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Conformational Dependence of *Anaplasma marginale* Major Surface Protein 5 Surface-Exposed B-Cell Epitopes†

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The *Anaplasma marginale* outer membrane is composed of immunogenic major surface proteins (MSPs) linked both covalently and noncovalently in multimeric complexes (M. C. Vidotto, T. C. McGuire, T. F. McElwain, G. H. Palmer, and D. P. Knowles, Infect. Immun. 62:2940–2946). Consequently, effective induction of antibody against surface-exposed MSP epitopes has been postulated to require maintenance of MSP secondary through quatenary structures. Using MSP5 as a model and the approach of epitope mapping with recombinant expressed full-length and truncated proteins, we demonstrated that the immunodominant surface epitope bound by monoclonal antibody (MAb) ANAF16C1 required disparate amino- and carboxy-terminal regions of MSP5, indicating the conformational dependence of this epitope. The required amino-terminal MSP5 region included the cysteines involved in intramolecular disulfide bonding. The dependence of the immunodominant epitope on disulfide bonding was confirmed by loss of MAb ANAF16C1 binding to MSP5 following disulfide bond reduction and covalent modification of the reduced sulfhydryl groups. The recognition of the MSP5 immunodominant epitope by antibody induced by protective immunization with *A. marginale* outer membranes was also conformationally dependent, as shown by the loss of epitope binding following serum adsorption with native but not reduced and denatured *A. marginale*. Importantly, the antibody response to all immunodominant MSP5 surface epitopes was restricted to conformationally dependent epitopes, since the binding of polyclonal anti-MSP5 antibody to the *A. marginale* surface could be blocked by adsorption with native but not denatured and reduced MSP5. These results confirm the importance of the secondary and tertiary structures of MSP epitopes as immune system targets and support the testing of immunogens which maintain the required conformation.

Anaplasma marginale is an arthropod-borne ehrlichial pathogen of cattle that invades and replicates in mature erythrocytes (7). Acute infection is characterized by high levels of rickettsemia ($>10^9$ infected erythrocytes/ml) and severe anemia, which frequently results in abortion or death (5, 7). Immunity against acute *A. marginale* rickettsemia is directed against outer membrane surface proteins, and infectivity can be neutralized with antibodies against surface exposed epitopes (18, 20, 21). Correspondingly, cattle immunized with *A. marginale* outer membranes develop significantly lower rickettsemia following challenge than do adjuvant-immunized controls (20, 22, 27). Sera from these immunized and protected cattle recognize six major surface proteins (MSPs), and antibody titers against MSP2 and MSP5 correlate with protection against challenge with the homologous strain (20, 22, 27). In contrast to protection induced by immunization with whole outer membranes or a native MSP1a/MSP1b complex, isolated recombinant-expressed MSPs, either alone or in combination, fail to induce comparable protection against rickettsemia (17, 18, 20, 27). Consequently, we have hypothesized that MSP conformation, as determined by secondary through quatenary structures, is a critical determinant in the efficacy of experimental vaccines (13, 20, 30).

The outer membrane is composed of MSPs linked both covalently and noncovalently in multimeric complexes (30). MSP5 and MSP2 occur in both monomeric intramolecularly disulfide-bonded and multimeric intermolecularly disulfide-bonded forms in the membrane: MSP5 as a dimer and MSP2 as a tetramer (19, 30, 31). Importantly, both MSP5 and MSP2 bear immunodominant B-cell epitopes and, in outer membrane-immunized cattle, the antibody titer correlates with protection against challenge with the homologous *A. marginale* strain (19, 27, 31). Based on our hypothesis, we would predict that the MSP2 and MSP5 immunodominant surface-exposed epitopes are conformationally dependent and require disulfide bonding to maintain epitope conformation. We chose to first test this prediction with intramolecularly disulfide bonded MSP5. MSP5, in contrast to the antigenically variable MSP2 (2, 19), is encoded by a single highly conserved gene and expresses invariant surface epitopes recognized by outer membrane-immunized as well as previously infected immune cattle (1, 6, 14). In this paper, we report the disulfide bond and conformational requirements of defined MSP5 surface-exposed epitopes and the results of testing whether antibody binding to the *A. marginale* surface requires maintenance of secondary and tertiary structures.

MATERIALS AND METHODS

Physical mapping. ANAF16C1 is an immunoglobulin G1 (IgG1) monoclonal antibody (MAb) directed against the *A. marginale* surface and binds MSP5 in all strains of *A. marginale*, *A. ovis*, and *A. centrale* tested (1, 6, 12, 14). *Escherichia coli* transformed with plasmid pAM104A expresses a full-length MSP5 polypeptide that is bound by MAb ANAF16C1 (31). Full-length and truncated *msp5* clones expressed as fusion partners with maltose binding protein (MBP) were used to identify the MSP5 region bound by MAb ANAF16C1. Briefly, the entire

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† This paper is dedicated to the memory of Devere Munodzana and to his family.

‡ Deceased.

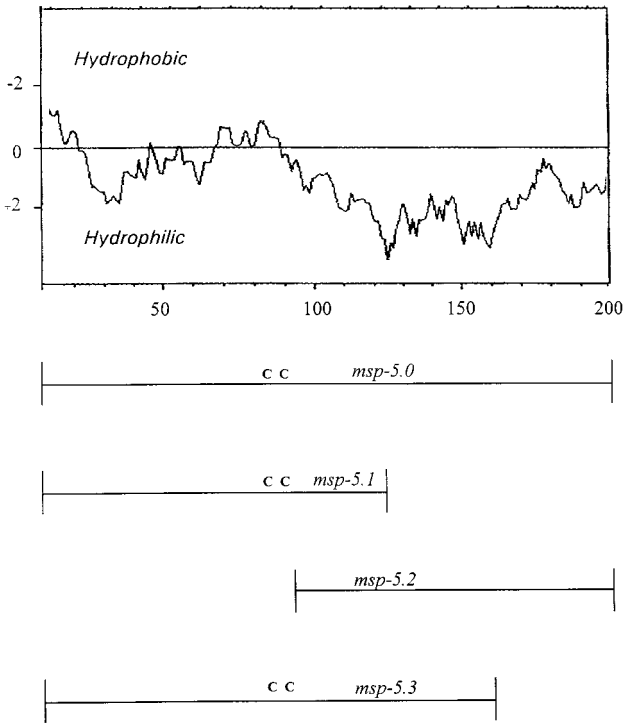

FIG. 1. Physical map of the recombinant MSP5 proteins relative to a transmembrane helical protein hydrophobicity-hydrophilicity profile of native MSP5. The map was generated with the Genetics Computer Group package from the University of Wisconsin. The *y* axis reflects the Goldman-Engelman-Steitz hydrophobicity scale over a window of 20 residues, and the *x* axis represents the amino acid position in MSP5. The proteins expressed by full-length (*msp-5.0*) or truncated (*msp-5.1*, *msp-5.2*, and *msp-5.3*) recombinant clones are plotted against the same *x* axis, and the positions of the two cysteines are indicated by the letter C.

msp5 open reading frame (nucleotides 118 to 753 based on the numbering of the original clone in pAM104A [31]) was amplified with forward and reverse primers incorporating *Xba*I recognition sites, digested, and ligated in frame into the *Xba*I site of the vector pMal-c2 (24). The plasmid encoding the full-length MSP5-MBP fusion was designated *msp5.0*, and the expressed protein was designated MSP5.0. The following truncated *msp5* clones were generated by the same strategy with site-specific forward and reverse primers: *msp5.1*, a 371-bp clone representing bp 118 to 488; *msp5.2*, a 356-bp clone representing bp 390 to 745; and *msp5.3*, a 483-bp clone representing bp 118 to 600. The sequences of all clones were verified by double-strand sequencing by primer extension with dideoxy chain termination (25). *E. coli* XL-1 Blue was transformed with each plasmid, and the expression of an MSP5-MBP fusion protein of the appropriate size was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of recombinant *E. coli* lysate and immunoblotting with detection by rabbit anti-MBP polyclonal antibody (3). The orientations of the full-length and truncated *msp5* constructs and the encoded proteins relative to the predicted conformation of native MSP5 are shown in Fig. 1. Each MSP5-MBP fusion protein was purified on individual amylose affinity columns following extraction as soluble proteins from recombinant *E. coli* (24). Briefly, 2×10^8 bacteria per ml of rich medium (1% tryptone, 0.5% yeast extract, 0.5% NaCl, 0.2% glucose), containing 100 μg of ampicillin per ml, was incubated in the same medium with the addition of 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 h at 37°C to induce fusion protein expression. Bacteria were disrupted by freezing and rapid thawing followed by sonication. The recombinant expressed proteins were collected in the supernatant and loaded on amylose columns with a binding capacity of 3 mg of MBP per ml of resin. The columns were washed and the recombinant fusion proteins were eluted as previously described (24). Eluted recombinant proteins were detected by immunoblotting with rabbit anti-MBP polyclonal antibody and then tested for reactivity with MAb ANAF16C1 by SDS-PAGE and immunoblotting (3). Antibody binding was detected by using horseradish peroxidase-labeled goat anti-rabbit IgG (for anti-MBP antibody) or goat anti-murine IgG (for MAbs) and enhanced chemiluminescence (3). Purified nonfusion MBP, and unrelated MBP fusion protein (MBP–*Babesia bovis* RAP-1 [26]), and uninfected erythrocytes were used as negative control antigens. *A. marginale*-infected erythrocytes and *E. coli* transformed with plasmid pAM104A were used as positive antigen controls (31). Normal rabbit serum and the IgG1 MAb Tryp1E1 were used as negative antibody controls.

Conformational sensitivity of MAb ANAF16C1 binding. Affinity-purified MSP5.0 was incubated, at 10 μg per treatment (in duplicate), with either 8 M urea, 60 mM dithiothreitol (DTT), or 300 mM iodoacetamide (IA), or one of the combinations DTT and IA; urea and IA; or urea, DTT, and IA. The urea and DTT treatments were performed at 56°C for 12 h, and the IA treatment was performed for 1 h at 25°C (4). An untreated sample was incubated identically and used as a positive control. Reactivity was determined by immunoblotting (3) with MAb ANAF16C1 or the negative control MAb Tryp1E1.

Cattle previously immunized with purified *A. marginale* outer membranes developed high titers of anti-MSP5 antibody and were shown to be protected against acute rickettsemia upon challenge (27, 31). Serum obtained postimmunization but prechallenge was adsorbed with either denatured and reduced (8 M urea, 60 mM DTT, 300 mM IA) or untreated, native Norton strain organisms (28). As controls, serum either was left unadsorbed or was adsorbed by the identical method with either denatured and reduced *E. coli* or untreated *E. coli*. Adsorption, performed at 25°C for 1 h, was repeated until there was no reactivity with the adsorbing antigen preparation as determined by immunoblotting. Each serum treatment was then tested for inhibition of MAb ANAF16C1 binding to recombinant MSP5.0 by a competitive inhibition enzyme-linked immunosorbent assay (ELISA) as described previously (1, 6). Briefly, individual wells in 96-well plates were coated with 1 μg of amylose-resin-purified MSP5.0 fusion protein in 100 μl of carbonate-bicarbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$ [pH 9.6]). The wells were incubated for 1 h at room temperature with 200 μl of blocking buffer (250 mM K_2HPO_4, 250 mM KH_2PO_4, 0.5% fraction V bovine serum albumin, 0.75% glycine, 1% sucrose) and then washed four times with phosphate-buffered saline (PBS; pH 7.2). The adsorbed and unadsorbed test sera were diluted in PBS–1% BSA, to a final dilution of 1:40,000, the dilution of unadsorbed serum that resulted in approximately 70% inhibition of MAb ANAF16C1 binding to MSP5.0. Adsorbed, diluted sera were added to triplicate wells in 100-μl aliquots, and the wells were incubated at room temperature for 1 h. The wells were washed four times with 200 μl of PBS per well and then incubated for 15 min at room temperature with horseradish peroxidase-conjugated MAb ANAF16C1 as described previously (1, 6). After four additional washes with PBS, 50 μl of 0.5-μg/μl *o*-phenylenediamine hydrochloride dihydrochloride in substrate buffer (0.2 M Na_2HPO_4, 0.1 M citric acid) was added to each well. The plates were incubated for 10 min, and the reactions were terminated with 25 μl of 2 N H_2SO_4. The results were expressed as percent inhibition (and standard deviation) of MAb ANAF16C1 binding to MSP5.0 (1, 6).

Conformational dependence of antibody binding to *A. marginale* MSP5 surface exposed epitopes. Calves were obtained at 1 day of age and raised in a tick- and fly-free facility at the Central Veterinary Laboratory, Harare, Zimbabwe. Before immunization, sera were shown to be unreactive with *A. marginale* by immunoblotting against whole-organism lysate (11) and by the competitive inhibition MSP-5.0 ELISA (1, 6). Five calves were immunized by subcutaneous inoculation of 50 μg of native MSP5, purified from *A. marginale* on a MAb ANAF16C1 affinity column as described previously (31), in saponin adjuvant. The immunization was repeated three times at 3- to 4-week intervals. Five adjuvant control calves were given saponin alone by using the identical schedule and route of inoculation. Sera were obtained 1 month after the last inoculation, and the anti-MSP5 titer was determined by the competitive inhibition ELISA. As described in Results, all sera from MSP5-immunized calves had high titers of anti-MSP5 antibody. Two of these sera were then adsorbed with amylose resin-purified MSP5.0 or denatured and reduced (8 M urea, 60 mM DTT, 300 mM IA) purified MSP5.0. As controls, these sera either were left unadsorbed or were adsorbed, by using the identical protocol, with either denatured and reduced MBP or untreated MBP. Adsorptions, performed at 25°C for 1 h, were repeated until there was no reactivity with the adsorbing antigen preparation as determined by immunoblotting. Unadsorbed and adsorbed sera were then tested for binding to native surface exposed MSP5 epitopes by agglutination of purified *A. marginale* as previously described (19).

RESULTS

Physical mapping. The physical maps of the full-length and truncated MSP5-MBP fusion proteins expressed in pMal-c2 are shown in Fig. 1. Each fusion protein was purified on individual amylose affinity columns and identified by SDS-PAGE and immunoblotting with detection by rabbit polyclonal antibody specific for the MBP fusion partner. Figure 2 shows the binding of anti-MBP antibody to the expected 65-kDa MSP5.0 fusion protein (lane 1) and to MBP alone (lane 2). The anti-MBP antibody also reacted with the truncated fusion proteins MSP5.1, MSP5.2, and MSP5.3 (data not shown) but not with purified *A. marginale* (lane 3). There was no binding of control normal rabbit sera to any of the MSP5-MBP fusion proteins

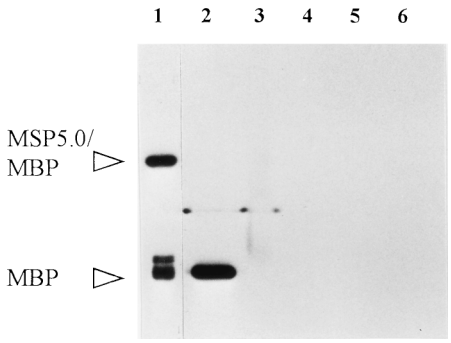

FIG. 2. Expression of recombinant MSP5.0. Lanes 1 and 4 contain MSP5.0 fused to MBP and purified on an amylose affinity column; lanes 2 and 5 contain MBP alone; and lanes 3 and 6 contain a lysate of *A. marginale*-infected erythrocytes. Lanes 1 to 3 were reacted with rabbit antiserum against MBP; lanes 4 to 6 were reacted with the same dilution of normal rabbit serum. The positions of the MSP5.0-MBP fusion protein and MBP alone are indicated in the left margin.

(MSP5.0 is shown in lane 4), MBP (lane 5), or *A. marginale* (lane 6).

Each recombinant MSP5 fusion protein was then tested for reactivity with MAb ANAF16C1 or the Tryp1E1-negative control MAb by immunoblotting. MSP5.0 was bound by MAb ANAF16C1 (Fig. 3, lanes 2 and 3) but not by an isotype control MAb, Tryp1E1 (lanes 6 and 7). This indicates that the presence of the MBP fusion partner does not alter recognition of the MSP5 epitope by MAb ANAF16C1. This MAb also bound *A. marginale* native MSP5 (lane 4). MAb ANAF16C1 did not react with the negative control *B. bovis* RAP-1–MBP fusion protein (lane 1). Of the truncated fusion proteins, only MSP5.3 was bound by MAb ANAF16C1 (Fig. 4, lane 4). ANAF16C1 did not bind MSP5.1 (lane 2), MSP5.2 (lane 3), or the negative control *B. bovis* RAP-1–MBP fusion protein (lane 1). This reactivity indicates that not only is the amino-terminal region (nucleotides 118 to 390, encoding the first 91 amino acids including the conserved cysteine residues) necessary for ANAF16C1 binding but that also some or all of the region composed of amino acids 125 to 161 (encoded by nucleotides 492 to 600) is also required. These data, without further mapping, are consistent with conformational dependence of the immunodominant epitope bound by MAb ANAF16C1. Nonfusion MSP5 expressed by *E. coli* containing plasmid p104A was used as a positive

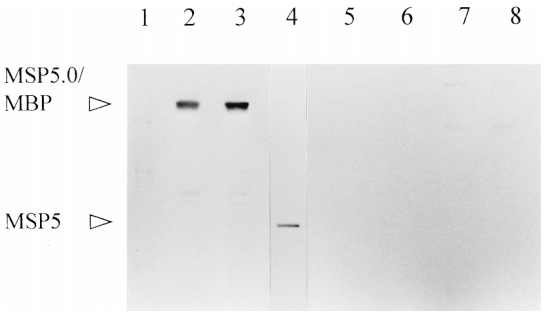

FIG. 3. MAb ANAF16C1 binds recombinant MSP5.0. Lanes 1 and 5 contain *B. bovis* RAP-1–MBP fusion protein as a negative antigen control; lanes 2, 3, 6, and 7 contain MSP5.0-MBP fusion protein (lanes 2 and 6 contain protein from a different column fraction from the protein in lanes 3 and 7); lanes 4 and 8 contain a lysate of *A. marginale*-infected erythrocytes. Lanes 1 to 4 were reacted with MAb ANAF16C1; lanes 5 to 8 were reacted with the isotype control MAb Tryp1E1. The positions of the MSP5.0-MBP fusion protein and the native MSP5 are indicated in the left margin.

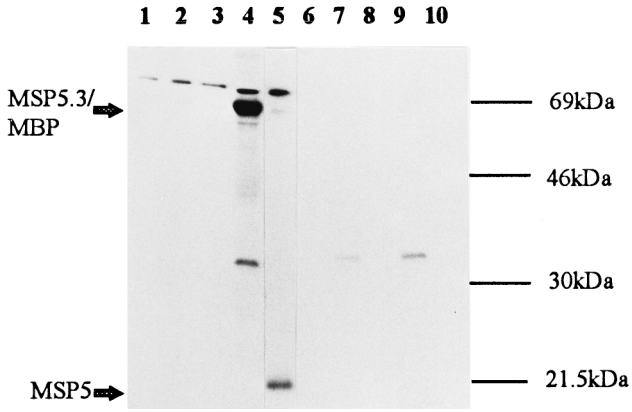

FIG. 4. MAb ANAF16C1 binds recombinant MSP5.3 but not MSP5.1 or MSP5.2. Lanes 1 and 6 contain *B. bovis* RAP-1–MBP fusion protein as a negative antigen control; lanes 2 and 7 contain MSP5.1-MBP fusion protein; lanes 3 and 8 contain MSP5.2-MBP fusion protein; lanes 4 and 9 contain MSP5.3-MBP fusion protein; and lanes 5 and 10 contain nonfusion MSP5 expressed by *E. coli* containing plasmid p104A (31). Lanes 1 to 5 were reacted with MAb ANAF16C1; lanes 6 to 10 were reacted with the isotype control MAb Tryp1E1. The positions of the MSP5.3-MBP fusion protein and nonfusion MSP5 are indicated in the left margin.

control and was bound, as expected, by MAb ANAF16C1 (Fig. 4, lane 5).

Conformational sensitivity of MAb ANAF16C1 binding. The conformational dependence of MSP5 was tested by treatment of purified MSP5.0 with denaturing and reducing agents followed by determination of MAb ANAF16C1 binding. Reduction of disulfide bonds with DTT followed by covalent modification of sulfhydryl groups with IA to prevent reoxidation completely abolished MAb binding (Fig. 5). This effect was probably due to the effect on disulfide bonding, since neither DTT nor IA alone had any detectable effect on the epitope (Fig. 5). This dependence on disulfide bonding is consistent with the epitope-mapping results, which showed a requirement for the amino-terminal half of MSP5, containing the conserved

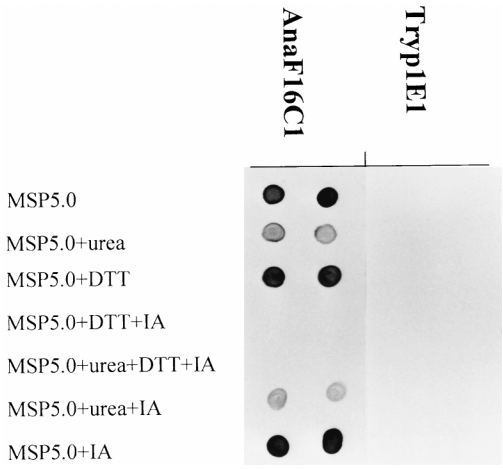

FIG. 5. MAb ANAF16C1 binding is sensitive to denaturation and reduction of MSP5. Purified MSP5.0 was either untreated or treated with 8 M urea (MSP5.0+urea); 60 mM dithiothreitol (MSP5.0+DTT); DTT and 300 mM IA (MSP5.0+DTT+IA); urea, DTT, and IA (MSP5.0+urea+DTT+IA); urea and IA (MSP5.0+urea+IA); or IA alone (MSP5.0+IA). Samples were reacted in duplicate with either MAb ANAF16C1 (left) or the negative control MAb Tryp1E1 (right).

TABLE 1. Binding of antibody induced by outer membrane immunization to the MSP5 immunodominant epitope is dependent on native conformation of *A. marginale*

Treatment of anti-outer membrane serum	% Inhibition of MAb ANAF16C1 binding[a]
Unadsorbed	70 ± 5
Adsorbed with denatured *E. coli* lysate	60 ± 11
Adsorbed with native *E. coli* lysate	63 ± 8
Adsorbed with denatured *A. marginale* lysate	56 ± 13
Adsorbed with native *A. marginale* lysate	20 ± 10

[a] Negative control serum from a nonimmunized, uninfected calf gave a background inhibition of 12 ± 3%.

cysteine residues. Treatment with 8 M urea, which denatures the protein secondary structure, resulted in a partial loss of MAb ANAF16C1 binding (Fig. 5). This effect is again consistent with a conformationally dependent epitope and may involve both the amino- and carboxy-terminal hydrophilic regions.

To test whether recognition of the MSP5 immunodominant epitope by antibody from outer membrane-immunized and protected cattle was also conformationally dependent, serum was adsorbed with native or reduced and denatured *A. marginale* lysate and then tested for the ability to inhibit MAb ANAF16C1 binding. Unadsorbed serum was diluted (1:40,000) to achieve 70% inhibition of MAb ANAF16C1 binding (Table 1). All test samples following adsorption were then tested at a final dilution of 1:40,000. As shown in Table 1, adsorption with native *A. marginale* significantly depleted bovine serum antibody inhibition of MAb ANAF16C1 binding. In contrast, adsorption with native *E. coli* or reduced and denatured *A. marginale* or *E. coli* did not significantly reduce the binding of the immune bovine serum to the MSP5 immunodominant epitope (Table 1). This indicates that the antibody response to this MSP5 epitope following effective outer membrane immunization is conformationally restricted.

Conformational dependence of antibody binding to *A. marginale* MSP5 surface-exposed epitopes. Immunization of cattle with native MSP5 induced high titers of antibody against the immunodominant MSP5 epitope, as determined by the competitive inhibition ELISA based on ANAF16C1 binding (data not shown). Sera from two of the MSP5-immunized cattle were then used to determine if recognition of MSP5 epitopes on the *A. marginale* surface was conformationally dependent. Unadsorbed sera had end-point agglutination titers of 512, while adsorption with native MSP5.0 diminished surface binding 32- and 64-fold, respectively, for each of the two test sera (Table 2). In contrast, adsorption with denatured and reduced MSP5.0 lysate either did not alter (anti-MSP5.0 serum 1) or only slightly diminished (anti-MSP5.0 serum 2) surface reactivity compared to negative control adsorptions with either native or reduced and denatured MBP (Table 2). Sera from the five cattle immunized with saponin alone had end-point agglutination titers of 4 or less (data not shown). These results indicate that the antibody response to MSP5, as presented on the *A. marginale* surface, is predominantly against conformationally dependent epitopes.

DISCUSSION

Why individual MSPs fail to induce protection at a level comparable to that induced by immunization with intact *A. marginale* outer membranes is unknown and represents an important gap in our knowledge needed to develop and improve vaccines against ehrlichial pathogens. Possible explanations, which are not mutually exclusive, include the following: (i) each MSP alone induces partially protective immunity, and the efficacy of the outer membrane complex simply reflects the sum of the individual components; (ii) the multimeric outer membrane complex enhances antigen presentation compared to soluble individual MSPs and generates a phenotypically different immune response; and (iii) induction of protection requires antibody to conformationally dependent epitopes on the *A. marginale* surface. The first possibility is not congruent with data showing that combinations of up to three MSPs do not consistently enhance protection compared to that afforded by immunization with individual MSPs (17, 18, 20, 23). In contrast, both the second and third explanations remain viable. As an entrée to investigating the importance of antibody against conformationally dependent epitopes, we analyzed the structural requirements of a highly conserved immunodominant epitope on MSP5. This epitope, defined by binding of MAb ANAF16C1, is conserved among all tested strains of *A. marginale*, *A. centrale*, and *A. ovis* and induces high titers of antibody in all infected species including cattle, sheep, and goats (1, 6, 14, 31). Initial physical mapping of the epitope with full-length and truncated recombinant expressed MSP5 indicated that residues encoded 5′ to nucleotide 390 (amino acid 91) as well as some or all of the region encoded by nucleotides 492 to 600 (amino acids 125 to 161) were required. Importantly, the required amino-terminal region included the conserved cysteines (31), consistent with the proposed importance of intramolecular disulfide bonding in the MSP conformation (30). The absolute dependence of the immunodominant epitope on disulfide bonding was confirmed by the loss of MAb ANAF16C1 binding to MSP5.0 following disulfide bond reduction and covalent modification of the reduced sulfhydryl groups. Interestingly, MAb binding was also reduced after urea treatment alone (Fig. 5). This suggests that secondary protein structure, apart from the tertiary requirements for intramolecular disulfide bonding, is also needed for epitope conformation, a finding consistent with the physical mapping results indicating contributions from two distant hydrophilic regions of MSP5. Whether amino acids in these disparate regions are juxtaposed to form the actual epitope (defined by binding to the complementarity determining regions of the antibody) or whether the epitope is encoded within one of the regions and the second is required only to provide correct secondary structure for binding is unknown. Both scenarios are consistent with the requirement for disulfide bonding in or adjacent to a hydrophobic segment interposed between two hydrophilic and presumed surface-exposed regions of MSP5 (Fig. 1).

The single MSP5 epitope defined by MAb ANAF16C1 binding was analyzed as a model for immunodominant MSP epitopes (11, 20, 30). The presence of conserved cysteines

TABLE 2. Binding of anti-MSP5 sera to the *A. marginale* surface requires reactivity with native, nondenatured epitopes

Treatment of anti-MSP5 serum	End-point agglutination titer for:	
	Serum 1	Serum 2
Unadsorbed	512	512
Adsorbed with denatured MBP	256	512
Adsorbed with native MBP	256	256
Adsorbed with denatured MSP5.0	256	128
Adsorbed with native MSP5.0	16	8

and disulfide bonds in MSP2 and MSP4 (15, 19, 30) suggests that conformational dependence may be a common feature among *A. marginale* MSPs. In addition, the presence of an MSP5 homolog in *Cowdria ruminantium* MAP-2 (8) and of MSP2 homologs in *C. ruminantium* MAP1 (19, 29) and *Ehrlichia chaffeenesis* OMP-1 (16) provides support for broad applicability of this model among ehrlichial pathogens.

Importantly, antibodies induced by outer membrane immunization, which results in high anti-MSP5 antibody titers that correlate with protection against homologous challenge (27), recognized the MSP5 immunodominant epitope in a conformationally dependent form, as shown by the results in Table 1. Furthermore, the polyclonal antibody induced by native MSP5 immunization also recognized predominantly conformationally dependent epitopes on the *A. marginale* surface. This indicates that the surface binding of antibody to all MSP5 immunodominant epitopes is conformationally dependent and is consistent with a requirement for native-protein secondary and tertiary structures in effective immunization.

In contrast to the secondary- and tertiary-structure requirements for MSP5 B-cell epitopes, the role of the quatenary structure remains unclear. Membrane MSP5 and MSP2 occur as both intramolecularly disulfide bonded monomers and intermolecularly disulfide linked multimers. Although monomeric MSPs, including MSP2 and MSP5, induce antibody against the *A. marginale* surface, complete neutralization of infectivity may require antibody directed against functional surface regions composed of two or more MSPs (13, 30). This possibility is suggested by the greater inhibition of *A. marginale* binding to the erythrocyte surface by antibodies generated against native organisms or a complex of MSP1a and MSP1b compared to antibody generated against MSP1a and MSP1b individually (9, 10). The importance of antibody against multiple MSPs is also supported by the complete neutralization of in vivo infectivity by antibody generated against the intact outer membranes (21). Whether intermolecular bonding of MSPs results in different B-cell epitopes from those resulting from intramolecularly bonded MSPs is unknown, although the high degree of conformational dependence shown in the present study suggests that changes in bonding pattern are likely to alter the surface-exposed epitopes. Consequently, defining the structural requirements of critical outer membrane epitopes is a priority and will support the development and testing of vaccines that maintain native MSP structure. These approaches include recombinant MSP immune system-stimulating complexes, expression of multiple recombinant MSPs in the outer membranes of live bacterial vectors, and direct immunization with DNA encoding MSPs.

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REFERENCES

1. **Echaide, S. T., D. P. Knowles, T. C. McGuire, G. H. Palmer, C. E. Suarez, and T. F. McElwain.** 1998. Detection of cattle naturally infected with *Anaplasma marginale* in an endemic region using nested PCR and recombinant MSP5-cELISA. J. Clin. Microbiol. **36:**777–782.
2. **Eid, G., D. M. French, A. Lundgren, A. F. Barbet, T. F. McElwain, and G. H. Palmer.** 1996. Expression of major surface protein 2 antigenic variants during acute *Anaplasma marginale* rickettsemia. Infect. Immun. **64:**836–841.
3. **Gallagher, S., S. E. Winston, S. A. Fuller, and J. G. R. Hurrell.** 1993. Immunoblotting and Immunodetection, p. 10.8.1–10.8.17. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 2. John Wiley & Sons, Inc., New York.
4. **Hotzel, I., W. C. Brown, T. F. McElwain, S. D. Rodriguez, and G. H. Palmer.** 1996. Dimorphic sequences of *rap-1* genes encode B and CD4+ T helper lymphocyte epitopes in the *Babesia bigemina* rhoptry associated protein-1 (RAP-1). Mol. Biochem. Prasitol. **81:**89–99.
5. **Kieser, S. T., I. S. Eriks, and G. H. Palmer.** 1990. Cyclic rickettsemia during persistent *Anaplasma marginale* infection of cattle. Infect. Immun. **58:**1117–1119.
6. **Knowles, D. P., S. Torioni de Echaide, G. H. Palmer, T. C. McGuire, D. Stiller, and T. F. McElwain.** 1996. Antibody against an *Anaplasma marginale* MSP-5 epitope common to tick and erythrocyte stages identifies persistently infected cattle. J. Clin. Microbiol. **34:**2225–2230.
7. **Losos, G. J.** 1986. Anaplasmosis, p. 742–795. *In* G. J. Losos (ed.), Infectious tropical diseases of domestic animals. Longman Press, Harlow, United Kingdom.
8. **Mahan, S. M., T. C. McGuire, S. M. Semu, M. V. Bowie, F. Jongejan, F. R. Rurangirwa, and A. F. Barbet.** 1994. Molecular cloning of a gene encoding the immunogenic 21-kDa protein of *Cowdria ruminantium*. Microbiology **140:**2135–2142.
9. **McGarey, D. J., and D. R. Allred.** 1994. Characterization of hemagglutinating components of the *Anaplasma marginale* initial body surface and identification of possible adhesins. Infect. Immun. **62:**4587–4593.
10. **McGarey, D. J., A. F. Barbet, G. H. Palmer, T. C. McGuire, and D. R. Allred.** 1994. Putative adhesins of *Anaplasma marginale* major surface polypeptides (MSP) 1a and 1b. Infect. Immun. **62:**4594–4601.
11. **McGuire, T. C., W. C. Davis, A. L. Brassfield, T. F. McElwain, and G. H. Palmer.** 1991. Identification of *Anaplasma marginale* long-term carrier cattle by detection of serum antibody to isolated MSP-3. J. Clin. Microbiol. **29:**788–793.
12. **McGuire, T. C., G. H. Palmer, W. L. Goff, M. I. Johnson, and W. C. Davis.** 1984. Common and isolate restricted antigens of *Anaplasma marginale* detected with monoclonal antibodies. Infect. Immun. **45:**697–700.
13. **Musoke, A. J., G. H. Palmer, T. F. McElwain, V. Nene, and D. McKeever.** 1996. Prospects for subunit vaccines against tick-borne diseases. Br. Vet. J. **152:**621–639.
14. **Ndung'u, L. W., C. Aguirre, R. R. Rurangirwa, T. F. McElwain, T. C. McGuire, D. P. Knowles, and G. H. Palmer.** 1995. Detection of *Anaplasma ovis* infection in goats using the MSP5 competitive inhibition ELISA. J. Clin. Microbiol. **33:**675–679.
15. **Oberle, S. M., and A. F. Barbet.** 1993. Derivation of the complete *msp-4* gene sequence of *Anaplasma marginale* without cloning. Gene **136:**291–294.
16. **Ohashi, N., N. Zhi, Y. Zhang, and Y. Rikihisa.** 1998. Immunodominant major outer membrane proteins of *Ehrlichia chaffeensis* are encoded by a polymorphic multigene family. Infect. Immun. **66:**132–139.
17. **Palmer, G. H., A. F. Barbet, G. H. Cantor, and T. C. McGuire.** 1989. Immunization of cattle with the MSP-1 surface protein complex induces protection against a structurally variant *Anaplasma marginale* isolate. Infect. Immun. **57:**3666–3669.
18. **Palmer, G. H., A. F. Barbet, W. C. Davis, and T. C. McGuire.** 1986. Immunization with an isolate-common surface protein protects cattle against anaplasmosis. Science **231:**1299–1302.
19. **Palmer, G. H., G. Eid, A. F. Barbet, T. C. McGuire, and T. F. McElwain.** 1994. The immunoprotective *Anaplasma marginale* major surface protein-2 (MSP-2) is encoded by a polymorphic multigene family. Infect. Immun. **62:**3808–3816.
20. **Palmer, G. H., and T. F. McElwain.** 1995. Molecular basis for vaccine development against anaplasmosis and babesiosis. Vet. Parasitol. **57:**233–253.
21. **Palmer, G. H., and T. C. McGuire.** 1984. Immune serum against *Anaplasma marginale* initial bodies neutralizes infectivity for cattle. J. Immunol. **133:**1010–1015.
22. **Palmer, G. H., D. Munodzana, N. Tebele, T. Ushe, and T. F. McElwain.** 1994. Heterologous strain challenge of cattle immunized with *Anaplasma marginale* outer membranes. Vet. Immunol. Immunopathol. **42:**265–273.
23. **Palmer, G. H., S. M. Oberle, A. F. Barbet, W. C. Davis, W. L. Goff, and T. C. McGuire.** 1988. Immunization with a 36-kilodalton surface protein induces protection against homologous and heterologous *Anaplasma marginale* challenge. Infect. Immun. **56:**1526–1531.
24. **Riggs, P.** 1994. Expression and purification of maltose-binding protein fusions, p. 16.6.1–16.6.14. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 2. John Wiley & Sons, Inc., New York, N.Y.
25. **Slatko, B. E., L. M. Albright, and S. Tabor.** 1994. DNA sequencing by the dideoxy method, p. 7.4.1–7.4.35. *In* F. M. Ausubel, R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith, and K. Struhl (ed.), Current protocols in molecular biology, vol. 1. John Wiley & Sons, Inc., New York, N.Y.
26. **Suarez, C. E., G. H. Palmer, D. P. Jasmer, S. A. Hines, L. E. Perryman, and**

T. F. McElwain. 1991. Characterization of the gene encoding a 60kD *Babesia bovis* merozoite protein with conserved and surface exposed epitopes. Mol. Biochem. Parasitol. **46:**45–52.
27. **Tebele, N., T. C. McGuire, and G. H. Palmer.** 1991. Induction of protective immunity using *Anaplasma marginale* initial body membranes. Infect. Immun. **59:**3199–3204.
28. **Tebele, N., and G. H. Palmer.** 1991. Crossprotective immunity between the Florida and a Zimbabwe stock of *Anaplasma marginale*. Trop. Anim. Health Prod. **23:**197–202.
29. **Van Vliet, A. H. M., F. Jongejan, M. Van Kleef, and B. A. M. Van Der Zeist.** 1994. Molecular cloning, sequence analysis, and expression of the gene encoding the immunodominant 32-kilodalton protein of *Cowdria ruminantium*. Infect. Immun. **62:**1451–1456.
30. **Vidotto, M., T. C. McGuire, T. F. McElwain, G. H. Palmer, and D. P. Knowles.** 1994. Intermolecular relationships of major surface proteins of *Anaplasma marginale*. Infect. Immun. **62:**2940–2946.
31. **Visser, E. S., T. C. McGuire, G. H. Palmer, W. C. Davis, V. Shkap, E. Pipano, and D. P. Knowles.** 1992. The *Anaplasma marginale msp5* gene encodes a 19-kilodalton protein conserved in all recognized *Anaplasma* species. Infect. Immun. **60:**5139–5144.

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