marginale*; VA, antiserum from a cow infected with a VA isolate of *A. marginale*; WA, antiserum from a cow infected with a WA isolate of *A. marginale*; SI, antiserum from a cow infected with an SI isolate of *A. marginale*; MAb AMG75C2, anti-MSP3 MAb. All antisera were used at a dilution of 1/300. The concentration of the MAb was 5 μg/ml. The numbers above each immunoblot indicate the pH taken at 1-cm distances along the length of the tube gel. Arrows indicate the isoelectric point of each 86-kDa band. The far right side of each gel shows an initial body preparation from an FL isolate of *A. marginale* electrophoresed in a single dimension and immunoblotted along with 2-D gel electrophoresis-focused initial bodies to indicate the 86-kDa MSP3. Molecular size standards (in kilodaltons) are given on the left of each immunoblot.

antigen at a pI of approximately 6.5. When the initial body preparation from the FL isolate was reacted with the anti-MSP3 MAb, major reactivity was seen in the area of the pH gradient of 5.6, but no reactivity was noted with antigens at a pI of 6.5 or 6.2 (Fig. 3).

Serum from an animal infected with a VA isolate showed reactivity similar to that of the MAb. However, serum from an animal infected with a WA isolate reacted with two or more antigens in an entirely different area of the pH gradient, having pIs of approximately 5.1 and 5.3 (Fig. 3). When these same initial body preparations were reacted with antiserum from an animal infected with an SI isolate, reactivity was noted in all three areas of the pH gradient, with approximate pIs of 6.5 to 6.2, 5.6, and 5.3 to 5.1 (Fig. 3). When a rabbit-anti-MSP3 polyclonal serum sample was used, reactivity was noted in areas of the pH gradient with pIs of 6.5 to 6.2 and 5.6 (Fig. 4).

Although the conditions seen here were not optimized to separate the 36-kDa proteins of *A. marginale* (MSP2), multiple spots were visualized at that apparent molecular size (Fig. 3). These spots were confirmed to be the MSP2 antigen by use of an anti-MSP2 MAb (ANF19E2), the reactivity of which has been described previously (26, 31, 32). Multiple spots with a molecular mass of 36 kDa were recognized by this MAb (Fig. 4). Some variations in the reactivities of the different antisera to MSP2 were seen. For example, in a homologous reaction there was reactivity with antigens with pIs of from 6.42 to 5.66. However, when the antisera were reacted with sera from animals infected with WA or SI isolates, reactivity with antigens with pIs of 6.42 to 5.36 was observed.

DISCUSSION

The current serological tests available for the diagnosis of *A. marginale* infection are based on antigens which are a crude mixture of *A. marginale* and erythrocyte proteins. This reduces both the sensitivities and specificities of various tests to unacceptable levels, particularly in detecting carrier cattle (11, 22, 23, 43). Because of this and because the erythrocyte stages of *A. marginale* have not been successfully maintained in erythrocyte culture, molecular techniques have been used to identify immunodominant proteins with acceptable sensitivities and specificities so that they can be used in recombinant form as purified test antigens. The ideal test antigen would be one which is not cross-reactive with antigens from other related organisms and which could detect acutely infected animals as well as carrier cattle infected with any of several strains of *A. marginale* from different geographic locations.

One antigen identified as immunodominant in the acutely infected animal as well as chronically infected carriers is the 86-kDa MSP3 protein from an FL isolate of *A. marginale* (25, 30). The FL isolate of *A. marginale* was used for antigen isolation because it has been found by adsorption studies to contain antigens common to both morphologic types of *A. marginale*, the tailed and the nontailed forms (12, 19). Experiments

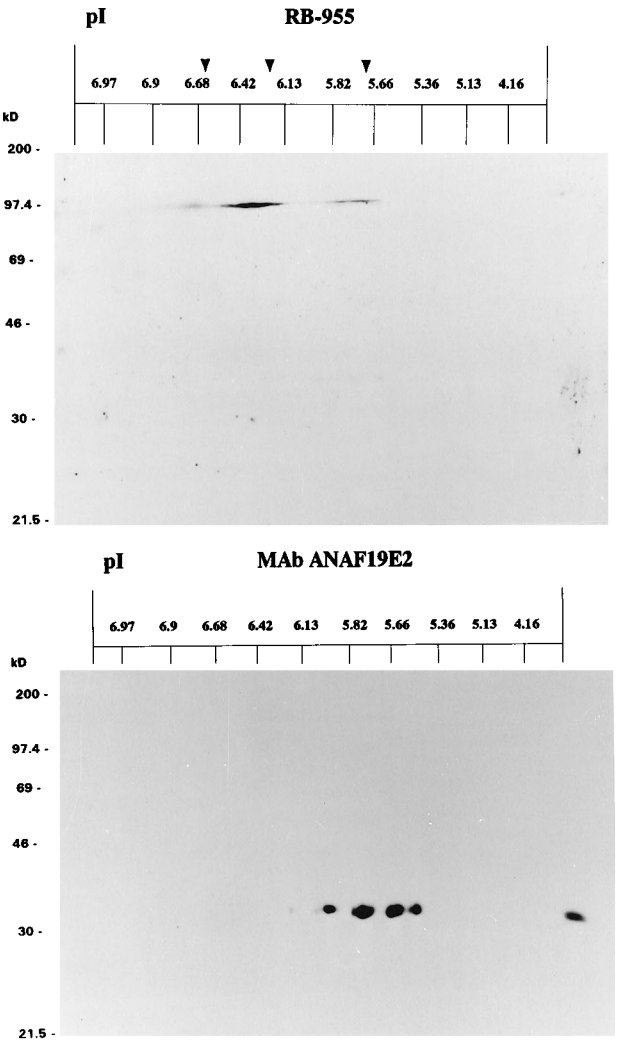

FIG. 4. Immunoblots with an initial body preparation of an FL isolate of *A. marginale* separated by 2-D gel electrophoresis. The letters centered above each immunoblot indicate the antibody used in the reaction. RB-955, rabbit anti-MSP3 polyclonal sera; MAb ANAF19E2, anti-MSP2 MAb. Rabbit anti-MSP3 was used at a dilution of 1/5,000. The concentration of the MAb was 5 μg/ml. The numbers above each immunoblot indicate the pH taken at 1-cm distances along the length of the tube gel. Arrows indicate the isoelectric point of each 86-kDa band. The far right side of each gel shows an initial body preparation from an FL isolate of *A. marginale* electrophoresed in a single dimension and immunoblotted along with 2-D gel electrophoresis-focused initial bodies to indicate the 86-kDa MSP3 and 36-kDa MSP2. Molecular size standards (in kilodaltons) are given on the left of each immunoblot.

with affinity-purified MSP3 showed excellent sensitivities; however, attempts to produce a recombinant form of MSP3 have resulted in inconsistent responses to immune sera from cattle (34). In addition, the specificity of this protein in detecting *A. marginale*-infected cattle needs to be fully defined. Previous experiments have determined that there is no cross-reactivity between MSP3 when it is reacted with sera from animals infected with *B. bovis*, *B. bigemina*, or an unidentified rickettsial agent isolated from an aborted calf fetus (25). However, by 16S rRNA sequencing analysis, *A. marginale* has recently been shown to be more closely related phylogenetically to *C. ruminantium* and the *Ehrlichia* spp., particularly *E. risticii*, *E. equi*, and *E. canis* (7, 45).

Our results confirm that cross-reactive antigens do exist between *Ehrlichia* spp., *C. ruminantium*, and *A. marginale*. The *Ehrlichia* spp. tested do not infect cattle; however, these results illustrate the potential for cross-reactivity between *A. marginale* and *Ehrlichia bovis*, particularly since cross-reactive antigens exist among many *Ehrlichia* spp. (15, 38, 44). This is a potential problem for serologic testing of cattle in areas where *A. marginale* and *E. bovis* coexist.

Antigenic similarities between *Anaplasma* spp., particularly *A. marginale* and *A. centrale*, have been identified (31, 40, 41). However, in the present study, *A. centrale* antiserum failed to react with *A. marginale* antigens (Fig. 1a). In previous experiments, strong reactivity was seen with the 36-kDa protein (MSP2) of an isolate of *A. marginale* from Israel when it was reacted with immune sera from a cow infected with *A. centrale* (41). In addition, epitopes common to both species were identified by using MAbs against the 36- and 105-kDa surface proteins of *A. marginale* (34). The discrepancy between the results of the present study and previously reported data may be explained by differences in the *A. marginale* isolates from different geographic locations that were used. Antigenic differences between different isolates of *A. marginale* have been well documented (4, 26).

In contrast, serum from sheep infected with *Anaplasma ovis* showed a strong positive reaction with MSP3 of *A. marginale* as well as the 36-kDa MSP2. In previous experiments, a panel of 18 MAbs reactive to various *A. marginale* proteins failed to react with *A. ovis*-infected erythrocytes (26). The difference in results may be explained by the limited number of epitopes recognized by these MAbs.

Size polymorphism was demonstrated in the MSP3 proteins in three of the four isolates from different geographic locations studied. Marked size polymorphism has been recognized in other major surface antigens of *A. marginale*, the MSP1α protein (29) and the MSP2 protein (35). Size variation of MSP1α among isolates from different geographic locations was explained by various numbers of tandem repeats within a single domain (1). Despite marked size variation, many of the surface epitopes remained conserved between strains. The MSP2 protein was found to be encoded by a multigene family, suggesting a potential mechanism for size polymorphism (32). The presence of variable numbers of tandem repeats or a multigene family are possible mechanisms of size variation of the MSP3 protein.

The present study demonstrates variations in the reactivities of immune sera from cattle infected with isolates from different geographic locations when they are reacted with the FL MSP3. Multiple 86-kDa antigens were seen by using immunoblots of preparations separated by 2-D electrophoresis (Fig. 3). The reactivities of antisera with these antigens varied depending on the geographic location of the isolate with which the cattle were infected. These results indicate that not only size polymorphism but also antigenic polymorphism exist between MSP3 antigens from different isolates. This illustrates the ability of the organism to antigenically alter this immunogenic surface protein, possibly in response to immune pressures from the host.

There are at least three possible explanations for the multiple 86-kDa antigens present in the FL isolate of *A. marginale*. These antigens may arise from (i) posttranslational modification of a protein transcribed from single-copy gene, (ii) several closely related genes from a multigene family, or (iii) entirely unrelated genes. The reactivity of the rabbit-anti-MSP3 serum with multiple 86 kDa antigens indicates that at least three of the MSP3 antigens share common epitopes, since this serum was made by injecting an MSP3 antigen which was affinity purified with MAb AMG75C2 (25). This would indicate these

antigens are likely the result of either posttranslational modification of a protein from a single-copy gene or transcription and translation of several closely related genes from a multigene family.

As mentioned above, studies have shown multigene families encoding major surface proteins of *A. marginale*. Between 7 and 10 similar gene copies encode the MSP2 gene of *A. marginale* (32). MSP2 is a 36-kDa protein, and our results obtained by 2-D gel electrophoresis and immunoblotting with an anti-MSP2 MAb illustrate multiple MSP2 antigens. These results support the findings for the MSP2 multigene family. In addition, the MSP1β subunit of the MSP1 gene of *A. marginale* also is encoded by a partially homologous multigene family (46). The genes in this family were shown to differ from each other by extensive deletions, insertions, and switching of sequences (46). Those previous experiments, along with the data presented in this report, demonstrate the ability of the organism to use multigene families to vary important immunogenic surface proteins. It has been proposed that this antigenic variation may play a role in the cyclic rickettsemia and persistent infection recognized in carrier cattle infected with *A. marginale* (16).

Work is being done in our laboratory to determine if a multigene family is responsible for the antigenic variation of MSP3. The results of these studies may be useful for studying antigenic variation in *A. marginale* and determining if antigenic variation of MSP2 or MSP3 occurs during rickettsemia cycles in persistent carriers. However, the cross-reactivity of this protein with sera from animals infected with *Ehrlichia* spp., the size polymorphism of MSP3 between isolates from different geographic locations, and the multiple 86-kDa antigens recognized by various antisera indicate that MSP3 is not a suitable candidate for use as a recombinant test antigen.

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