CD4\(^+\) T Lymphocytes from Calves Immunized with *Anaplasma marginale* Major Surface Protein 1 (MSP1), a Heteromorphic Complex of MSP1a and MSP1b, Preferentially Recognize the MSP1a Carboxyl Terminus That Is Conserved among Strains

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Native major surface protein 1 (MSP1) of the ehrlichial pathogen *Anaplasma marginale* induces protective immunity in calves challenged with homologous and heterologous strains. MSP1 is a heteromorphic complex of a single MSP1a protein covalently associated with MSP1b polypeptides, of which at least two (designated MSP1F1 and MSP1F3) in the Florida strain are expressed. Immunization with recombinant MSP1a and MSP1b alone or in combination fails to provide protection. The protective immunity in calves immunized with native MSP1 is associated with the development of opsonizing and neutralizing antibodies, but CD4\(^+\) T-lymphocyte responses have not been evaluated. CD4\(^+\) T lymphocytes participate in protective immunity to ehrlichial pathogens through production of gamma interferon (IFN-\(\gamma\)), which promotes switching to high-affinity immunoglobulin G (IgG) and activation of phagocytic cells to produce nitric oxide. Thus, an effective vaccine for *A. marginale* and related organisms should contain both T- and B-lymphocyte epitopes that induce a strong memory response that can be recalled upon challenge with homologous and heterologous strains. This study was designed to determine the relative contributions of MSP1a and MSP1b polypeptides, which contain both variant and conserved amino acid sequences, in stimulating memory CD4\(^+\) T-lymphocyte responses in calves immunized with native MSP1. Peripheral blood mononuclear cells and CD4\(^+\) T-cell lines from MSP1-immunized calves proliferated vigorously in response to the immunizing strain (Florida) and heterologous strains of *A. marginale*. The conserved MSP1-specific response was preferentially directed to the carboxy-terminal region of MSP1a, which stimulated high levels of IFN-\(\gamma\) production by CD4\(^+\) T cells. In contrast, there was either weak or no recognition of MSP1b proteins. Paradoxically, all calves developed high titers of IgG antibodies to both MSP1a and MSP1b polypeptides. These findings suggest that in calves immunized with MSP1 heteromorphic complex, MSP1a-specific T lymphocytes may provide help to MSP1b-specific B lymphocytes. The data provide a basis for determining whether selected MSP1a CD4\(^+\) T-lymphocyte epitopes and selected MSP1a and MSP1b B-lymphocyte epitopes presented on the same molecule can stimulate a protective immune response.

Anaplasmosis is an important disease of livestock caused by the tick-transmitted rickettsial pathogen *Anaplasma marginale*, which invades and replicates within erythrocytes, resulting in high levels of rickettsialemia, hemolytic anemia, and often death. Protection against disease and infection can be achieved by immunization with outer membranes or purified outer membrane proteins (12, 35, 44). Among these, major surface protein 1 (MSP1) is an immunoprotective heteromeric complex of high-molecular-weight MSP1a and MSP1b proteins (6, 14, 30, 31). Immunization with purified native MSP1 induces protection against homologous and heterologous strain challenge, as shown by the significant reduction in rickettsialemia and anemia (14, 30, 31). Furthermore, MSP1 was recognized by memory CD4\(^+\) T lymphocytes in *A. marginale* outer membrane protein-immunized cattle that were completely protected against the development of rickettsialemia following challenge (12).

The MSP1 complex is composed of a single MSP1a polypeptide that is covalently linked, via disulfide bonds, to MSP1b polypeptides (6, 29, 46). MSP1a, encoded by a single *msp1a* gene, is invariant within a strain but varies in size among strains (3, 29). The size variation in MSP1a among strains results from the presence at the amino (N) terminus of the protein of variable numbers of a semiconserved 28- or 29-amino-acid (aa) serine-rich repeat, which contains a neutralization-sensitive epitope (3, 36). This epitope, defined by monoclonal antibodies (MAb) ANA22B1 and ANA15D2, is conserved among all strains (3, 27). Conserved serine-rich motifs have also been identified in the repeat units of several high-molecular-weight proteins of the agent of human granulocytic ehrlichiosis (HGE) *Ehrlichia phagocytophila*, which has recently been reclassified as *Anaplasma phagocytophila* (17), and related organisms *Ehrlichia chaffeensis* and *Ehrlichia canis* (23, 42, 48, 49).

MSP1b is encoded by two or more *msp1b* genes in the Florida (FL) strain (5, 6, 14, 47). The originally identified MSP1b1 (5), which we designated MSP1bF1 to indicate its FL strain origin (14), and a second protein, MSP1b2 (47), which we designated MSP1bF3 (14), were each expressed in the
MSP1 complex (47). It is not known whether additional \textit{msp1/β} transcripts F2 and F4 identified in the FL strain are also expressed (14). The \textit{msp1/β} genes and their encoded proteins are very closely related, most likely reflecting their origination by gene duplication (14, 47). The MSP1β polypeptides share a highly conserved core sequence with five discrete blocks of variation, which are predicted to be surface exposed. However, there appears to be minimal variation in these MSP1β copies between strains (14). This finding is consistent with the observation that MSP1β B-cell epitopes recognized by either MAb or polyclonal antibodies from MSP1-immunized and protected calves are conserved among all strains examined (24, 30).

Acquired immunity to ehrlichial pathogens involves both neutralizing antibody and gamma interferon (IFN-γ)-mediated activation of phagocytic cells, which kill the organisms via nitric oxide or related molecules (2, 4, 35, 37, 43). In \textit{A. marginale} MSP1-immunized calves protected against challenge, high titers of antibody were induced which were similar for MSP1a and MSP1b (30). Antibody specific for the MSP1 complex, MSP1a, or MSP1b inhibits the binding of \textit{A. marginale} to erythrocytes (24, 25), suggesting an in vivo role for neutralizing antibody (24) and optimal opsonization and subsequent organism killing requires the induction of high-affinity immunoglobulin G (IgG), and optimal opsonization and subsequent organism killing requires the induction of high-affinity IgG2 subclass (in cattle) and macrophage phagocytosis (15). Efficient neutralization likely requires the induction of high-affinity immunoglobulin G (IgG), and optimal opsonization and subsequent organism killing requires the induction of both the IgG2 subclass (in cattle) and macrophage activation (26, 35). As in other species, these effector mechanisms are dependent on major histocompatibility complex (MHC) class II-restricted, antigen-specific, IFN-γ-secreting CD4+ T lymphocytes (10, 18). Thus, an effective recombinant or DNA MSP1 vaccine should include both strain-conserved helper T-lymphocyte epitopes and B-lymphocyte epitopes important for eliciting neutralizing and opsonizing antibody.

In contrast to immunization with the native MSP1 complex, immunization with recombinant MSP1a and MSP1b alone or in combination failed to provide protective immunity in spite of the induction of high antibody titers (33; T. C. McGuire, unpublished observations). The reasons for the failure of the recombinant vaccines are not known. However, possible explanations include the use of only a single MSP1b (F1) polypeptide in the immunogen and the lack of covalent association in the heteromeric complex. The presence of serine-rich repeats within MSP1a that vary in number and sequence between MSP1a, or MSP1b inhibits the binding of \textit{A. marginale} to erythrocytes (24, 25), suggesting an in vivo role for neutralizing antibody in blocking initial steps in invasion. Additionally, antibody to MSP1 opsonizes live organisms for macrophage-mediated phagocytosis (15). Efficient neutralization likely requires the induction of high-affinity immunoglobulin G (IgG), and optimal opsonization and subsequent organism killing requires the induction of both the IgG2 subclass (in cattle) and macrophage activation (26, 35). As in other species, these effector mechanisms are dependent on major histocompatibility complex (MHC) class II-restricted, antigen-specific, IFN-γ-secreting CD4+ T lymphocytes (10, 18). Thus, an effective recombinant or DNA MSP1 vaccine should include both strain-conserved helper T-lymphocyte epitopes and B-lymphocyte epitopes important for eliciting neutralizing and opsonizing antibody.

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MATERIALS AND METHODS

\textit{Anaplasma} strains and preparation of homogenates and MSP1 antigen. The \textit{A. marginale} strains used in this study are designated by their original location of isolation, and include FL, Washington’/Clarkston (WA/C), Washington’/Okanogan, Virginia (VA), South Idaho, and St. Maries, Idaho (St. M). A strain of \textit{Anaplasma ovis} isolated in Idaho was also used. These have been described or referenced previously (11, 12, 27, 39). \textit{Anaplasma} strains were maintained as liquid nitrogen cryopreserved stablates of infected bovine erythrocytes in dimethylsulfoxide-phosphate-buffered saline (PBS). Antigen was prepared for in vitro assays by resuspending organisms in PBS in the presence of protease inhibitors and homogenization either by sonication or by two passages through a French pressure cell (SLM Instruments, Urbana, Ill.) (12). Native MSP1 protein was isolated from the FL strain of \textit{A. marginale} by MAb affinity chromatography using MAb ANA15D2 or ANA22B1 (30, 31).

Recombinant MSP1 proteins and peptides. Recombinant MSP1a (FL strain) was expressed in vaccinia virus (28), and recombinant MSP1bF1 was expressed in \textit{Escherichia coli} (6). Proteins were isolated by affinity chromatography (31) using MAb ANA15D2 (MSP1a) and MAb AMA-1 (MSP1b). Recombinant MSP1b proteins F2 to F4 were expressed in \textit{E. coli} as His-tagged fusion proteins and purified by affinity to Ni²⁺-charged columns (Novagen, Milwaukee, Wis.) as described (14). The C-terminal region of MSP1a from the FL strain encompassing aa 242 to 767, which lacks the N-terminal repeat region, was prepared as a recombinant maltose-binding fusion protein. This region was amplified by PCR from genomic DNA using forward primer 3'-TATGAAATCTGATTTGGGCGCA-5' and reverse primer 5'-ATATAGATTTTCTACCCCGCGCC-3', and the PCR product was ligated into the pMAL C.2 vector (New England Biolabs, Beverly, Mass.) following EcoRI digestion of the vector and amplicon as described in the manufacturer’s protocol. \textit{E. coli} XL-1 Blue (Stratagene, La Jolla, Calif.) carrying the recombinant plasmid, designated B164, and encoding the maltose-binding protein (MBP) fused to the C region of MSP1b was grown at 37°C in Luria broth containing 2% glucose under constant agitation. Protein expression was induced with isopropylβ-D-thiogalactopyranoside (IPTG) at 37°C, and the cells were harvested by centrifugation at 2,000 x g. Pelleted bacteria were resuspended in lysis buffer (200 mM NaCl, 1 mM EDTA, 20 mM Tris [pH 7.4]) containing 0.5% Nonidet P-40 and subjected to one cycle of freeze-thawing and sonication. The lysate was centrifuged for 10 min at 15,000 x g, and the recombinant protein was purified from the supernatant by affinity chromatography on amyllose resin columns (New England Biolabs) as described by the manufacturer. Purified fusion protein was diazylated extensively against PBS and stored at −20°C. MBP purchased from New England Biolabs was used as a control antigen. Protein concentrations were determined by the Bradford assay (Bio-Rad, Hercules, Calif.).

Peptides that compose the N-terminal repeat region of MSP1a were synthesized by Gerhardt Munske (Laboratory for Biotechnology and Bioanalysis I, Washington State University, Pullman, Wash.). Peptide B (ADSSSAGGQQEESVSVOSSQDSATSSQSLG) is tandemly repeated seven times in the N terminus of the FL strain of \textit{A. marginale} MSP1a (3). Peptide A (DDSSSAGGQQEESVSVOSSQDSATSSQSLG) is found as a single variant of the peptide B repeat in the N-terminal region of MSP1a (FL). A 29-aa Babesia bigemina rhoptry-associated protein-1 (RAP1) peptide, FSLNLLRNLFLGGDDKNA LHGFQKYFYFM, was used as a control peptide antigen in proliferation assays. All antigens were resuspended in PBS and stored at −20°C.

Immunization of calves with MSP1. Three 6-month-old Holstein calves were immunized intramuscularly four times at 2-week intervals with 20 μg per injection of native MSP1 emulsified in complete Freund’s adjuvant for the first injection and in incomplete Freund’s adjuvant for subsequent injections. The bovine rhophagy antigen (BoLA-A) class I alleles of the calves were determined by serological typing (16), and DRB1 alleles were determined by PCR-fragment restriction length polymorphism analysis of exon 2 (45). The BoLA-DQ haplotypes were inferred from BoLA-A and DRB3 typing on the basis of haplotypes defined in the Seventh International BoLA Workshop (21; also see the BoLA Nomenclature Web Site [http://www2.rzbin.ac.bau/bo/]). The DRB3-DQ haplotypes are as follows: for call 87, DRB3*16-DQA1*1A-DQB1*1C/DRB3*13-DQA1*10-DQB1*10; for call 96, DRB3*22-DQA1*9B-DQB1*9B/DRB3*3-DQA1*10-DQB1*10; for call 95, DRB3*24-DQA1*1A-DQB1*1/DRB3*23-DQA1*7D-DQB1*7A; and for cow G4, DRB3*18*23. For cow G4 (Charolais) the DQ alleles were not inferred because of insufficient information on this breed.
Reactivity of sera from MSP1-immunized calves with native MSP1 and recombinant MSP1a and MSP1b proteins. Peptidemunization sera and sera obtained from immunized calves 2 weeks following the last inoculation of antigen were tested for reactivity to MSP1 proteins by immunoblotting (14). Sera were adsorbed extensively with E. coli lysates. A known positive serum from MSP1-immunized animal B541 was used as a control (14). Briefly, 2 μg of antibody affinity-purified MSP1, recombinant MSP1a C region, or MSP1b was electro-phoresed in a 4 to 20% gradient gel containing sodium dodecyl sulfate, transferred to nitrocellulose, and then incubated with 10-fold dilutions (1:13,000 to 1:300,000) of bovine IgG. Bound IgG was detected using a 1:2,000 dilution of peroxidase-conjugated protein G (Zymed Laboratories, Inc., San Francisco, Calif.) and developed by enhanced chemiluminescence using the ECL reagent (Amersham, Arlington Heights, Ill.) according to the manufacturer’s protocol. Student’s one-tailed t test was used to determine significant differences between antibody titers in the different groups. A dot blot assay was performed to detect antibody specific for the 29-aa repeat, peptide B, as described (3). Peptide (1 μg) was applied to nitrocellulose and incubated with serially diluted sera, and IgG was detected as described for immunoblots.

A. marginale-specific T-lymphocyte lines and clones. Short-term T-lymphocyte lines were repeatedly established from peripheral blood mononuclear cells (PBMC) of A. marginale-immunized calves 87, 93, and 96 from shortly after immunization to more than 1 year later. In all experiments, cell lines were propagated by stimulation with homogenate prepared from the FL strain of A. marginale, with native MSP1, or with alternate stimulations with the two antigens. Briefly, 4 × 10^6 PBMC were cultured per well in 24-well plates (Costar, Cambridge, Mass.) in a volume of 1.5 ml of complete RPMI 1640 medium (9) with 1 to 10 μg of A. marginale homogenate or MSP1 per ml. After 7 days and weekly thereafter, cells were subcultured to a density of 7.5 × 10^5 cells/well and cultured with 2 × 10^5 irradiated (3,000 rad) autologous PBMC as a source of antigen-presenting cells (APC) with or without antigen, which was often given on alternate weeks to lower background proliferation. T-lymphocyte lines were maintained for up to 5 weeks, and cells were assayed for antigen-dependent proliferation 7 days following the last stimulation. In most experiments γ6 T lymphocytes were depleted by incubating either PBMC or cell lines with γ6 T-cell receptor 1–specific MAb CACT 61A and complement as described (11). T-lymphocyte clones were obtained from MSP1-specific cell lines by limiting dilution (13). Clones were propagated with A. marginale homogenate and 10% bovine T-cell growth factor (8). Frequencies of positive wells were 10 to 26% (1 cell per well) and 4 to 9% (0.3 cells per well).

Cell surface phenotypic analysis. Differentiation markers on T-lymphocyte lines and clones were analyzed by fluorescence-activated cell sorting (12). The MAB used were specific for bovine CD2 (MAb MUC2A), CD3 (MAb MM1A), CD4 (MAb CACT 138A), CD8 (MAb CACT 80C and BAT 82B), and the T-cell receptor (MAb CACT 61A) purchased from the Washington State University Monoclonal Antibody Center, Pullman.

Lymphocyte proliferation assays. Proliferation assays were carried out in replicate wells of round-bottomed 96-well plates (Costar) for 3 to 5 days when using PBMC or for 3 to 4 days when using short-term T-lymphocyte lines or T-lymphocyte clones, as described (11–13). PBMC (2 × 10^5) were cultured in replicate wells with antigen in a total volume of 100 μl of complete RPMI 1640 medium. T-lymphocyte lines and clones (3 × 10^5 cells) were cultured in duplicate or triplicate wells in a total volume of 100 μl of complete medium containing antigen and 2 × 10^5 APC. APC consisted of irradiated PBMC from the autologous donor or from calves either sharing one DRB3-DQ haplotype (half-matched) or mismatched at the DRB3-DQ locus. Antigens consisted of homogenate (0.2 to 25 μg/ml) prepared from different strains of A. marginale or A. ovis, native MSP1 protein, recombinant MSP1a and MSP1b proteins, and 0.1 to 10 μg of peptide per ml. Membranes prepared from uninfected red blood cells (URBC) and recombinant MBP were used as negative control antigens. Cells were radiolabeled for the last 18 h of culture with 0.25 μCi of [3H]thymidine, harvested using an automated cell harvester (TomTec, Orange, Conn.), and counted with a liquid scintillation counter. Results are presented as the mean cpm of replicate cultures ± 1 standard deviation (SD), or for ease of presentation, as the stimulation index (SI), which represents the mean cpm of replicate cultures of cells plus antigen divided by the mean cpm of replicate cultures of cells plus medium or URBC. The Student’s t test was used to determine statistically significant differences in proliferation induced by using different antigens or APCs. A p value of 0.05 was considered significant.

Detection of IFN-γ in supernatants of T-lymphocyte lines. Cell lines were cultured for 1 week with A. marginale and for 1 week without antigen and then were restimulated for 72 h with autologous APC and 10 μg of MSP1 C region-MBP fusion protein per ml, and supernatants were tested for IFN-γ production by enzyme-linked immunosorbent assay (ELISA). Controls consisted of supernatants from cell lines stimulated with MBP. The bovine IFN-γ assay was performed using an ELISA kit (BOVIGAM; CSL Limited, Parkville, Victoria, Australia) according to the manufacturer’s protocol. The IFN-γ activity in culture supernatants diluted 1:4 to 1:1,000 was determined by comparison with a standard curve obtained with a supernatant from a Mycobacterium bovis purified protein derivative (PPD)-specificTh lymphocyte clone that contained 440 U of IFN-γ per ml (previously determined by the neutralization of vesicular stomatitis virus [12]). In our assay, 1 U corresponds to 1.7 ng of IFN-γ (7). The results are presented as units of IFN-γ per ml. Student’s one-tailed t test was used to determine the significance of IFN-γ produced by cell lines stimulated with MSP1 C region antigen or MBP.

RESULTS

MSP1a- and MSP1b-specific antibody responses. It was previously shown that MAb (ANA15D2 and ANA22B1) specific for the neutralizing epitope on the repeat region of MSP1a reacted with all isolates (31), although antibody responses in MSP1-immunized calves directed against this epitope were not reported. Therefore, titers of antibody to MSP1 and its subunits, including the MSP1a repeat region, were determined. Strong antibody responses were observed against the immunizing native MSP1 complex (Table 1). Furthermore, the titers of antibody directed against either the C-terminal region of MSP1a, which lacks the N-terminal serine-rich repeats, or MSP1b were not significantly different (P = 0.2). The use of protein G to detect bound immunoglobulin indicates that the antibody is composed mainly of IgG. Interestingly, antibodies were also present to the 29-aa peptide repeat (peptide B) which has seven copies in MSP1a of the FL strain and contains the neutralizing antibody epitope defined by MAb ANA15D2 and ANA22B1 (3, 31). For comparison, a known positive serum from cow B541 that was immunized previously with MSP1 from the FL isolate (14) also responded strongly to all antigens, whereas preinfection sera from all calves were negative (data not shown). Thus, sera from calves immunized with the MSP1 complex consisting of covalently associated MSP1a and MSP1b proteins recognize both proteins and at least two epitopes on MSP1a. One epitope is located in the N-terminal repeat that contains the neutralization-sensitive B-cell epitope EASTS(S/Q)ASTSS (3), and at least one epitope is present on the C-terminal region of MSP1a.

<table>
<thead>
<tr>
<th>Cow or cell no.</th>
<th>MSP1</th>
<th>MSP1a C region</th>
<th>MSP1a peptide B</th>
<th>MSP1b</th>
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<tr>
<td>Calves</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>87</td>
<td>300,000</td>
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</tr>
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<td>1,000,000</td>
<td>100,000</td>
<td>100,000</td>
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</table>

* Following the final immunization of calves 87, 93, and 96, titers of antibody to MSP1, MSP1a, and MSP1b were determined. A known positive antiserum from MSP1-immunized cow B541 served as a positive control. Titers were determined by end point titration by using serial dilutions of protein G-purified immunoglobulin in immunoblots using native MSP1 and recombinant MSP1b (F2), or the nonrepeat C-terminal region of MSP1a (MSP1a-C region), or in dot blots using 1 μg of MSP1a peptide B per spot. Preimmunization sera were negative when tested at 1:3,000 and 1:10,000 dilutions (data not shown).
MSP1 immunization induced a strain-conserved long-lasting memory T-lymphocyte response. To evaluate T-lymphocyte responses following MSP1 immunization, lymphocyte proliferative responses to *A. marginale* were monitored during and after immunization with MSP1 (FL strain). PBMC obtained prior to immunization did not respond to *A. marginale* (data not shown). However, *A. marginale*-specific PBMC proliferative responses were consistently observed in all three calves shortly after the fourth immunization. Strong responses to MSP1 and the homologous *A. marginale* FL strain homogenate were still present at 14 months after immunization (Fig. 1), which were significantly greater than the response to control uninfected erythrocyte (URBC) antigen (*P* < 0.05).

To determine the presence of T-lymphocyte epitopes on MSP1 that were conserved among *A. marginale* strains, homogenates from six different strains were compared for stimulation of PBMC from calves immunized with MSP1. All strains tested stimulated significant (*P* < 0.05) levels of proliferation compared with URBC, and these levels were comparable to those induced by the immunizing FL strain (Fig. 2). Results are presented for 5 µg of antigen per ml, for which the SI ranged from 22.0 to 61.3 for calf 87, 8.1 to 42.7 for calf 93, and 6.8 to 14.4 for calf 96, which were representative of two experiments.

To ascertain that CD4+ T cells responded to MSP1, PBMC depleted of γδ T cells were used to establish short-term T-lymphocyte lines by stimulation with *A. marginale* homogenate and/or MSP1 isolated from the FL strain. After 2 or 3 weeks of culture, the cell lines consisted predominantly of CD4+ T cells, with 1 to 8% CD8+ cells and 4 to 8% γδ T cells. As observed with PBMC, the CD4+ T-lymphocyte-enriched lines obtained approximately 1 year following immunization repeatedly proliferated to all four strains of *A. marginale* tested. However, these cell lines did not respond to *A. ovis* (Table 2). Together, these data indicated that T-lymphocyte epitopes that were conserved among *A. marginale* strains were not conserved in *A. ovis*.

T-lymphocyte responses in MSP1-immunized calves were preferentially directed against MSP1a. Since calves mounted strong antibody responses to both MSP1a and MSP1b (reference 30 and Table 1), it was of interest to determine whether both proteins were recognized by memory T lymphocytes. The proliferative responses against antibody affinity-purified recombinant MSP1a and MSP1bF1 proteins by PBMC from MSP1-immunized calves were compared (Fig. 3). In experiments repeated at least three times, strong, dose-dependent and significant (*P* < 0.05) levels of proliferation were observed in response to to MSP1a that were comparable to proliferation induced by *A. marginale* homogenate. In contrast, MSP1b failed to stimulate significant recall responses compared with negative control URBC antigen. There was also no significant proliferation when three different Ni²⁺ affinity-purified His-tagged MSP1b (F2 to F4 [reference 14]) proteins were tested (data not shown).

To exclude the possibility that PBMC may contain a low frequency of MSP1b-specific T lymphocytes, memory T lymphocytes were expanded by short-term culture with *A. marginale* or MSP1. However, when these CD4+ T-lymphocyte-enriched and γδ T-lymphocyte-depleted cell lines were examined, MSP1a was again the dominant subunit that evoked a memory response. In multiple assays performed with independently derived cell lines cultured from 1 to 3 weeks with *A. marginale* and/or MSP1, lines from calves 87 and 96 responded significantly (*P* < 0.05) only to MSP1a. Representative data are presented for a 2-week line from calf 87 (Fig. 4A) and for a 1-week line from calf 96 (Fig. 4C). In addition, MSP1b-specific cell lines could not be propagated by in vitro culture with MSP1bF1 (data not shown). Similarly, the dominant response by T-lymphocyte lines from calf 93 was to MSP1a (Fig.
T-lymphocyte responses to MSP1a were presented by three different MHC class II haplotypes. To determine whether the response to MSP1a was MHC class II haplotype restricted, autologous APC, APC matched for one DRB3-DQ haplotype, or mismatched APC were used to present antigen to CD4+ T-cell lines. Cell lines from all three calves responded to A. marginale, MSP1, and MSP1a when autologous APC or APC matched for one DRB3-DQ haplotype were used, but the response to antigen was severely reduced or absent in the presence of DRB3-DQ-mismatched APC (results are presented in Fig. 5 for MSP1 and MSP1a). The presentation of antigen by DRB3-DQ-mismatched G4 APC was significantly less than presentation by autologous APC (P < 0.05), with the exception of 96 T cells and MSP1a (P = 0.08). The response to antigen presented by APC matched for one DRB3-DQ haplotype was not significantly different than the response to autologous APC. The proliferative response to T-cell growth factor was unaffected by using different APC, and there was no proliferation to MSP1b (data not shown). Based on the DRB3 typing of the APC donor cattle, all three DRB3 alleles (+3, +16, and +22) and/or closely linked DQ alleles presented antigen to primed T cells. The results presented in Fig. 5 are similar to those obtained in a second experiment where the haplotype-mismatched APC from calf 95 had the DRB3 *24 and *23 alleles (data not shown).

T-lymphocyte responses to MSP1a were preferentially targeted to the unique C-terminal region. Because B-lymphocyte epitopes were identified in both the N-terminal repeat (peptide B) and C-terminal nonrepeat region of MSP1a (Table 1), it was of interest to determine which of these regions contained T-lymphocyte epitopes. Whereas the C-terminal region of MSP1a expressed as an MBP fusion protein stimulated significant proliferation of PBMC of all animals, the N-terminal peptide B was only recognized by PBMC from calf 87. In multiple experiments, PBMC from calf 87 but not calves 93 or 96 responded to the 29-aa peptide B, which constitutes seven of the eight repeated peptides (Table 4). Peptide A, which varies in amino acid sequence from peptide B, did not stimulate any PBMC (data not shown). Short-term lymphocyte lines in culture for 1 to 3 weeks from calf 87 also responded significantly (P < 0.01) to the N-terminal repeat peptide B (Fig. 6A) but did not respond to peptide A (data not shown). However, short-term T-lymphocyte lines from all calves proliferated significantly in response to the C-terminal region of MSP1a and to MSP1 (Fig. 6A) (P < 0.01). In contrast, there was no response to MBP (data not shown). Relatively high levels of

### Table 2. Short-term T-lymphocyte lines respond to different A. marginale strains but not to A. ovis

<table>
<thead>
<tr>
<th>Calf no.</th>
<th>FL</th>
<th>VA</th>
<th>WA/C</th>
<th>St. M</th>
<th>A. ovis</th>
</tr>
</thead>
<tbody>
<tr>
<td>87</td>
<td>15,957 ± 1,347&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9,015 ± 3,384</td>
<td>18,096 ± 2,557&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>93</td>
<td>31,586 ± 13&lt;sup&gt;b&lt;/sup&gt;</td>
<td>17,550 ± 1,729&lt;sup&gt;b&lt;/sup&gt;</td>
<td>24,882 ± 535&lt;sup&gt;b&lt;/sup&gt;</td>
<td>26,477 ± 2,237&lt;sup&gt;b&lt;/sup&gt;</td>
<td>&lt;BR&gt;&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>96</td>
<td>17,644 ± 2,336&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8,997 ± 1,558</td>
<td>20,870 ± 2,209&lt;sup&gt;b&lt;/sup&gt;</td>
<td>11,994 ± 134</td>
<td>&lt;BR&gt;&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup> T lymphocytes (3 × 10<sup>6</sup>) from cell lines in culture for 2 weeks were tested in a 3-day proliferation assay with APC (2 × 10<sup>5</sup>) and antigen prepared from the indicated strains of A. marginale or A. ovis. Results are presented for 10 μg of antigen per ml, after subtracting the background response to medium, and are representative of two or three experiments per animal.

<sup>b</sup> Responses were significantly greater (P < 0.05) than those with 10 μg of URBC per ml.

<sup>c</sup> <BR>, less than background response to medium.
IFN-γ were also produced by the T-cell lines in response to the C-terminal portion of MSP1a, which among individual cell lines ranged from 123 to 282 U/ml (Fig. 6B) and 136 to 1,032 U/ml in a second assay (data not shown). Control MBP stimulated significantly less IFN-γ in all three lines (P < 0.03), ranging from 0 to 4 U/ml (Fig. 6B).

**DISCUSSION**

MSP1 is a candidate vaccine antigen of *A. marginale*, and an effective vaccine should include both Th and B-cell epitopes. In this and earlier studies (30, 31), all MSP1-immunized calves produced high titers of antibody against both MSP1a and MSP1b subunits of the heteromeric complex. We extend this finding to show that MSP1a contains at least two B-lymphocyte epitopes recognized by immune bovine sera. One or more epitopes which induced high levels of antibody reside in the nonrepeat C-terminal region of MSP1a. An additional B-cell epitope is found within the N-terminal serine-rich tandemly repeated peptide (peptide B) of MSP1a known to contain a strain-conserved neutralization-sensitive epitope defined by MAb (3, 27, 29, 31). Antibody to MSP1 could function in vivo to block erythrocyte invasion or to facilitate organism uptake by macrophages (15, 24, 25). However, it is unlikely that antibody alone is sufficient to protect cattle against anaplasmosis, since passively administered sera from immune donors which had high titers of antibody failed to provide protection against *A. marginale* challenge (20). Furthermore, in a murine model of HGE, antibody reduced the level of rickettsemia but did not afford complete protection against challenge (43). Together, these studies indicated the importance of additional effector mechanisms against genogroup II ehrlichial pathogens. For this reason, we investigated T-lymphocyte recognition of MSP1a and MSP1b polypeptides by CD4+ T cells in calves immunized with native MSP1.

In this study of MSP1-immunized calves, MSP1a was recognized preferentially by CD4+ T lymphocytes from all calves, whereas MSP1b was transiently recognized by T lymphocytes from a single calf. Because MSP1b is encoded by a multigene family, which in the FL strain consists of two expressed proteins and two potentially expressed proteins (14, 47), all known and potential antigenically variant MSP1b proteins were tested for the ability to elicit a memory T-lymphocyte response. Only MSP1bF1 stimulated T-lymphocyte proliferation of a single cell line from one calf. Although limited dilution cloning of this cell line yielded two clones that responded specifically to

**TABLE 3. Summary of responses of T-lymphocyte clones to MSP1a and MSP1b**

<table>
<thead>
<tr>
<th>Calf no.</th>
<th>No. of clones that responded to the indicated antigen</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MSP1 MSP1a MSP1b</td>
</tr>
<tr>
<td>87</td>
<td>7 7 0</td>
</tr>
<tr>
<td>93</td>
<td>21 19 2</td>
</tr>
<tr>
<td>96</td>
<td>20 20 0</td>
</tr>
</tbody>
</table>

a Two cell lines from calf 87 cultured for 2 or 4 weeks with *A. marginale* were cloned by limiting dilution. PBMC from calves 93 and 96 were depleted of γδ T cells, stimulated with *A. marginale* for 1 week and with MSP1 for 1 week, and cloned by limiting dilution. In a separate cloning experiment, PBMC from calf 96 were depleted of γδ T cells, cultured with *A. marginale* for 3 weeks, and cloned by limiting dilution.

b Clones were tested in proliferation assays with MSP1 (5 μg/ml) and MSP1a or MSP1bF1 (2 μg/ml). The data represent total numbers of MSP1-specific clones from individual animals. A clone was considered to respond positively if the SI in response to the antigen was >3.0.
MSP1b, it was not possible to reproducibly generate MSP1b-specific T-lymphocyte lines from this calf. Nevertheless, these data indicate that the lack of MSP1bF1 response is not due to the poor quality of the antigen.

The dominant MSP1a-targeted response was observed in calves that expressed three different MHC class II haplotypes. Three DRB3-DQ haplotypes were capable of presenting antigen, indicating that the preferential recognition of MSP1a is not limited to a restricted set of MHC class II proteins. Based on DRB3-DQ allelic frequencies in Holstein populations, approximately 50% of Holstein or Friesian cattle in a given herd would be predicted to have at least one of the DRB3-DQ haplotypes evaluated in this study (reference 40 and H. A.
TABLE 4. Proliferation of PBMC from MSP1-immunized calves against the N-terminal repeat region (peptide B) and the C-terminal region of MSP2

<table>
<thead>
<tr>
<th>Expt no. and antigen</th>
<th>Proliferation (SI) by PBMC from calf no.abc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>87</td>
</tr>
<tr>
<td>1</td>
<td></td>
</tr>
<tr>
<td>A. marginale</td>
<td>57.3</td>
</tr>
<tr>
<td>MSP1a</td>
<td>61.4</td>
</tr>
<tr>
<td>MSP1a peptide B</td>
<td>33.1</td>
</tr>
<tr>
<td>RAP1 peptide</td>
<td>3.0</td>
</tr>
<tr>
<td>2</td>
<td></td>
</tr>
<tr>
<td>A. marginale</td>
<td>27.1</td>
</tr>
<tr>
<td>MSP1</td>
<td>22.8</td>
</tr>
<tr>
<td>MSP1a C-region</td>
<td>24.8</td>
</tr>
<tr>
<td>MBP</td>
<td>2.6</td>
</tr>
</tbody>
</table>

a Results are presented as the SI comparing the mean cpm of triplicate cultures of PBMC cultured with antigen and those cultured with control URBC antigen. Data are presented for optimal antigen concentrations, which were 25 μg/ml for A. marginale, 5 μg/ml for MSP1a, or 10 μg/ml for peptide B or control peptide derived from the B. bigemina RAP1 protein, versus 5 μg/ml for control URBC (experiment 1) and 10 μg/ml for antigen (calf 87 and 96) or 2 μg/ml for antigen (calf 93) versus 10 or 2 μg/ml for URBC, respectively (experiment 2). An SI of >3.0 was considered significant.

Lewin, unpublished observations). Thus, MSP1a should be broadly recognized by these breeds.

The T-cell epitope(s) in MSP1a recognized by all calves is located in the C region of MSP1a. The C region is highly conserved among strains of A. marginale, whereas the N region is composed of tandem repeats of a serine-rich 28- or 29-aa peptide that vary slightly in sequence within and between strains (3). Because T lymphocytes from only one calf proliferated to the N-terminal repeat region (peptide B), the sequence conservation in the C terminus likely explains the response of CD4+ T-cell lines from MSP1 (FL)-immunized calves to multiple strains of A. marginale. However, A. ovis was not recognized by A. marginale MSP1a-specific T-cell lines. We have also been unable to amplify MSP1a from A. ovis genomic DNA using primers derived from the A. marginale sequence, suggesting that the sequence is not highly conserved between Anaplasma species (G. H. Palmer, T. C. McGuire, and W. C. Brown, unpublished observations). In contrast, the A. marginale MSP2 is highly conserved between the species and, as a component of the same A. ovis homogenate antigen used in the present study, stimulated proliferation of A. marginale MSP2 specific T-cell lines and clones (11).

The dominant proliferative T-cell response to the C region of MSP1a was mirrored by a strong IFN-γ response. A similar positive correlation between CD4+ T-lymphocyte proliferation and IFN-γ production in response to MSP2 and MSP2-derived peptides was recently demonstrated (11). The production of IFN-γ in response to candidate vaccine antigens of A. marginale and related ehrlichiae is an important consideration for vaccine development (35). In calves and mice, IFN-γ production was associated with protection against A. marginale or HGE, respectively (2, 12). Through production of IFN-γ, CD4+ T cells are critical for activating macrophages to secrete nitric oxide (1, 22, 41), which is inhibitory for ehrlichial pathogens (4). Furthermore, in cattle, IFN-γ promotes isotype switching to IgG2, the best opsonin (10, 18, 26). Thus, induction of both strong memory CD4+ T-lymphocyte proliferative and IFN-γ responses by the strain-conserved C region of MSP1a provides a rationale for identifying Th-cell epitopes within this region for inclusion in a vaccine for A. marginale.

The undetectable or weak CD4+ T-lymphocyte responses to MSP1b coupled with the induction of high titers of MSP1b-specific IgG antibody suggests that MSP1a-specific CD4+ T lymphocytes function as helper cells to promote isotype switching in MSP1b-specific B cells. Since MSP1a and MSP1b are covalently associated in the native protein (46), it is possible that during cognate T-cell-B-cell interactions, B cells specific for MSP1b recognize the MSP1 complex, and through surface immunoglobulin receptor-mediated endocytosis, process and present MSP1a peptides to specific Th cells (19). Disulfide bonding between MSP1a and MSP1b polypeptides may be required for correct processing and presentation of T-lympho-

FIG. 6. Proliferation and IFN-γ production by MSP1-specific lymphocytes in response to the C-terminal region of MSP1a. (A) PBMC were cultured for 1 week with A. marginale and tested for proliferation in a 3-day assay with antigen (0.4, 2, or 10 μg/ml) in triplicate cultures. Results are presented as the mean cpm ± 1 SD (error bars) of triplicate cultures stimulated with the optimal concentration of antigen, which was 10 μg/ml for calves 87 and 96 and 2 μg/ml for calf 93 lymphocytes, after subtracting the background cpm for cells cultured without antigen, and are representative of at least three experiments. (B) Cell lines were cultured for 1 week with A. marginale and for 1 week without antigen and then were restimulated for 72 h with autologous APC and 5 μg of MSP1a C-region or control MBP antigen per ml, and supernatants were tested for IFN-γ production by ELISA. Additional controls consisted of supernatants from APC stimulated with antigen.
cyte epitopes on MSP1a and/or cognate MSP1a-specific T-cell–MSP1b-specific B-cell interactions that will elicit a recall response upon challenge. If this hypothesis is true, the failure of recombinant MSP1a and MSP1b proteins to induce protective immunity may be due to the lack of covalent association. An alternative explanation for the failure of the recombinant proteins to induce protective immunity is the use of a single MSP1b (F1) protein in these earlier studies, which could be important if variant epitope-specific antibody is involved in protection. Experiments designed to induce immunity with either recombinant MSP1a covalently associated with recombinant proteins including all MSP1b variants or a hybrid protein containing defined MSP1a-specific Th-cell epitopes and MSP1b-specific B-cell epitopes could test these possibilities.

Although a true homologue of A. marginale MSP1 has not been identified in other ehrlichiae, high-molecular-mass proteins which contain multiple serine-rich repeats have been described for E. chaffeensis, E. canis, and the agent of HGE (42, 48, 49). The function of these proteins is not known, but like MSP1, they appear to be surface exposed (6, 34, 38) and are antigenic in naturally infected individuals (30, 42, 48). The serine-rich repeats are believed to be sites for a novel form of glycosylation (23), which could explain why the proteins that contain these repeats migrate on polyacrylamide gels with a molecular mass higher than that predicted by sequence alone. Interestingly, E. coli-expressed proteins were also glycosylated and contained the same composition of carbohydrate-drates as the native proteins (23). A similar glycosylation pattern of A. marginale MSP1a is predicted from the presence of serine-rich repeats and from the finding that, for a given strain, MSP1a has an apparently higher molecular mass than that predicted by the sequence (3). Studies are in progress to determine whether MSP1a is glycosylated. Whether antibody directed against carbohydrate epitopes on the serine-rich repeat regions of ehrlichial outer membrane proteins is important for neutralizing infectivity remains to be determined.

In summary, we have identified MSP1a as the major immunogenic component of the protective A. marginale MSP1 heteromeric complex for CD4+ T lymphocytes. Importantly, the MSP1a-specific response is preferentially directed against the C-terminal region, which is highly conserved among A. marginale strains. This knowledge, together with induction of strong, memory CD4+ T-lymphocyte proliferative and IFN-γ responses by the C region of MSP1a in cattle with a broad representation of MHC class II haplotypes, provides the basis for including MSP1a C-terminal region T-lymphocyte epitopes in a vaccine. The epitopes recognized by MSP1a-specific CD4+ T-lymphocyte lines and clones are currently being defined.

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