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Intermolecular Relationships of Major Surface Proteins of *Anaplasma marginale*

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Immunization with *Anaplasma marginale* membranes containing major surface proteins (MSPs) induces protective immunity against clinical disease (N. Tebele, T. C. McGuire, and G. H. Palmer, Infect. Immun. 59:3199–3204, 1991). For use in design of a recombinant antigen subunit vaccine for *A. marginale*, intermolecular relationships of known *A. marginale* MSPs were analyzed. Under nonreducing conditions, MSP-2 and MSP-5 occur as multimers. A large (>300-kDa-molecular-mass), nonreduced protein complex contained MSP-1a linked by disulfide bonds to MSP-1b and by noncovalent bonds to MSP-5. MSP-2 was also noncovalently bound to this complex. The nearest neighbor membrane proteins were identified by cross-linking reactions followed by immunoblotting with anti-MSP antibodies. A cross-linked aggregate retained in the stacking gel contained MSP-1a, MSP-1b, MSP-2, MSP-3, MSP-4, and MSP-5. Collectively, the data indicate that MSP-2 and MSP-5 occur as monomers and disulfide-bonded multimers. The MSP-1 complex occurs as both disulfide-bonded and noncovalently associated MSP-1a and MSP-1b, and MSP-2 and MSP-5 are noncovalently associated with MSP-1. Also, MSP-1, MSP-2, MSP-3, and MSP-4 are nearest neighbors, and MSP-5 is noncovalently associated with this cross-linked complex.

Anaplasma marginale, a rickettsia that causes severe disease in cattle throughout temperate and tropical regions worldwide, is transmitted either biologically by ixodid ticks or mechanically by needles and biting flies (27). This rickettsia replicates in erythrocytes of infected cattle, resulting in anemia, weight loss, abortion, and death (1). Cattle that survive clinical disease are persistently infected with *A. marginale* (30) and are protected against subsequent homologous challenge (9).

The Florida strain of *A. marginale* induces postinfection immunity against heterologous challenge (9, 14, 28, 34) and has been used to identify surface proteins for vaccine development. Four major surface proteins (MSPs) from the Florida strain were recognized by neutralizing polyclonal antibody (23) and designated MSP-1 (105 kDa), MSP-2 (36 kDa), MSP-3 (86 kDa), and MSP-4 (31 kDa) (4, 13, 17, 20, 22, 24). MSP-5 was originally identified in outer membrane preparations of the Norton Zimbabwe strain and subsequently shown to be conserved in other strains, including Florida (31). MSP-1 is a complex of two polypeptides with molecular masses of 105 and 100 kDa (4), designated MSP-1a and MSP-1b, respectively (18). MSP-1a exhibits marked size polymorphism among different strains of *A. marginale* (18) and is encoded by a single-copy gene (2). In contrast, MSP-1b is encoded by a multicopy gene and has minimal interstrain polymorphism (3). MSP-2 and MSP-3 are encoded by multicopy genes (19), whereas MSP-4 and MSP-5 are encoded by single-copy genes (16, 33).

Cattle immunized with an outer membrane fraction of *A. marginale* are protected from clinical disease following homologous challenge (31). Sera from these protected cattle recognize MSP-1, MSP-2, MSP-4, and MSP-5, and antibody titers against the MSPs correlate with protection against anemia (31). In contrast to the protection induced by immunization with outer membranes, vaccination with isolated MSPs induces incomplete protection (20, 21, 24). In this context, we hypothesized that the intermolecular relationships of MSPs are important for induction of complete protective immunity and initiated the analysis reported here.

To determine intermolecular relationships among *A. marginale* MSPs, we analyzed nonreduced MSP complexes from sodium dodecyl sulfate (SDS)-disrupted organisms and complexes isolated by monoclonal antibody affinity (MAb) chromatography from deoxycholate-disrupted *A. marginale*. Also, we determined the spatial arrangement of MSPs in the membrane by using homobifunctional cross-linking reagents, which cross-link neighboring polypeptides through amino groups (6, 7, 11, 26, 35).

MATERIALS AND METHODS

Organisms. The Florida strain of *A. marginale* maintained as a stabilate (28) was used to infect a splenectomized calf. When the rickettsemia reached >50%, the infected blood was collected aseptically. *A. marginale* was isolated from infected erythrocytes as previously described (23). Briefly, 20 ml of blood with >50% rickettsia-infected erythrocytes was washed five times with phosphate-buffered saline (PBS) and centrifuged at 16,000 × *g* for 20 min. At each wash an upper layer containing both leukocytes and erythrocytes was removed. The washed-erythrocyte suspension was sonicated for 2 min at 50 W on ice and centrifuged for 20 min at 20,000 × *g*. The pellet was resuspended in PBS and sonicated again for 30 s at 50 W. After centrifugation at 20,000 × *g* for 20 min, the *A. marginale* pellet was resuspended in PBS (23). The presence of intact organisms was visualized with Giemsa stain, and the protein

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TABLE 1. *A. marginale* MSPs

MSP identified	Molecular mass (kDa)	Antibody
MSP-1a	105	MAb ANA22B1
MSP-1b	100	MAb AMR36A6
MSP-2	31	MAb ANAF19E2
MSP-3	86	MAb AMG76B1
MSP-3	86	MAB AMG75C2
MSP-4	31	Rabbit anti-MSP-4 serum[a]
MSP-5	19	MAb ANAF16C1
Isotype control	NA[b]	MAb IgG1
Isotype control	NA	MAb IgG2a
Isotype control	NA	MAb IgG3

[a] Rabbit serum recognizing recombinant MSP-4 (16).
[b] NA, not applicable.

concentration was determined by a detergent-compatible protein assay (Bio-Rad, Hercules, Calif.).

MSP complexes. MSP components were identified in both SDS-solubilized *A. marginale* and immunoaffinity-purified MSP complexes.

(i) Solubilized *A. marginale*. *A. marginale* was suspended in electrophoresis sample buffer (0.025 M Tris-hydrochloride, 2% SDS, 15% glycerol, 2.5% 2-mercaptoethanol, pH 6.8) with or without 2-mercaptoethanol, boiled for 3 min, and electrophoresed in an SDS–7.5 to 17.5% polyacrylamide gradient slab gel with a 5% polyacrylamide stacking gel. Gels were either silver stained (Bio-Rad) or set up for immunoblotting. Protein complexes containing MSPs were retained at the top of the stacking gel (TSG) and the top of the resolving gel (TRG). Acrylamide sections containing these complexes were cut in small pieces and placed in PBS for 18 to 24 h at 4°C. After centrifugation at 16,000 × *g* for 2 min, the nonreduced protein complexes were solubilized in electrophoresis sample buffer and evaluated by immunoblotting. The polypeptides were transferred onto nitrocellulose as described previously (32), and the membranes were blocked in PBS-Tween 20 containing 5% milk. Individual MSPs were detected with previously defined MAbs (MSP-1a, MAb ANA22B1; MSP1-b, AMR36A6; MSP-2, ANA19E2; MSP-3, AMG76B1 and AMG75C2; MSP-5, ANAF16C1) (Table 1). MSP-4 was detected with rabbit antiserum to recombinant MSP-4 (16). Bound antibody was detected with either peroxidase-conjugated donkey anti-murine or anti-rabbit immunoglobulin G (IgG) (Jackson Immunoresearch Laboratories, Inc., West Grove, Pa.) and enhanced chemiluminescence (Amersham International, Amersham, United Kingdom). Control antibodies used were normal rabbit serum or IgG1 and IgG2a isotype MAbs directed against unrelated antigens.

(ii) Immunoaffinity-purified MSP complexes. *A. marginale*-infected erythrocytes, obtained from a frozen stabilate which was thawed and washed three times with PBS containing protease inhibitors, were solubilized with 0.5% deoxycholate in proteinase inhibitor buffer (50 mM Tris [pH 8.0], 5 mM EDTA, 5 mM iodoacetamide, 0.1 mM *Nα-p*-toxyl-L-lysine chloromethyl ketone [TLCK], and 1 mM phenylmethylsulfonyl fluoride). Purification and coupling of anti-MSP MAbs against MSP-1, MSP-2, MSP-3, and MSP-5 to CN-bromide-activated Sepharose 4B (Pharmacia LKB Biotechnology, Piscataway, N.J.) were performed as described previously (21). Native MSPs were eluted from the affinity column with TEN (0.02 M Tris, 0.005 M EDTA, 0.1 M NaCl, pH 7.6) containing 0.5% deoxycholate and 2 M potassium thiocyanate (21). Following dialysis against PBS, the purity of eluted MSPs was evaluated by SDS-polyacrylamide gel electrophoresis (PAGE) followed by silver staining (Bio-Rad) and immunoblotting.

Nearest-neighbor analysis of MSPs. The homobifunctional cross-linkers disuccinimidyl suberate (DSS), dithiobis(succinimidylpropionate) (DSP), and dithiobis(sulfosuccinimidylpropionate) (DTSSP) (Pierce Chemical Co., Rockford, Ill.) form covalent bonds with amino groups separated by a distance of 12 Å (1.2 nm) (7). Also, DSP and DTSSP have an internal disulfide bond which can be cleaved by 2-mercaptoethanol (11, 29). Solutions of DSS and DSP were prepared in dimethyl sulfoxide at a concentration of 10 mM. Ten millimolar DTSSP was prepared in a pH 7.8 cross-linking buffer of 20 mM $NaPO_4$ and 20 mM NaCl. In preliminary experiments, DSS, DSP, or DTSSP was used at a final concentration of 0.1 to 5 mM with 50 μg of *A. marginale* in 1.0 ml of cross-linking buffer. From these experiments, 0.2 mM of cross-linker was selected for all further studies. After incubation for 30 min at room temperature, the reaction was stopped with 50 mM Tris buffer, pH 7.4. The suspension was centrifuged at 16,000 × *g* for 15 min, and the pellet was suspended in PAGE sample buffer.

RESULTS

MSP complexes. (i) Solubilized whole *A. marginale*. MSPs were solubilized with sample buffer and separated by SDS-PAGE under reducing conditions (RC) and nonreducing conditions (NRC) and immunoblotted with MAbs to MSP-1a, MSP-1b, MSP-2, MSP-3, and MSP-5 and with monospecific rabbit antibodies to MSP-4. *A. marginale* separated under NRC contained large complexes at the TRG that reacted in immunoblots with MAb to MSP-1a (Fig. 1, lane 2). MAb to MSP-1a also reacted with a 105-kDa protein and seven smaller polypeptides under both RC as previously described (2) and NRC (Fig. 1, lanes 1 and 2). MAb to MSP-1b reacted with large nonreduced protein complexes in the TSG and the TRG (Fig. 1, lanes 2 and 4, arrowheads) and also reacted with a 100-kDa protein and large complexes separated under both RC and NRC (Fig. 1, lanes 3 and 4). MAb to MSP-2 reacted with monomer MSP-2 (36 kDa) under RC (Fig. 1, lane 5) and with monomer and a 144-kDa protein under NRC (Fig. 1, lane 6, arrowhead). This 144-kDa protein may represent a tetramer of MSP-2. Also, MSP-2 purified by MAb affinity matrix and analyzed by silver staining contained only proteins of 36 and 144 kDa (data not shown). A 21-kDa protein bound both MAb to MSP-2 (Fig. 1, lane 5) and the isotype control MAb IgG2a (Fig. 1, lane 15). MAb to MSP-5 reacted with monomer MSP-5 (19 kDa) under RC and with the monomer and a 38-kDa protein under NRC (Fig. 1, lanes 11 and 12). The 38-kDa protein that bound the MAb (ANAF16C1) to MSP-5 (Fig. 1, lane 12, arrowhead) was eluted from the SDS-PAGE gel, separated under RC, and shown to react as a 19-kDa protein with MAb ANAF16C1 (data not shown). MAb to MSP-3 and monospecific rabbit antibody to MSP-4 reacted with only monomeric forms (86 and 31 kDa, respectively) under both RC and NRC (Fig. 1, lanes 7 to 10). Isotype control IgG2a bound a 21-kDa protein under RC (Fig. 1, lane 15), while isotype control IgG1 MAb was unreactive (Fig. 1, lanes 13 and 14).

To determine the composition of the large nonreduced protein complexes reacting with MAbs to MSP-1a and MSP-1b, bands were removed from the TRG and TSG and analyzed. The protein complexes were eluted and reseparated by SDS-PAGE under RC and NRC and immunoblotted with the described antibodies to MSPs. Proteins eluted from the TRG under NRC and separated under both RC and NRC included

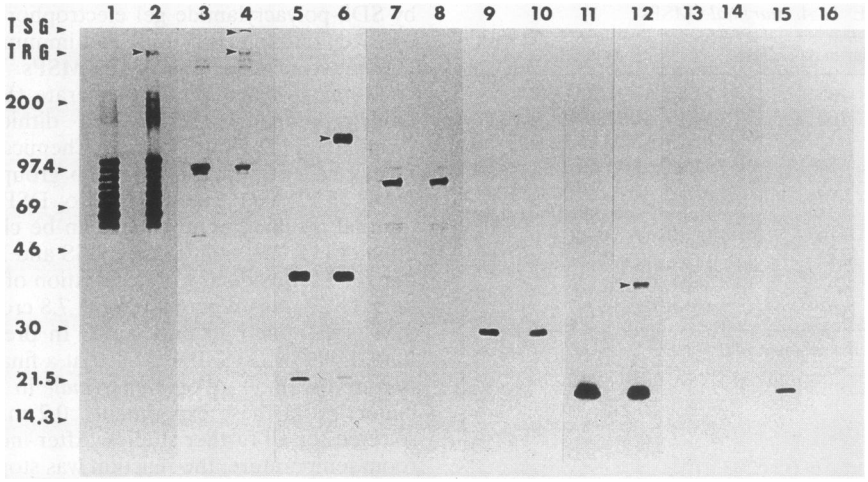

FIG. 1. Identification of native MSP *A. marginale* complexes. Odd-numbered lanes contain reduced proteins, while proteins in even-numbered lanes are nonreduced. Lanes 1 and 2, reacted with MAb ANA22B1 to MSP-1a; lanes 3 and 4, reacted with MAb AMR36A6 to MSP-1b; lanes 5 and 6, reacted with MAb ANAF19E2 to MSP-2; lanes 7 and 8, reacted with pooled MAbs AMG76B1 and AMG75C2 to MSP-3; lanes 9 and 10, reacted with rabbit antiserum to MSP-4; lanes 11 and 12, reacted with MAb ANAF16C1 to MSP-5; lanes 13 and 14, reacted with IgG1 isotype control MAb TRYP1E1; lanes 15 and 16, reacted with IgG2a isotype control MAb 44/37.15. Sizes are given on the left in kilodaltons.

proteins of 105 and 19 kDa that reacted in immunoblots with MAbs to MSP-1a and MSP-5 (Fig. 2, lanes 1, 2, 11, and 12). Proteins eluted from the TSG under NRC and separated under both conditions included proteins of 105, 100, and 19 kDa reacting with MAbs to MSP-1a, MSP-1b, and MSP-5, respectively (Fig. 3, lanes 1 to 4, 11, and 12). In addition, the proteins eluted included a 144-kDa protein reactive with MAb to MSP-2 under NRC (Fig. 3, lane 5) and large nonreduced protein in the TSG reacting with monospecific antibody to MSP-4 (Fig. 3, lane 9).

(ii) Immunoaffinity-purified MSP complexes. To further evaluate MSP complexes, proteins purified by MAb affinity chromatography were evaluated. The proteins purified from the ANA22B1 MAb (anti-MSP-1a) affinity matrix and separated by SDS-PAGE under RC and NRC reacted in immunoblots with MAbs to MSP-1a, MSP-1b, MSP-2, and MSP-5 (Fig. 4, lanes 1 to 6, 9, and 10). MAb to MSP-1a reacted with a 105-kDa protein and seven smaller polypeptides under RC, while MAb to MSP-1b reacted with a major protein at 100 kDa and one of slightly smaller size under RC (Fig. 4, lanes 1 and 3). MAb to MSP-2 reacted with a 36-kDa protein under RC and NRC (Fig. 4, lanes 5 and 6). MAb to MSP-5 reacted with a protein of 19 kDa under RC and NRC (Fig. 4, lanes 9 and 10). MAb to MSP-3 did not react with any of the proteins isolated from the MSP-1a MAb affinity matrix (Fig. 4, lanes 7

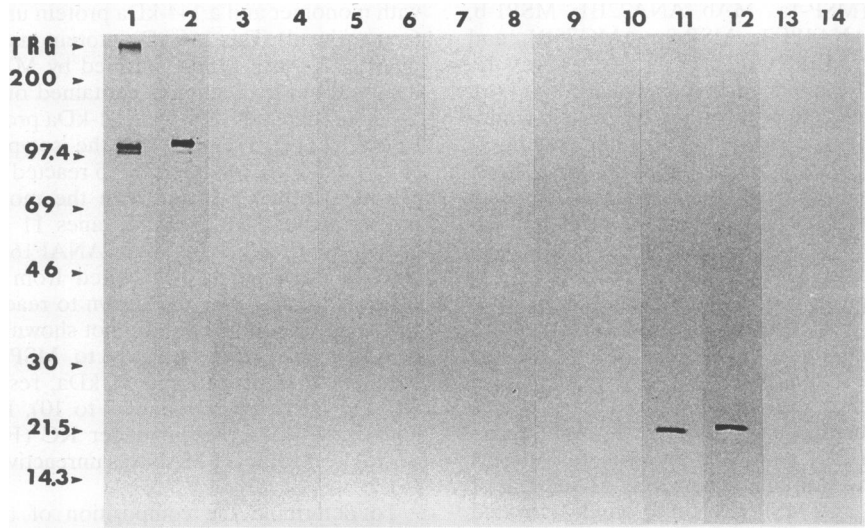

FIG. 2. Immunoblot of native MSP complex eluted from the TRG and rerun under NRC and RC. All lanes contain the eluted complex; odd-numbered lanes contain nonreduced proteins, while even-numbered lanes contain reduced proteins. Lanes 1 and 2, reacted with MAb ANA22B1 to MSP-1a; lanes 3 and 4, reacted with MAb AMR36A6 to MSP-1b; lanes 5 and 6, reacted with MAb ANAF19E2 to MSP-2; lanes 7 and 8, reacted with pooled MAbs AMG76B1 and AMG75C2 to MSP-3; lanes 9 and 10, reacted with rabbit antiserum to MSP-4; lanes 11 and 12, reacted with MAb ANAF16C1 to MSP-5; lanes 13 and 14, reacted with IgG2a isotype control MAb 44/37.15. Sizes are given on the left in kilodaltons.

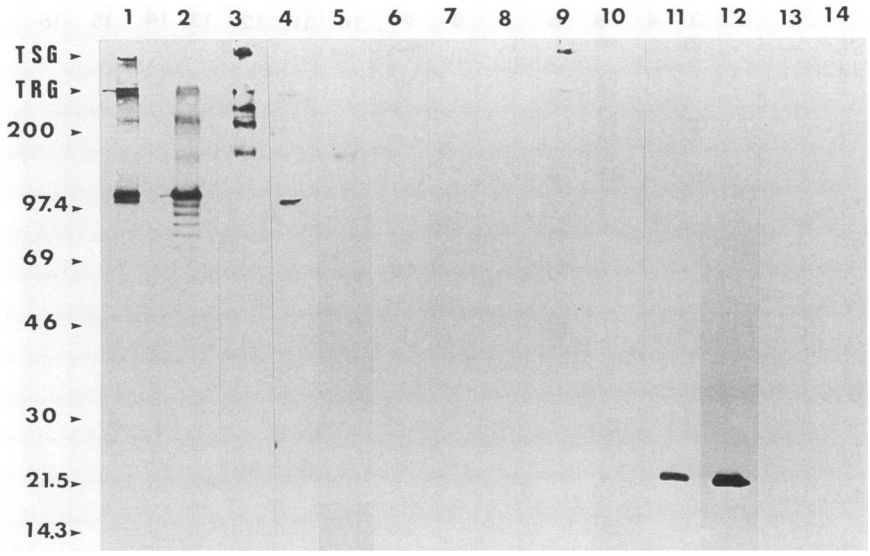

FIG. 3. Immunoblot of native MSP complex eluted from the TSG and rerun under NRC and RC. All lanes contain the eluted complex; odd-numbered lanes contain nonreduced proteins, while even-numbered lanes contain reduced proteins. Lanes 1 and 2, reacted with MAb ANA22B1 to MSP-1a; lanes 3 and 4, reacted with MAb AMR36A6 to MSP-1b; lanes 5 and 6, reacted with MAb ANAF19E2 to MSP-2; lanes 7 and 8, reacted with pooled MAbs AMG76B1 and AMG75C2 to MSP-3; lanes 9 and 10, reacted with rabbit antiserum to MSP-4; lanes 11 and 12, reacted with MAb ANAF16C1 to MSP-5; lanes 13 and 14, reacted with IgG2a isotype control MAb 44/37.15. Sizes are given on the left in kilodaltons.

and 8). Bands of 24, 28, and 78 kDa in lanes 5 to 10 are also reactive with control MAb IgG3 in lanes 11 and 12.

Similar evaluation of proteins purified from affinity matrices of MAbs ANAF19E2 (anti-MSP-2) and AMG75C2 (anti-MSP-3) resulted in identification of only MSP-2 and MSP-3, respectively. Also, MSP-2 purified by MAb affinity chromatography and analyzed by silver staining contained only proteins of 36 and 144 kDa (data not shown). However, MAbs to MSP-5 and MSP-1a reacted with proteins of 19 and 100 kDa, respectively, in immunoblots of protein purified from the MAb ANAF16C1 (anti-MSP-5) affinity matrix (data not shown).

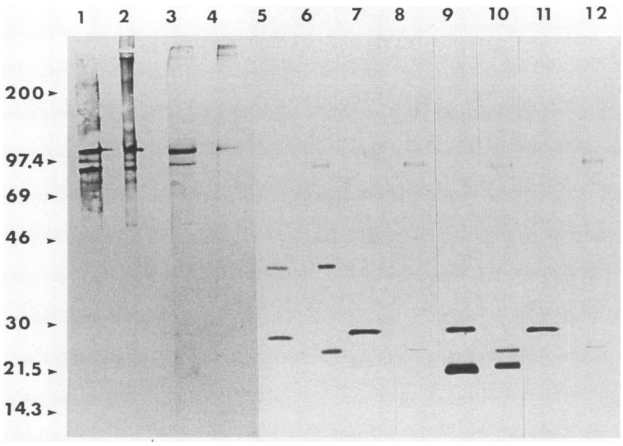

FIG. 4. Immunoblot of immunoaffinity-purified MSP-1. Odd numbered lanes were run under RC, while even-numbered lanes were run under NRC. Lanes 1 and 2, reacted with MAb ANA22B1 to MSP-1a; lanes 3 and 4, reacted with MAb AMR36A6 to MSP-1b; lanes 5 and 6, reacted with MAb ANAF19E2 to MSP-2; lanes 7 and 8, reacted with pooled MAbs AMG76B1 and AMG75C2 to MSP-3; lanes 9 and 10, reacted with MAb ANAF16C1 to MSP-5; lanes 11 and 12, reacted with IgG3 isotype control MAb. Sizes are given on the left in kilodaltons.

Nearest-neighbor analysis of MSPs. To determine nearest-neighbor relationships among the known MSPs, isolated *A. marginale* was treated with 0.2 mM DTSSP, DSS, or DSP. The MSPs were separated by SDS-PAGE under RC and NRC and immunoblotted with antibodies to MSPs. Results with the three cross-linkers were similar (data not shown). Consequently, all further studies were done with DTSSP, which contains a disulfide bond cleavable under RC. After separation of DTSSP-treated MSPs under NRC, a high-molecular-mass band in the TSG reacted with MAbs to MSP-1a, MSP-1b, MSP-2, and MSP-3 and with monospecific antibody to MSP-4 (Fig. 5, lanes 1, 3, 5, 7, and 9) but not with MAb to MSP-5 (Fig. 5, lane 11). In addition, DTSSP treatment resulted in another high-molecular-mass band in the TRG which reacted with MAbs to MSP-1a, MSP-1b, and MSP-2 and with monospecific antibody to MSP-4 (Fig. 5, lanes 1, 3, 5, and 9). RC resulted in the appearance of a 105-kDa band and several smaller bands reacting with MAb to MSP-1a (Fig. 5, lane 2); a 100-kDa band reacting with MAb to MSP-1b (lane 4); 144-, 72-, and 36-kDa bands reacting with MAb to MSP-2 (lane 6); an 86-kDa band and three higher-molecular-mass bands reacting with MAb to MSP-3 (lane 8); a 31-kDa band and two other bands at the TRG reacting with monospecific rabbit antibody to MSP-4 (lane 10); and one 19-kDa band reacting with MAb to MSP-5 (lane 12). Isotype control IgG1 and IgG2a MAbs were unreactive with complexes formed by cross-linking MSPs (Fig. 5, lanes 13 to 16).

To further define the MSPs in the cross-linked (DTSSP) complexes, the bands in the TRG and TSG were isolated and the protein complexes were eluted. The eluted complexes were reseparated by SDS-PAGE under RC and NRC and evaluated by immunoblotting. Separation of the complexes from the TRG under RC resulted in a predominant band at 105 kDa identified by MAb to MSP-1a (Fig. 6A, lane 2); bands of 36, 72,

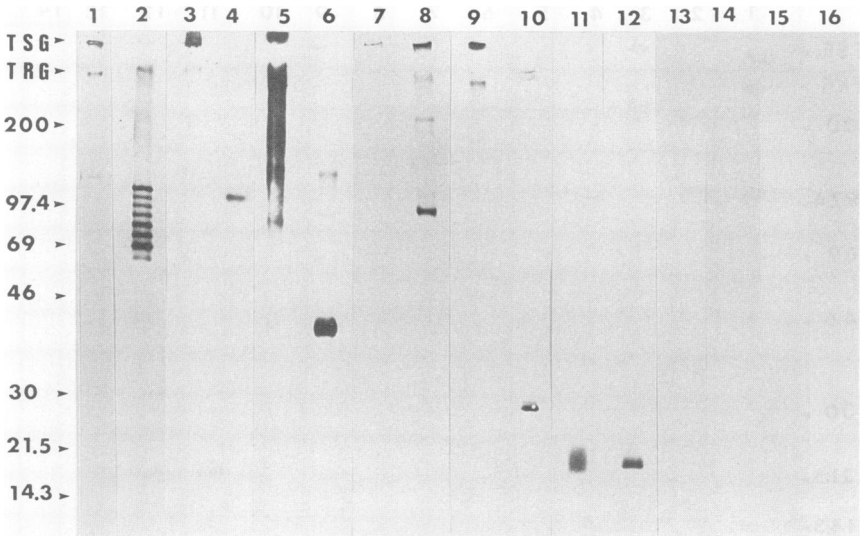

FIG. 5. Immunoblot of MSPs cross-linked with DTSSP. All lanes contain *A. marginale* treated with DTSSP; odd-numbered lanes were run under NRC, while even-numbered lanes were run under RC. Lanes 1 and 2, reacted with MAb ANA22B1 to MSP-1a; lanes 3 and 4, reacted with MAb AMR36A6 to MSP-1b; lanes 5 and 6, reacted with MAb ANAF19E2 to MSP-2; lanes 7 and 8, reacted with pooled MAbs AMG76B1 and AMG75C2 to MSP-3; lanes 9 and 10, reacted with rabbit antiserum to MSP-4; lanes 11 and 12, reacted with MAb ANAF16C1 to MSP-5; lanes 13 and 14, reacted with IgG2a isotype control MAb 44/37.15; lanes 15 and 16, reacted with normal rabbit serum. Sizes are given on the left in kilodaltons.

and 108 kDa identified by MAb to MSP-2 (lane 4); and a band of 19 kDa identified by MAb to MSP-5 (lane 6). There were no bands identified by MAb to MSP-1b or MSP-3 or by monospecific antibody to MSP-4 (data not shown). Separation of the band from the TSG under RC resulted in a single band of 100 kDa identified by MAb to MSP-1b (Fig. 6B, lane 2), a band of 86 kDa and three higher-molecular-mass bands identified by MAb to MSP-3 (lane 4), and a single band of 31 kDa identified by monospecific antibody to MSP-4 (lane 6). Also, the protein complex eluted from the TSG contained MSP-1a, MSP-2, and MSP-5 (data not shown).

DISCUSSION

Previous work demonstrated that immune serum containing antibody against MSPs can neutralize *A. marginale* infectivity

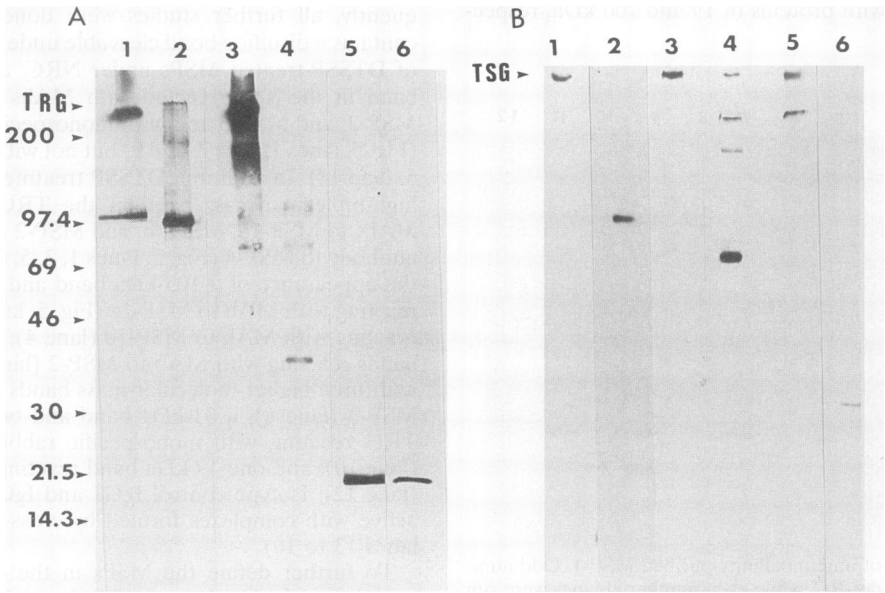

FIG. 6. Immunoblot of cross-linked MSP complexes. (A) All lanes contain the nonreduced linked complex eluted from the TRG; odd-numbered lanes were run under NRC, while even-numbered lanes were run under RC. Lanes 1 and 2, reacted with MAb ANA22B1 to MSP-1a; lanes 3 and 4, reacted with MAb ANAF19E2 to MSP-2; lanes 5 and 6, reacted with MAb ANAF16C1 to MSP-5. (B) All lanes contain the nonreduced linked complex eluted from the TSG; odd-numbered lanes were run under NRC, while even-numbered lanes were run under RC. Lanes 1 and 2, reacted with MAb AMR36A6 to MSP-1b; lanes 3 and 4, reacted with pooled MAb AMG76B1 and AMG75C2 to MSP-3; lanes 5 and 6, reacted with rabbit antiserum to MSP-4. Sizes are given on the left in kilodaltons.

(23). In addition, an *A. marginale* membrane fraction can induce protective immunity against rickettsemia and anemia that correlates with anti-MSP antibody titers (31). However, immunization with isolated MSPs has induced only partial protection against challenge (20, 21, 24). One explanation for these data is that immune responses to epitopes on multiple MSPs are required for protective immunity. A preliminary immunization trial using a mixture of three different MSPs did not result in enhanced protective immune responses compared with the responses in cattle immunized with individual MSPs (8). The premise initiating the present work was that some of the epitopes necessary for the induction of protective immunity require quaternary conformation.

B-cell epitopes are often dependent on native conformation, may be few in number, and are frequently constructed from discontinuous regions of the protein brought into juxtaposition by molecular folding (10). One example of the role of tertiary conformation to epitope configuration was demonstrated with influenza virus hemagglutinin (5). Twenty peptides, corresponding to 75% of the hemagglutinin molecule, were synthesized, and antibody raised to 18 of these peptides reacted with the native hemagglutinin molecule. However, antibody raised to the native hemagglutinin did not bind any of the 20 peptides (5). While it is apparent that some epitopes are dependent on tertiary conformation, the importance of quaternary conformation to epitope configuration is not clear.

The work presented here was initiated to define intermolecular relationships of the currently known MSPs of *A. marginale*. The purpose of this work, in the context of our hypothesis concerning the potential role of intermolecular relationships of MSPs in the induction of protective immunity, is to define these relationships for use in design of *A. marginale* subunit recombinant vaccines.

The findings concerning the intermolecular relationships of the six known MSPs of *A. marginale* yielded by this work are in three categories: (i) disulfide bonding, (ii) intermolecular noncovalent bonding, and (iii) nearest neighbors. Disulfide bonding and noncovalent bonding of MSPs were examined under RC and NRC by SDS-PAGE. Nearest-neighbor analysis of MSPs was done before and after treatment with protein cross-linkers. The cross-linking reagents used were membrane impermeable and cleavable in order to provide information about membrane protein subunits on the outer surface of the rickettsial membrane (29). Such cross-linking reagents have been used to analyze nearest-neighbor relationships of proteins in viruses (25), on the outer membrane of *Neisseria gonorrhoeae* (15), and in cellular organelles such as ribosomes (12).

Disulfide-bonded MSP-1a and MSP-1b were found in the eluted nonreduced TSG complex analyzed under NRC (Fig. 3, lanes 1 and 3). This complex was not present in the TSG under RC. These data do not differentiate between direct disulfide bonding of MSP-1a and MSP-1b and disulfide bonding via another unidentified protein within the complex. However, since the MSP-1 subunits are nearest neighbors, the disulfide linkage is probably directly between MSP-1a and MSP-1b. Previously, MSP-1a and MSP-1b were believed to occur primarily as a noncovalently bound complex on the basis of studies that showed that the molecular masses of MSP-1a and MSP-1b were unchanged under RC or NRC (4). The collective data now show that MSP-1a and MSP-1b occur as both a disulfide-bonded complex and a noncovalently bound complex.

Disulfide-bonded multimers of MSP-2 (Fig. 1, lane 6) and MSP-5 (Fig. 1, lane 12) were found under NRC. The molecular masses of the multimers, 144 kDa for MSP-2 and 38 kDa for MSP-5, indicate that these multimers most likely represent tetramers of MSP-2 (monomers of 31 kDa) and dimers of MSP-5 (monomers of 19 kDa). However, the data do not rule out the possibility of multimeric linkage between MSP-2 or MSP-5 with other, as yet uncharacterized proteins. The presence of disulfide bonding involving MSP-1a, MAP-1b, MSP-2, and MSP-5 is consistent with the presence of cysteines in the deduced amino acid sequence from the genes encoding these MSPs (2, 3, 19, 33). MSP-3 (86 kDa) and MSP-4 (31 kDa) were detected only as monomers. Although MSP-4 contains cysteines (16), the available MSP-3 DNA sequence does not contain codons for cysteines (19).

Noncovalently bonded MSPs were shown by immunoblotting of eluted *A. marginale* protein complexes (TSG and TRG) after separation under both NRC and RC and immunoblotting of MSPs purified by MAb affinity chromatography under both NRC and RC. Collectively these analyses showed the presence of noncovalent bonds between MSP-1a and MSP-1b (Fig. 4, lanes 1 to 4) as previously described (4) and noncovalent bonding of monomeric MSP-2 (Fig. 4, lanes 5 and 6) and monomeric MSP-5 (Fig. 4, lanes 9 and 10) with the MSP-1 complex. Native MSP-5 purified by affinity chromatography contained noncovalently associated MSP-1a. This finding indicates that the noncovalent bonding of MSP-5 with the MSP-1 complex is via MSP-1a. Although MSP-5 was shown to be within the eluted TRG and TSG complexes (Fig. 2 and 3), anti-MSP-5 MAb did not detect MSP-5 within initial TRG and TSG complexes from solubilized whole *A. marginale* (Fig. 1, lane 12). The inability to detect MSP-5 in the TRG and TSG complexes from solubilized whole *A. marginale* may reflect inaccessibility of the epitope defined by MAb ANAF16C1 in these complexes.

Analysis of MSPs cross-linked with DTSSP showed MSP-1, MSP-2, MSP-3, and MSP-4 as nearest neighbors. A high-molecular-mass cross-linked complex in the TSG reacted with antibodies to all MSPs (Fig. 5, lanes 1, 3, 5, 7, and 9) except anti-MSP-5 antibody (Fig. 5, lane 11). A high-molecular-mass cross-linked complex in the TRG reacted with antibodies to MSP-1a, MSP-1b, MSP-2, and MSP-4 (Fig. 5, lanes 1, 3, 5, and 9). Eluted TSG DTSSP complex contained all MSPs, including MSP-5. MSP-5 was also present in the eluted TRG DTSSP complex (Fig. 6A, lanes 5 and 6). MSP-1b, MSP-3, and MSP-4 were not detected in the eluted TRG DTSSP complex. Although the eluted cross-linked TRG complex contains MSP-5, the data do not indicate that MSP-5 is cross-linked to other MSPs in the TRG DTSSP complex. In contrast, the data (Fig. 5, lanes 11 and 12, and Fig. 6A, lanes 5 and 6) indicate MSP-5 is only noncovalently associated with the cross-linked TRG complex. If MSP-5 were cross-linked within the eluted TRG DTSSP complex, monomeric MSP-5 would be expected to be reduced or not present under NRC (Fig. 5, lane 11; Fig. 6A, lane 5).

Collectively, the data presented here indicate that MSP-1a and MSP-1b associate by both noncovalent and disulfide bonds. MSP-2 and MSP-5 occur as monomers and disulfide-bonded multimers which probably represent tetramers and dimers, respectively. There are noncovalent bonds between MSP-1, MSP-2, and MSP-5, and the association of MSP-1 and MSP-5 is through MSP-1a. As defined by cross-linking with DTSSP, MSP-1, MSP-2, MSP-3, and MSP-4 are nearest neighbors. Although MSP-5 is noncovalently associated with MSP-1a, MSP-5 is not a nearest neighbor as defined by cross-linking with DTSSP.

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