

Expression of Major Surface Protein 2 Variants with Conserved T-Cell Epitopes in *Anaplasma centrale* Vaccinates

Varda Shkap,¹ Thea Molad,¹ Kelly A. Brayton,² Wendy C. Brown,² and Guy H. Palmer^{2*}

Department of Parasitology, Kimron Veterinary Institute, Bet Dagan, Israel,¹ and Program in Vector-Borne Diseases, Washington State University, Pullman, Washington²

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Major surface protein 2 (MSP-2), identified as a protection-inducing immunogen against *Anaplasma marginale* challenge, is an immunodominant outer membrane protein with orthologues in all examined *Anaplasma* species. Although immunization with live *Anaplasma centrale* has long been used to induce protection against acute disease upon challenge with virulent *A. marginale*, its MSP-2 structure and whether MSP-2 variants are generated during persistence of the vaccine strain was unknown. In this study, we showed that the *A. centrale* vaccine strain persisted for a minimum of 4 years postvaccination and generated sequential MSP-2 variants. Comparison of amino acid sequences encoded by *A. centrale* *msp-2* transcripts from the initial postimmunization period and from sequential time points during persistence of the vaccine strain revealed a central hypervariable domain flanked by conserved amino and carboxy-terminal regions. This structure corresponded to that shown in *A. marginale* MSP-2, where the central hypervariable region encodes variant B-cell epitopes in the extracellular domain and the flanking transmembrane domains are rich in CD4⁺-T-cell epitopes. Importantly, at least four CD4⁺-T-cell epitopes are conserved between the two species, a finding consistent with *A. marginale* challenge triggering a recall response of CD4⁺ T cells induced by *A. centrale* vaccination. The genomic arrangement is conserved between *A. centrale* and *A. marginale* with multiple *msp-2* pseudogenes and a single operon-linked expression site for the full-length *msp-2*. This conservation of both genomic structure for generating MSP-2 variants and the CD4⁺-T-cell epitopes between these two genetically distinct *Anaplasma* species indicates that they present a similar repertoire of MSP-2 epitopes to the immune system and that this similarity may be responsible for all or part of the *A. centrale* vaccine efficacy.

The identification of *Anaplasma marginale* as a specific infectious pathogen of cattle in 1908 was the first description of what are now recognized as ehrlichiae (28, 29). Remarkably, within 4 years of this initial description, Sir Arnold Theiler described the use of a related organism, *Anaplasma centrale*, as a live vaccine to minimize the severity of *A. marginale* infection (30). In experiments carried out from 1909 to 1911, Theiler described the need to establish an *A. centrale* infection and allow recovery from this mild form of the disease prior to field exposure to ticks carrying *A. marginale* (30). Furthermore, he established that *A. centrale* vaccination did not prevent *A. marginale* infection upon challenge but rather resulted in lower levels of *A. marginale* rickettsemia and a reduction in severity of clinical signs compared to unvaccinated controls. Based on these findings, *A. centrale* live vaccines were used to protect cattle imported from England to South Africa in 1909 and have since remained in use in tropical and subtropical regions (15, 24, 25, 33).

Current mechanistic understanding of immunity to *A. marginale* has been derived both from experimental vaccine trials, which identified major surface protein 2 (MSP-2) as a protection-inducing immunogen (22), and from the study of infection-induced immunity. After recovery from acute *A. marginale* infection, during which rickettsemia levels exceed 10⁹ organisms per ml, cattle remain persistently infected but are immune

to high-level rickettsemia and disease if subsequently challenged with homologous or heterologous strains (15, 23). Persistent infection is asymptomatic and characterized by cyclic waves of rickettsemia (12), with each cycle reflecting the emergence and replication of *A. marginale* organisms expressing an antigenically variant MSP-2 (8, 9, 20). Analysis of *A. marginale* MSP-2 antigenic variants has revealed highly conserved amino and carboxy-terminal regions with the central one-third of the protein being hypervariable (7–9). This central region represents the primary surface domain and encodes the immunodominant but hypervariable B-cell epitopes (7–9). In contrast, the conserved flanking regions are rich in T-cell epitopes and induce strong recall CD4⁺-T-cell responses against MSP-2 regardless of the variation in the central surface domain (3, 5). This conserved anamnestic T-cell response is postulated to provide rapid T-cell help for primary B-cell responses to the hypervariable surface epitopes and control the emergent variants prior to reaching the high rickettsemia levels responsible for clinical disease (3, 20). This role for the conserved T-cell epitopes is supported by the rapid generation of variant-specific immunoglobulin G2 antibody after emergence of a new MSP-2 variant and the control of rickettsemia during persistent *A. marginale* infection to levels 100 to 1,000 times below the threshold for clinical disease (4, 8, 9).

Does Theiler's *A. centrale* vaccine work by mimicking natural persistent infection? If this is correct, *A. centrale* should persist in vaccinates and generate MSP-2 variants that mimic the antigenic structure of those expressed by *A. marginale*. Specifically, this would comprise a diverse set of MSP-2 central extracellular domains and highly conserved flanking regions

* Corresponding author. Mailing address: Department of Veterinary Microbiology and Pathology, Washington State University, Pullman, WA 99164-7040. Phone: (509) 335-6033. Fax: (509) 335-8529. E-mail: gpalmer@vetmed.wsu.edu.

that contain CD4⁺-T-cell epitopes shared between *A. centrale* and *A. marginale*. In this study, we report testing these hypotheses with *A. centrale*-immunized cattle.

MATERIALS AND METHODS

***A. centrale* vaccine and cattle immunization.** The *A. centrale* vaccine strain was originally obtained from South Africa in 1952 and has since been used as the standard vaccine in Israel (24). The procedures on vaccine quality control, determination of dose, thawing, and administration have been described in detail (24). Briefly, all calves were screened for *A. marginale* infection prior to vaccination by using serology, microscopic examination of Giemsa-stained blood smears, and PCR (24, 32). The 3- to 4-month-old calves were then inoculated intravenously with 1.0 ml of vaccine containing erythrocytes infected with 10⁸ live *A. centrale*. The study population included three groups maintained under tick-free conditions: (i) two Holstein calves (no. 337 and 410) examined during acute rickettsemia after *A. centrale* vaccination, (ii) two Holstein calves (no. 405 and 429) during acute rickettsemia after *A. centrale* vaccination and sequentially for 12 months after vaccination, and (iii) eight Holstein dairy cows (no. 376, 378, 468, 477, 478, 573, 578, and 773) examined at a single time point 30 to 49 months postvaccination. Blood was obtained from individual vaccinates at various time points postinoculation, and the persistence of the vaccine strain was determined by subinoculation of 50 ml of blood into seronegative, susceptible calves that were monitored by microscopic detection of *A. centrale*-infected erythrocytes (13, 15) or by using *A. centrale*-specific PCR.

***A. centrale* PCR.** A one-stage nested PCR for *A. centrale* was developed based on the *mpb-58* sequence and was shown to be capable of specific discrimination between *A. centrale* and *A. marginale* (V. Shkap, T. Molad, L. Fish, and E. Pipano, Abstr. 2nd Int. Vet. Vaccines Diagn. Conf., abstr. P52, 2000). The one-step nested PCR has a detection limit of ca. 500 *A. centrale*-infected erythrocytes per ml of blood, corresponding to a level of 0.00001% infected erythrocytes in the peripheral blood. Briefly, the external forward and reverse primers were, respectively, 5'-TGCACCGTCCTGCCGTGACC and 5'-AACCCACGC GGGCAGCTTGA. The internal forward and reverse primers were 5'-TCCAG TAACAAGCAGTTC and 5'-TGAACCTACGGTACACAT, respectively. The two rounds of amplification were separated by limiting the amount of the external primers. The reaction was performed in a final reaction volume of 25 µl containing a 0.004 µM concentration of each external primer, a 0.5 µM concentration of each internal primer, a 0.2 mM concentration of each deoxynucleoside triphosphate, 1× PCR buffer (10 mM Tris-HCl, pH 8.4; 50 mM KCl; 0.08% Nonidet P-40), 2.5 mM MgCl₂, and 0.75 U of *Taq* DNA polymerase. Cycling conditions were as follows: 1 cycle of preheating at 95°C for 3 min, 14 cycles of 94°C for 50 s, 60°C for 50 s, and 72°C for 50 s; 26 cycles of 94°C for 30 s, 49°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 5 min. Amplicons were analyzed by electrophoresis in 1.5% agarose gels and staining with 0.015% ethidium bromide.

Cloning and sequencing of full-length *msp-2* cDNA. The *msp-2* transcripts were identified from *A. centrale* acute rickettsemia or from subsequent peaks of persistent rickettsemic cycles within individual vaccinates. Cycles were identified by using the quantitative *msp-5* PCR as previously described in detail (8). At the peak of each rickettsemic cycle, total RNA was extracted from whole blood and reverse transcribed by using random hexamers, as described previously (8, 9). Full-length *msp-2* cDNA clones were generated by PCR with primers derived from the 5' and 3' ends of *msp-2*. The forward primer was 5'-TTGGATCCAT GAGTGCTGTAAGTAATAGGAAGC and the reverse primer was 5'-TCTCT CGAGCTAGAAGGCAAACCTAACACCCA. These primers were derived from the conserved 5' and 3' ends of the open reading frame of the existing full-length genomic clones, DF5 *msp-2* and 11.2 *msp-2* (7, 21). To amplify only the 600-bp central hypervariable region of *msp-2*, two primers flanking this region were used: 5'-GTGGATCCGGAGGAGCAAGGGTTGAAGTA and 5'-AGCTCGAGTTACCACCGATACC-AGCACAA (8). These sequences are conserved among *msp-2* genes and transcripts of different *A. marginale* and *A. ovis* strains (3, 8, 9, 21, 26) and represent nucleotide positions 376 to 396 and positions 967 to 945, respectively, as numbered in the 11.2 *msp-2* sequence (21). PCR products were ligated into pCR2.1 by using the TA Cloning Kit (Invitrogen). Competent *Escherichia coli* XL1-Blue was transformed with the ligated vector and plated with 5 mM IPTG (isopropyl-1-β-D-thiogalactopyranoside) and BCIP (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) for blue-white screening. Presence of *msp-2* inserts in plasmids from transformed colonies was confirmed by restriction digests or PCR, as previously described (8, 9). Plasmid DNA was extracted from each clone and sequenced in both directions.

Sequence analysis. For alignment and presentation of amino acid sequences, CLUSTAL W (31) and the Boxshade Server (<http://www.ch.embnet.org/software>

TABLE 1. Detection of *A. centrale* vaccine strain persistence

<i>A. centrale</i> vaccinee (animal no.)	Time postvaccination (mo)	Detection of <i>A. centrale</i>
405	5	Nested <i>mpb58</i> PCR
429	11	Nested <i>mpb58</i> PCR
773	30	Subinoculation into calf 369
578	35	Subinoculation into calf 336
573	37	Subinoculation into calf 334
376	41	Subinoculation into calf 359
378	41	Subinoculation into calf 355
468	48	Subinoculation into calf 351
477	49	Subinoculation into calf 350
478	49	Subinoculation into calf 362

/BOX_form.html) were used. The GenBank accession numbers for the *A. centrale msp-2* transcripts are AY040556 to AY040563.

Southern blot detection of genomic *A. centrale msp-2* and the *msp-2* expression site. DNA was extracted from 10⁹ *A. centrale* and from the South Idaho strain of *A. marginale* and digested by using *Fsp*I as previously described for *A. marginale* (1). After electrophoresis on a 0.7% agarose gel and transfer to a nylon membrane by standard techniques, the membrane was hybridized sequentially with each of three probes: (i) full-length *orf4* from the expression site, (ii) *msp-2* (bp 375 to 965), and (iii) the 5' terminus of *msp-2* (bp 2 to 335). The probes were generated by PCR by using the following corresponding primer sets: (i) for *orf4*, 5'-TTGCACACACTACCTCTTC and 5'-GCCGTACATAGAGCTCACCC; (ii) for *msp-2*, 5'-TGGAGGAGCAAGGGTTGAAGT and 5'-TTACCACCGA TACCAGCACAA; and (iii) for the 5' terminus *msp-2*, 5'-TGAGTGCTGTAA GTAATAGG and 5'-CTATCCTTGAAGCTAATCTTGGGTTTC. Labeling of the probes with digoxigenin, hybridization with DIG Easy Hyb (Roche, Inc.) overnight at 42°C, washing (twice with 2× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate]-0.1% sodium dodecyl sulfate at room temperature and twice in the same buffer at 60°C), and detection by enhanced chemiluminescence was done as previously described (1, 2). The filter was stripped between hybridizations with each of the probes by using two washes in 0.2 M NaOH-0.1% sodium dodecyl sulfate at 37°C for 15 min.

Peptide stimulation of MSP-2 specific CD4⁺-T-cell lines. The derivation and characterization of the short-term T-cell lines used in this study have been described in detail elsewhere (3). Briefly, these T-cell lines were derived from three MSP-2-immunized calves with different class II haplotypes (calf no. 59 [DRB3 22/7, DQA 9B/2, and DQB 9B/2], calf no. 60 [DRB3 22/23, DQA 9B/7D, and DQB 9B/7A], and calf no. 61 [DRB3 8/8, DQA 12/12, and DQB 12/12]). After stimulation with *A. marginale* for 1 week and depletion of γδ and CD8⁺ T cells by treatment with monoclonal antibody and complement (3), the cell lines were 94 to 99% CD4⁺ T cells. Proliferation assays were conducted in replicate wells of 96-well plates by using the T-cell lines (3 × 10⁴ cells per well) in complete RPMI 1640 medium containing antigen and, as antigen-presenting cells, irradiated autologous peripheral blood mononuclear cells (2 × 10⁵ cells per well), as previously described (3-5). The tested peptide antigens were derived from MSP-2 sequences that stimulated CD4⁺-T-cell lines from each of the three calves of different major histocompatibility complex (MHC) class II haplotypes (3) or else represented the polymorphic sequence identified in *A. centrale* during the present study. These sequences are shown in Fig. 5. The peptides were tested at concentrations from 0.4 to 10.0 µg/ml. As positive control antigens, *A. marginale* lysate, purified native MSP-2, and the *A. marginale* peptide P12 (positions 312 to 341) were tested at the same concentrations. As negative control antigens, lysates of uninfected erythrocytes or an *A. marginale* 30-mer peptide (P1) that does not contain a T-cell epitope (3) was tested at the same concentrations. Cells were radiolabeled during the last 18 h of a 3- to 4-day stimulation period by using 0.25 µCi of [³H]thymidine, collected by using an automated cell harvester, and counted with a liquid scintillation counter. The results are presented as the mean counts per minute of replicate cultures ± 1 standard deviation.

RESULTS

Persistence of the *A. centrale* vaccine strain. *A. centrale* was detected in blood obtained from each of the 10 Holstein dairy cows vaccinated 5 to 49 months earlier (Table 1). Blood from each of the eight vaccinates tested transmitted *A. centrale* upon subinoculation into individual susceptible, seronegative calves

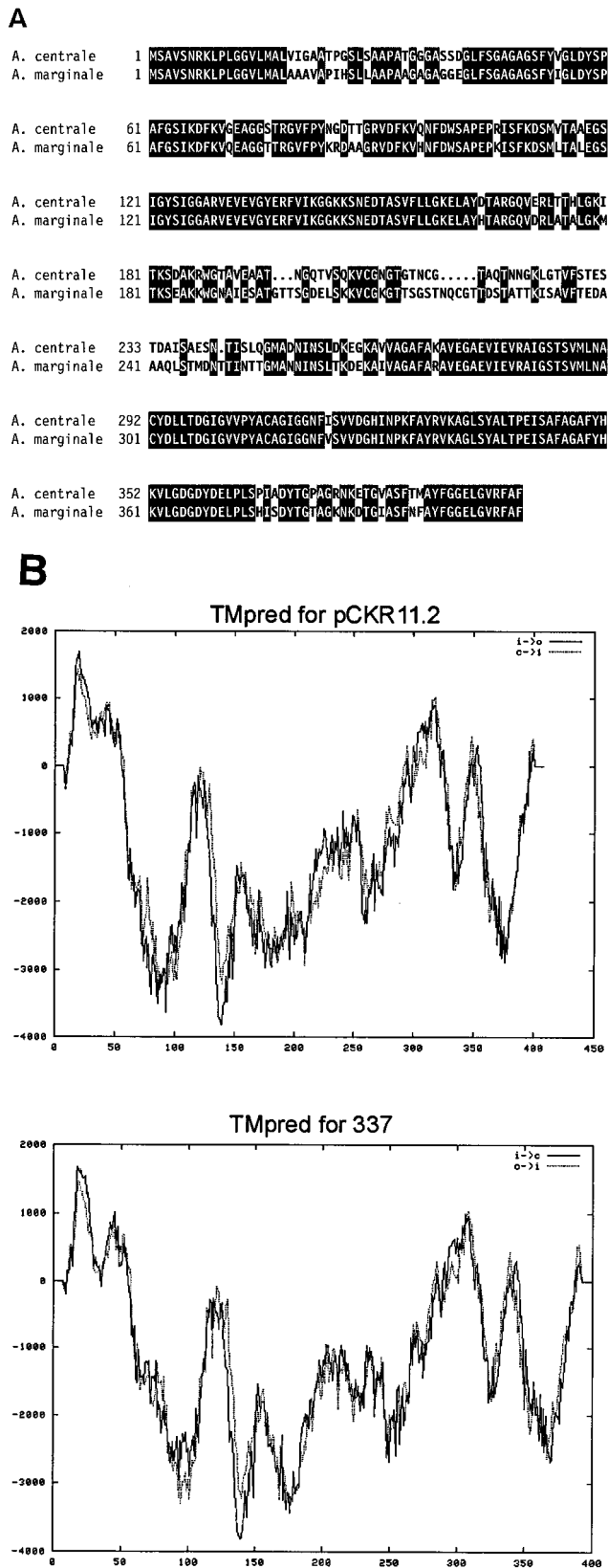


FIG. 1. Comparison of the full-length *A. marginale* 11.2 MSP-2 (21) with *A. centrale* MSP-2 obtained postimmunization from vaccinate 337. (A) Amino acid sequence alignment. Areas of amino acid substitutions, insertions, and deletions are indicated by a white background,

(Table 1). Each of the recipient calves developed microscopically detectable *A. centrale* rickettsemia within 40 days of inoculation. The two vaccinates that were tested only using the *A. centrale* *mpb58* PCR (no. 405 and 429) were positive and were used to evaluate expression of *msp-2* transcripts during persistence as described in detail below. Two additional vaccinates (no. 337 and 410) were examined only during the acute infection after *A. centrale* vaccination, as described below, and were not further examined for persistence.

Sequence and structure of MSP-2 expressed after *A. centrale* vaccination. The MSP-2 expressed by *A. centrale* in the acute infection after vaccination were identified in the blood of vaccinated animals 337 and 410. The predicted *A. centrale* MSP-2 sequence, derived from a full-length *msp-2* transcript obtained by reverse transcription-PCR from the blood of vaccinated animal 337, was compared to *A. marginale* 11.2 MSP-2 (the initial *A. marginale* *msp-2* sequenced [21]). The overall primary (Fig. 1A) and secondary (Fig. 1B) structure of the encoded MSP-2 is common between the *A. centrale* vaccine strain and *A. marginale*. While overall there is 77% identity between the vaccine strain MSP-2 and *A. marginale*, alignment revealed that this conservation was primarily in the amino- and carboxy-terminal domains (Fig. 1A). There is 83% identity in the region from amino acids 1 to 189 between *A. centrale* and *A. marginale* and 92% identity in the region from amino acid 272 to the carboxy terminus (numbering based on 11.2 MSP-2 [21]). The predicted membrane-spanning structure of these amino- and carboxy-terminal regions is highly conserved between *A. marginale* and *A. centrale* (Fig. 1B). In contrast, the central extracellular domain was variable between *A. centrale* and *A. marginale* 11.2, with only 44% identity in the region between amino acids 190 and 271. This variation is generated by deletions and substitutions in *A. centrale* MSP-2 relative to *A. marginale* MSP-2 (Fig. 1A) and does alter the predicted structure (Fig. 1B).

Examination of a second *A. centrale* vaccinate, animal 410, in the acute infection after vaccination revealed two distinct full-length *msp-2* transcripts. One of these, 410A, encoded a MSP-2 very similar to that in animal 337 but the second, 410B, encoded a variant MSP-2 (Fig. 2). Alignment of the amino acid sequences encoded by these two transcripts with the sequence from vaccinate 337 revealed absolute conservation of the amino-terminal domain, a single amino acid substitution in the carboxy-terminal domain, and a highly variable region between amino acid 181 and approximately amino acid 265 (Fig. 2). This region corresponds to the *A. marginale* MSP-2 hypervariable extracellular domain (7, 8, 26), and the changes among the *A. centrale* MSP-2 variants reflected insertions, deletions, and substitutions (Fig. 2).

Expression of *msp-2* variants in *A. centrale* vaccinates. To address whether variation in the central extracellular domain is

and areas of amino acid identity have a black background. (B) Secondary structure prediction. The predicted membrane orientation calculated by using TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) is plotted on the y axis versus the amino acid position on the x axis for *A. marginale* 11.2 MSP-2 (upper panel) and *A. centrale* vaccinate 337 MSP-2 (lower panel). The solid line ($i > 0$) is the calculation performed from the N to the C terminus; the dashed line ($0 > i$) is the calculation performed from the C to the N terminus.

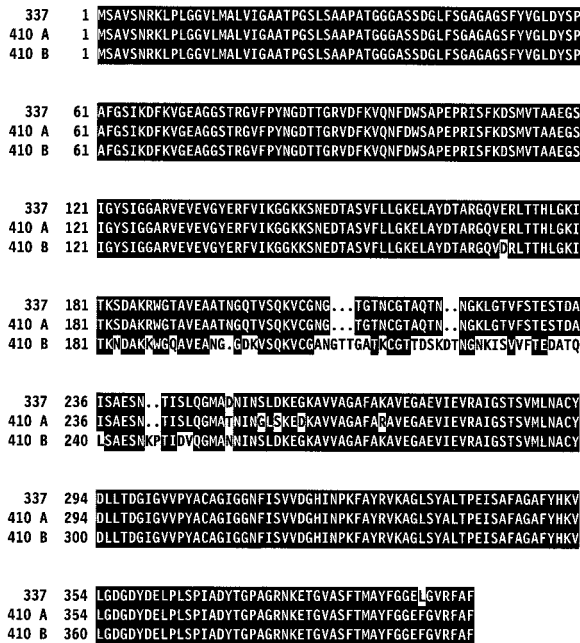


FIG. 2. Alignment of the full-length *A. centrale* MSP-2 sequence obtained postimmunization from vaccinate 337 with the two MSP-2 sequences obtained postimmunization from vaccinate 410. The amino acid identity and substitutions, insertions, and deletions are indicated as in Fig. 1A.

generated during persistence of the vaccine strain, the central 600-bp portion of *msp-2* transcripts was sequenced from vaccinated animal 429 during acute infection following vaccination (1 May 2000) and at the peaks of the first two cycles of persistent rickettsemia (6 June 2000 and 31 August 2000). Consistent with results from *A. marginale* infection in which multiple variants emerge during persistence, a total of 37 *msp-2* transcripts were identified. Alignment of the encoded MSP-2 amino acid sequences revealed a predominant region of variation extending from position 197 to position 270. The identity in the hypervariable region ranged from 75 to 95% when the peak 1 and peak 2 MSP-2 types were compared. Alignment of the two most different encoded MSP-2 types from peaks 1 and 2 illustrates the substitutions, insertions, and deletions that typify the hypervariable regions (Fig. 3). To verify that variation was consistently generated in the *A. centrale* vaccines, *msp-2* transcripts were sequenced from three peaks of rickettsemic cycles in vaccinated animal no. 405 (peak 1, 5 May 2000; peak 2, 8 June 2000; peak 4, 8 October 2000 [peak 3 was not examined]). A total of 28 different *msp-2* transcripts were identified. These revealed the same pattern of central extracellular domain variation, typified by 72% identity between the most divergent MSP-2 types of peaks 1 and 2 and 73% identity between MSP-2 types of peaks 2 and 4 (accession no. AY040561 to AY040563 [data not shown]).

Genomic structure of *A. centrale msp-2*. Genomic DNA was extracted from the vaccine strain and digested with *FspI* to excise the entire expression site locus (1). This locus is composed of an promoter and an operon of four genes, *orf4*, *orf3*, *orf2*, and *msp-2* (1). There are no *FspI* sites within this locus in either *A. centrale* or *A. marginale*. Probing the digested DNA



FIG. 3. Alignment of the *A. centrale* MSP-2 hypervariable regions between clones from two sequential persistent rickettsemic peaks. Peak 1-1 and peak 2-1 each represent a single MSP-2 type from, respectively, the first or second persistent rickettsemic cycle of vaccinate 429. Sequences are aligned starting from amino acid position 126 and extending to position 326 for peak 1-1 and to position 333 for peak 2-1. The amino acid identity and substitutions, insertions, and deletions are indicated as in Fig. 1A.

with the *orf4* probe identified the operon expression locus in *A. centrale* and, as previously reported (1, 2), in *A. marginale* (Fig. 4). The same blot was stripped and rehybridized by using a *msp-2* probe (bp 375 to 695) that includes the highly conserved region from bp 375 to 550 present in all described *msp-2* genes, including the pseudogenes that lack a complete 5' end, as well as the expression site complete *msp-2* gene (1). This second hybridization revealed multiple fragments in both *A. centrale* and *A. marginale*, a pattern previously reported for *A. marginale* DNA cleaved with *FspI* or additional restriction enzymes that cleave outside of the *msp-2* coding sequence (1, 21). In contrast to the multiple *msp-2* sequences detected by the bp-375-to-695 probe, stripping and then rehybridizing with a probe representing the 5' terminus of *msp-2* (bp 2 to 335)

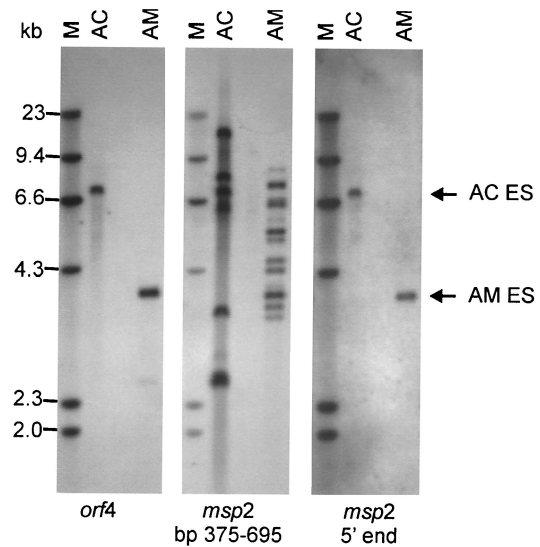


FIG. 4. Genomic structure of *msp-2*. Genomic DNA from either the *A. centrale* vaccine strain (AC) or *A. marginale* (AM) was digested with *FspI* and, by using a Southern blot, hybridized sequentially with probes representing *orf4*, *msp-2* (bp 375 to 695), and *msp-2* 5' end (bp 2 to 335). The blot was stripped between hybridizations. The expression site (ES) defined by *orf4* is indicated in the right margin. The molecular size markers (M) are indicated in the left margin.

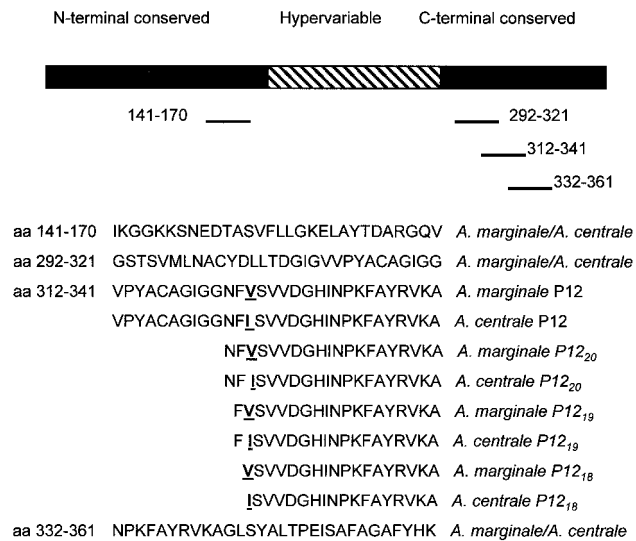


FIG. 5. Structure of MSP-2 and location of CD4⁺-T-cell epitopes. The central hypervariable region (cross-hatched) is flanked by conserved N- and C-terminal regions (solid). The positions of the CD4⁺-T-cell epitopes recognized by cattle with at least three different MHC class II haplotypes (3) are indicated by solid lines, and the amino acid positions (numbering based on 11.2 MSP-2 [21]). The sequences of the peptides representing the epitopes are provided for both *A. marginale* and *A. centrale*. The tested peptides include the 312-to-341 P12 peptide and 18-, 19-, and 20-mer peptides representing the different sequences of *A. marginale* and *A. centrale*. The amino acid substitution is indicated by an underlined boldface letter.

identified only the single band corresponding to the operon expression site (Fig. 4). This demonstrates the presence of multiple *msp-2* pseudogenes, lacking the 5' end of full-length *msp-2*, in *A. centrale* as well as in *A. marginale*. There was only a single *msp-2* gene complete with a 5' end, and this sole full-length gene was in the operon expression site defined by *orf4* (Fig. 4).

Conservation of CD4⁺-T-cell epitopes between *A. centrale* and *A. marginale* MSP-2. Notable features of the MSP-2 structure in *A. centrale* and *A. marginale* include the hypervariable extracellular domain and the flanking amino- and carboxy-terminal membrane domains (Fig. 1). Although the amino- and carboxy-terminal domains are, in general, conserved between *A. marginale* and *A. centrale* MSP-2 (Fig. 1A), the occurrence of amino acid substitutions in *A. centrale* relative to *A. marginale* may affect the CD4⁺-T-cell epitopes identified in these regions. Mapping of the CD4⁺-T-cell epitopes within the amino- and carboxy-terminal domains of *A. marginale* MSP-2 identified a minimum of four epitopes that were recognized by cattle with three different MHC class II haplotypes (3). Comparison of the sequences between *A. marginale* and the vaccine strain of *A. centrale* (Fig. 5) revealed that three of these epitopes were completely conserved and the fourth (designated P12 [3]), represented by amino acids 312 to 341, had a single amino acid substitution in *A. centrale* (V→I). This 312-to-341 region contains a dominant CD4⁺-T-cell epitope in *A. marginale* MSP-2 that induces both strong proliferation and gamma interferon production (3). Stimulation of MSP-2-specific CD4⁺-T-cell lines with the parent P12 (312 to 341), the *A. marginale* P12₁₈ (324 to 341 peptide) represented in *A. marginale*,

or the *A. centrale* P12₁₈ peptide with the V→I substitution found in *A. centrale* revealed similar proliferation (Fig. 6). Notably, T-cell lines from each of three cattle (animals 59, 60, and 61) with different MHC class II haplotypes responded to the peptide derived from either *A. marginale* or *A. centrale* (Fig. 6). There were no significant differences in the proliferative responses to the P12, *A. marginale* P12₁₈, and *A. centrale* P12₁₈ peptides by any of the T-cell lines. In addition, each T-cell line responded similarly to *A. marginale* P12₁₉ (323 to 341 peptide), *A. marginale* P12₂₀ (322 to 341 peptide), *A. centrale* P12₁₉ peptide, and *A. centrale* P12₂₀ (data not shown), indicating that the V→I substitution found in *A. centrale* had no effect on the epitope at either terminal or subterminal positions. All cell lines responded to *A. marginale* lysate (Fig. 6) and native MSP-2 (data not shown) but not to either a control peptide P1 (Fig. 6) or uninfected erythrocyte lysate (data not shown).

DISCUSSION

A. centrale is genetically distinct from *A. marginale* based upon analysis of the 16S rRNA gene sequences of both organisms (11). The question of whether *A. centrale* is a subspecies of *A. marginale*, as originally suggested by Theiler (30), or a separate species is unresolved (6, 11). Nonetheless, there are clear differences in other genes, antigens, and virulence between the two organisms (14, 16, 19, 27). The presence of a closely related MSP-2 ortholog in *A. centrale* is unsurprising given that both *Anaplasma ovis* and *Anaplasma phagocytophila*, the agent of human granulocytic ehrlichiosis, also contain a MSP-2 ortholog (3, 10, 17, 18, 35). The overall structure of these MSP-2 orthologs is retained in each of these *Anaplasma* species—conservation in the amino- and carboxy-terminal thirds of the protein with a highly variable domain in the central extracellular domain (3, 10, 17, 18, 35). Originally shown in *A. marginale* persistently infected cattle, variation is generated in this central hypervariable MSP-2 domain, while the amino- and carboxy-terminal regions are essentially invariant (3, 8, 9, 26). The MSP-2 ortholog of *A. phagocytophila* demonstrates a similar pattern of variation in the central domain when strains are compared (10, 16, 35). In the present work, we have shown that the *A. centrale* vaccine strain also generates sequential variation in the central domain, whereas the flanking regions are highly conserved. Comparison of the encoded MSP-2 sequences obtained during acute and persistent infection after *A. centrale* vaccination revealed substitutions, deletions, and insertions in the central hypervariable domain. This pattern of variation and the degree of variation, as little as 72% identity between sequential time points, is similar to the variation identified in the *A. marginale* central extracellular domain and has been shown to result in B-cell epitope variation (9, 10, 26). Thus, persistence of the *A. centrale* vaccine strain would be expected to induce a population of B-cell responses against MSP-2 variants, including those that may cross-react with *A. marginale* variants. This is consistent with prior data demonstrating that cross-reactive antibody from persistent infection, resulting from infection with either the *A. centrale* vaccine strain or *A. marginale*, is primarily directed at MSP-2 (19, 34).

The amino- and carboxy-terminal thirds of MSP-2 are not

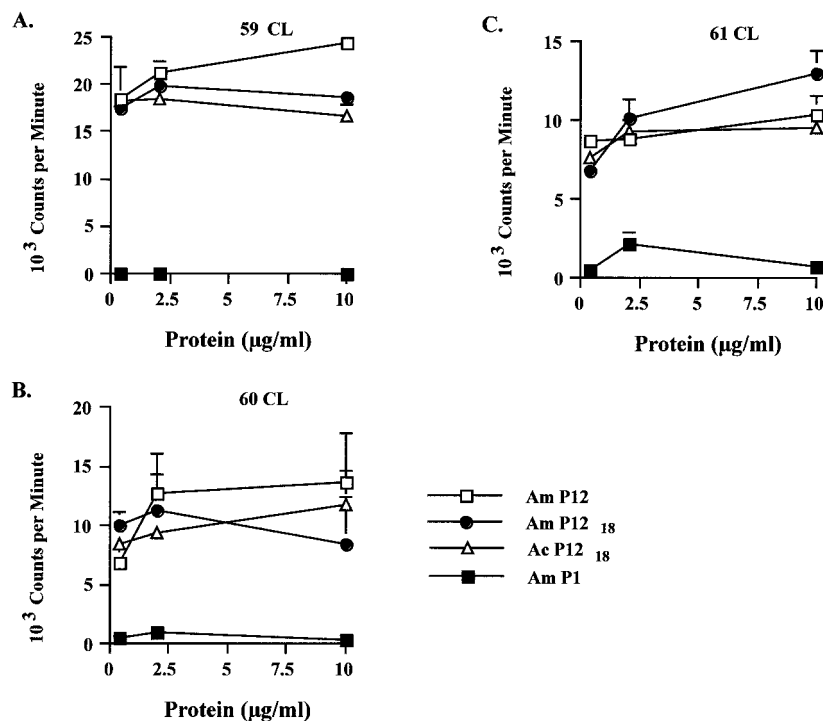


FIG. 6. Conservation of MSP-2 CD4⁺-T-cell epitopes between *A. marginale* and the *A. centrale* vaccine strain. MSP-2-specific cell lines from each of three calves (A, animal 59; B, animal 60; and C, animal 61) were stimulated with a 30-mer peptide representing *A. marginale* MSP-2 amino acids 312 to 341 (Am P12) previously shown to contain a CD4⁺-T-cell epitopes (3), an 18-mer peptide representing *A. marginale* MSP-2 amino acids 324 to 341 (Am P12₁₈), an 18-mer peptide representing the same region but with a single amino acid substitution in *A. centrale* MSP-2 (Ac P12₁₈), or a 30-mer peptide representing an *A. marginale* sequence that does not contain a T-cell epitope (P1 [3]). All peptides were tested over a concentration range of 0.4 to 10 µg per ml. The results are presented as mean counts per minute of replicate cultures ± 1 standard deviation.

identical between *A. centrale* and *A. marginale*, although they are conserved among MSP-2 variants within each species. In *A. marginale* this conservation is reflected by the ability of CD4⁺-T-cell lines and clones against these regions to recognize all strains and is consistent with the priming and continual restimulation of CD4⁺ T cells to provide B-cell help for production of variant-specific antibody during persistent infection (3, 5). CD4⁺-T-cell epitopes mapped in *A. marginale* MSP-2 include four that are recognized by T cells of cattle with three different MHC class II haplotypes (3). Three of these epitopes are within sequences that are identical between *A. centrale* and *A. marginale* (amino acids 141 to 170, 292 to 321, and 332 to 361), while the variation in the fourth (amino acids 312 to 341) was represented by a single amino acid substitution at position 324 that did not alter the epitope. This epitope conservation suggests that *A. marginale* challenge would stimulate recall responses in *A. centrale*-vaccinated cattle. In contrast, the polymorphic region from amino acid positions 19 to 41 in the amino terminus, in which 12 of 23 residues differ between *A. centrale* and *A. marginale* (Fig. 1A), does not appear to contain a CD4⁺-T-cell epitope recognized by any of the MHC class II alleles represented in the calves (3). There are some CD4⁺-T-cell epitopes in the amino- and carboxy-terminal thirds that map to sequences that are polymorphic between *A. centrale* and *A. marginale*, e.g., the 30-mer peptide representing amino acid positions 372 to 401. This peptide, recognized by CD4⁺-T-cell lines from some but not all of the MHC class II haplotypes (3), has eight substitutions in *A. centrale* relative to *A.*

marginale (Fig. 1A). Whether multiple conserved CD4⁺-T-cell epitopes would be sufficient to trigger protective recall responses even though other CD4⁺-T-cell epitopes are polymorphic is an intriguing question but has not yet been addressed.

A. marginale MSP-2 variants are generated by the recombination of pseudogenes, each composed of the hypervariable domain flanked by segments of the conserved 5' and 3' regions, into a single expression site (1, 2). The expression site itself is an operon composed of a single promoter followed by four open reading frames: *orf4*, *orf3*, *orf2*, and finally *mSP-2* (1, 2). Consistent with a conserved pattern of MSP-2 variation, *A. centrale* maintains this genomic structure with multiple *mSP-2* pseudogenes but with only a single full-length *mSP-2* gene that maps to the *orf4*-bearing expression site. Amplification and sequencing of this *mSP-2* expression site locus in *A. centrale* (data not shown) identifies the presence of the promoter, *orf4*, *orf3*, and *orf2* as predicted.

The efficacy of *A. centrale* vaccination is dependent upon actual infection of the recipient and cannot be reproduced by inoculation with killed whole organisms. This is consistent with the need to generate sequential MSP-2 variants during infection and to induce both a population of variant-specific B cells and CD4⁺ T cells to conserved epitopes capable of controlling the high-level rickettsemia upon *A. marginale* challenge. The efficacy of this vaccine regimen, now in use for more than 90 years, suggests that strategies to mimic these induced immune responses are a key to development of an effective, non-blood-based vaccine.

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