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(54) **RECOMBINANT ANTIGEN MSP1A FROM ANAPLASMA MARGINALE TO REDUCE INFECTIONS IN TICKS, VACCINE COMPOSITIONS AND METHODS OF USE**

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(58) **Field of Search** 424/184.1, 265.1, 424/191.1, 266.1, 93.1, 235.1

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(57) **ABSTRACT**

Vaccines and methods for inducing an immune response in a ruminant. The vaccine composition including pathogen and tick-derived antigens and a carrier or diluent. The method for inducing an immune response in a ruminate to provide immune protection which reduces the infection of ticks by *A. marginale* and/or prevents the transmission of the pathogen includes the steps of administering to the ruminant an effective amount of the vaccine composition having at least one antigen member of the group comprising at least one antigen member of the group comprising (i) recombinant MSP1a surface protein antigen of *A. marginale*, (ii) a subunit of recombinant MSP1a surface protein antigen of *A. marginale* and (iii) recombinant MSP1a surface protein antigen or subunits thereof in combination with antigen preparation derived from *A. marginale* infected cultured tick IDE8 cells and/or other pathogen and tick-derived antigens, and a carrier or diluent.

7 Claims, 1 Drawing Sheet

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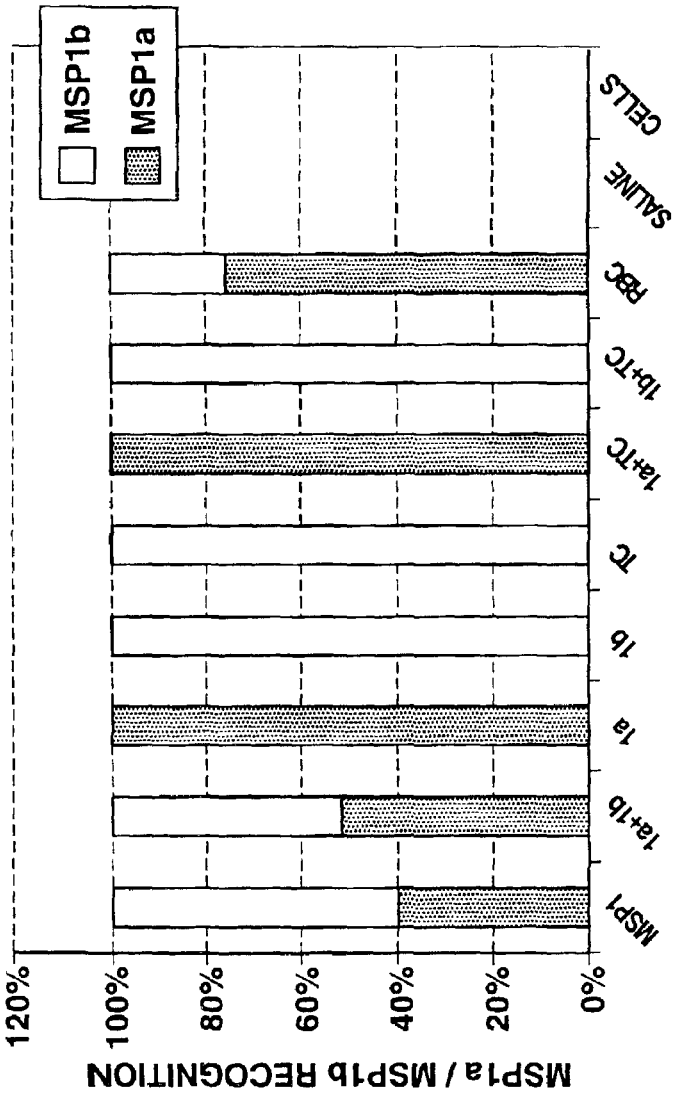
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SALINE
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100%
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40%
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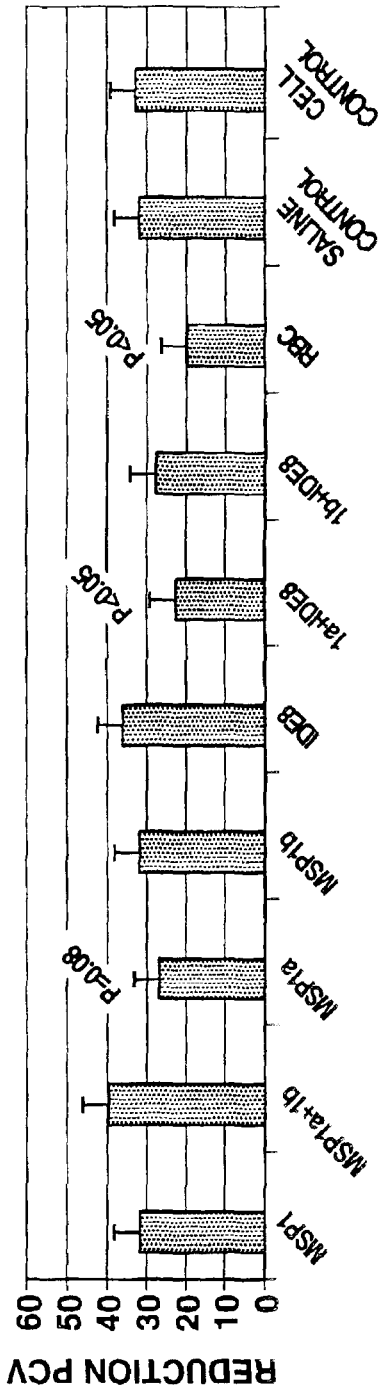
MSP1a / MSP1b RECOGNITION

Fig. 1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60
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REDUCTION PCV

Fig. 2

RECOMBINANT ANTIGEN MSP1A FROM ANAPLASMA MARGINALE TO REDUCE INFECTIONS IN TICKS, VACCINE COMPOSITIONS AND METHODS OF USE

CROSS REFERENCE TO RELATED APPLICATION

This application is a continuation-in part of prior filed, copending U.S. patent application Ser. No. 10/002,636, filed Oct. 26, 2001 which claims the benefit of U.S. provisional patent application Ser. No. 60/244,333, filed Oct. 30, 2000, both of which are hereby incorporated by reference.

BACKGROUND OF THE INVENTION

1. Technical Field

The present invention relates to recombinant *Anaplasma marginale* major surface protein (MSP)1a, related vaccines and methods useful to reduce infections in ticks and affect the biological transmission of the pathogen of the species *A. marginale.*

2. Background

Anaplasmosis is a tick-borne disease of cattle caused by *Anaplasma marginale* (Rickettsiales: Anaplasmataceae). The only known site of development of *A. marginale* in cattle is within erythrocytes [1]. The number of infected erythrocytes increases logarithmically during infection and removal of infected erythrocytes by phagocytic cells of the reticuloendothelial system often results in development of anemia and icterus without hemoglobinemia and hemoglobinuria [2]. While mechanical transmission of *A. marginale* occurs when infected blood is transferred from infected to susceptible animals by biting flies or blood-contaminated fomites, biological transmission is effected by feeding ticks. Approximately 20 species of ticks have been incriminated as vectors worldwide [3, 4]. Cattle that recover from acute infection remain persistently infected and develop life-long immunity against clinical disease, but they serve as reservoirs of infection for mechanical and/or biological transmission by ticks.

The development of *A. marginale* in ticks is complex and coordinated with the tick feeding cycle [5, 6, 8]. In the cycle of *A. marginale* that was described in male ticks transferred from infected to susceptible hosts, the first site of infection occurs in tick gut cells. After the ticks feed a second time, many other tick tissues become infected, including the salivary glands from where the rickettsiae are transmitted to cattle during feeding. Male ticks become persistently infected with *A. marginale* and are able to transmit *A. marginale* to multiple hosts [6, 7, 8].

Major surface protein (MSP)1a is one of six MSPs that have been described on *A. marginale* derived from bovine erythrocytes [9]. MSP1a forms the MSP1 complex with MSP1b [10, 11]. MSP1a is encoded by a single gene, msp1α, which is conserved during the multiplication of the bacterium in cattle and ticks [12, 13]. This protein is variable in molecular weight among geographic isolates because of varying numbers of tandem 28 or 29 amino acid repeats located in the amino terminal portion of the protein [11, 14, 15]. MSP1a was shown to be an adhesin for bovine erythrocytes and for both native and cultured tick cells using recombinant *E. coli* expressing MSP1a in microtiter hemagglutination and adhesion recovery assays and by microscopy [16, 17, 18]. Furthermore, MSP1a was shown to effect infection and transmission of *A. marginale* by *Dermacentor* spp. ticks [19] and was also shown to be involved in bovine immunity to *A. marginale* infection [20, 21, 22, 26]. See also U.S. Pat. No. 10/002,636, incorporated herein by reference.

Recently, we demonstrated that infection of *A. marginale* for cultured tick cells was inhibited by antibodies against recombinant MSP1a [23, 24]. While antisera from cattle naturally infected with *A. marginale* did not inhibit *A. marginale* infection, antibodies produced in rabbits and cattle immunized with the recombinant MSP1a effected inhibition of *A. marginale* infection for the cultured tick cells [24]. This inhibitory effect has also been demonstrated using antibodies against a synthetic MSP1a repeated peptide, and this data provided additional evidence that MSP1a plays a role in adhesion of *A. marginale* to tick cells [15].

Vaccination is the most efficient and economical method for control of anaplasmosis, and development of effective vaccines has been a priority of the cattle industry worldwide [9]. Infected bovine erythrocytes have been the only source of vaccine antigen until recently when a tick cell culture system was developed for propagation of *A. marginale* and provides an alternative antigen source. The cell culture-derived *A. marginale* is currently being tested as antigen for use in vaccine development [20, 22]. See also U.S. Pat. No. 5,869,335, incorporated herein by reference.

Thus far, vaccines using erythrocyte or cell culture-derived antigens have effected reduction of clinical disease but have not prevented infection of cattle [9, 20, 22, 25, 27, 28, 37]. Also, antibodies in cattle immunized with erythrocyte-derived *A. marginale* have not caused reduction of *A. marginale* infections in ticks [7].

The desired result of a vaccine for the control of anaplasmosis is to have a protection effect on the multiplication of *A. marginale* in the bovine host and a blocking effect on the transmission of the pathogen by the tick vector. Existing vaccines and experimental vaccines, however, including formulations using the recombinant MSP1a, the MSP1 complex and partially purified parasites from infected erythrocytes and cultured tick cells (see U.S. Pat. Nos. 5,549,898; 5,869,335 and 10/002,636 incorporated herein by reference) have not demonstrated any effect on the infection of the tick vector by the pathogen. Therefore, it is desirable to develop vaccines against anaplasmosis with protection effect on the multiplication of *A. marginale* in the bovine host and an effect on the transmission of the pathogen by the tick vector.

A better understanding of the present invention, its several aspects, and its advantages will become apparent to those skilled in the art from the following detailed description, taken in conjunction with the attached figures, wherein there is described the preferred embodiment of the invention, simply by way of illustration of the best mode contemplated for carrying out the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a graphical illustration of the immune response against MSP1a and MSP1b determined by Western blot analysis of sera derived from immunized cattle and controls generated in connection with the experimental results reported herein.

FIG. **2** is a graphical illustration of the reduction in PCV achieved by various combinations of antigens and controls in connection with the experimental results reported herein.

DETAILED DESCRIPTION OF THE INVENTION

Before explaining the present invention in detail, it is important to understand that the invention is not limited in

its application to the details of the embodiments and steps described herein. The invention is capable of other embodiments and of being practiced or carried out in a variety of ways. It is to be understood that the phraseology and terminology employed herein is for the purpose of description and not of limitation.

In accordance with the present invention there is provided a new vaccine against the rickettsial cattle pathogen *A. marginale* through the use of discrete recombinant MSP1a and polypeptides derived from MSP1a containing the immunoprotective and functional regions that are expressed in *E.coli*. In one aspect, only recombinant MSP1a or immunoprotective and functional regions thereof are utilized as the antigenic component of the vaccine. In another aspect, recombinant MSP1a or subunits thereof are utilized in combination with other antigen preparations, particularly antigen preparations derived from *A. marginale*-infected cultured tick IDE8 cells. Another aspect of the present invention relates to recombinant *A. marginale* major surface protein (MSP)1a, related vaccines and methods useful to reduce infections in ticks and affect the biological transmission of the pathogen of the species *A. marginale*.

MSP1a and MSP1b are isolated from *A. marginale* initial bodies as a complex of two noncovalently linked, antigenically distinct polypeptides. It is possible that the association between MSP1a and MSP1b in the surface protein complex allows the parasite to more effectively bind to erythrocyte and/or tick cell components. MSP1a could be the essential subunit in the recognition of the tick cell receptor, while the binding to the erythrocyte receptor could be mediated primarily by MSP1b or by both protein subunits through the binding of distinct erythrocyte components. Additionally, the association between MSP1a and MSP1b could stabilize and/or properly conform the MSP1 complex [29].

MSP1a is encoded by a single monocystronic gene, msp1α, which is polymorphic among geographical isolates of *A. marginale* [30, 31, 32]. *A. marginale* isolates differ in the number of 28–29 amino acids tandem repeats within the MSP1a polypeptide [31, 32], which contain a neutralization-sensitive epitope [33, 31]. However, the sequence of msp1α does not change during the multiplication of the parasite in the bovine host and the tick vector. The second MSP1 subunit, MSP1b, is encoded by at least two monocystronic genes, msp1β1 and msp1β2 [34]. These loci are polymorphic between and within populations of *A. marginale* from different geographical regions and life cycle stages but conserve a high degree of similarity. Sequence diversity is mainly due to point mutations in variable regions, perhaps due to selective immune pressure. The genetic structure of msp1α together with the vital function of codified polypeptides permits the inclusion of recombinant MSP1a polypeptides, or its functional domains, in vaccine formulations against *A. marginale*.

The experiments described and examples provided hereinafter demonstrate that cattle immunized with recombinant MSP1a alone or in combination with tick cell culture derived *A. marginale* are unexpectedly better protected against *A. marginale* infection as demonstrated by a lower reduction in packed cell volume (PCV) and lower peak parasitemia (PPE) than cattle immunized with the MSP1 complex, a combination of uncomplexed MSP1a and MSP1b surface protein antigens, the MSP1b antigen alone, cell culture derived *A. marginale*, or cell culture derived *A. marginale* combined with MSP1b. Indeed, only erythrocyte-derived *A. marginale* appears to confer like protection.

EXAMPLE 1

Preparation of Recombinant *E. coli* Expressing MSP1a and Preparation of Antigen

The msp1α gene was cloned by PCR from the Oklahoma isolate of *A. marginale* derived from infected erythrocytes. DNA was extracted from 1 ml stored blood samples containing infected bovine erythrocytes collected during high parasitemia employing 250 μL Tri Reagent (Sigma) and following manufacturer's recommendations. Extracted DNA was resuspended in 100 μL water. The msp1α gene was amplified from 1 μL DNA by PCR using 10 pmol of each primer MSP1aP: 5'GCATTACAACGCAACGCTTGAG3' (SEQ. ID NO: 1) and MSP1a3: 5'GCTTTACGCCGCCGCCTGCGCC3' (SEQ. ID NO: 2) in a 50-μL volume PCR employing the Access RT-PCR system (Promega). Reactions were performed in an automated DNA thermal cycler (Eppendorf) for 35 cycles. After an initial denaturation step of 30 sec at 94° C., each cycle consisted of a denaturing step of 30 sec at 94° C. and an annealing-extension step of 2.5 min at 68° C. The program ended by storing the reactions at 4° C. PCR products were electrophoresed on 1% agarose gels to check the size of amplified fragments. The amplified fragments were resin purified from PCR reactions (Wizard Promega) and cloned into pGEM-T vector (Promega) for sequencing both strands (Core Sequencing Facility, Department of Biochemistry and Molecular Biology, Noble Research Center, Oklahoma State University).

For high level expression of MSP1a, msp1α coding region was amplified from per1 (msp1α in pGEM-T vector) plasmid DNA by PCR using the primers 5'CCGCTCGAGATGTTAGCGGAGTATGTGTCC3' (SEQ. ID NO: 3) and 5'GAAGATCTCGCCGCCGCCTGCGCC3' (SEQ. ID NO: 4). The msp1α amplification product was digested with XhoI and BglII and inserted into the cloning site of pFLAG-CTC expression vector (Sigma). Recombinant plasmid was named pFLC1a. In this construct, the inserted gene is under the control of the inducible tac promoter and yield full-length MSP1a polypeptide, with a C-terminal fusion of a FLAG marker octapeptide. The fidelity and orientation of the construct was verified by sequencing. For expression of MSP1a recombinant polypeptides, pFLC1a expression plasmid was transformed into *E. coli* K-12 (strain JM109). Transformed *E. coli* strains were inoculated in LB containing 50 μg/ml Ampicillin and 0.4% glucose. Cultures were grown at 37° C. to OD_{600nm}=0.4. IPTG was then added to 0.5 mM final concentration, and incubation continued during 4 h, for induction MSP1a expression. Cells were collected by centrifugation and membranes extracted after sonication and centrifugation. MSP1b was cloned, expressed and purified in a similar way. Doses of 5 ml containing 100 μg recombinant antigens were used for vaccination in subsequent studies.

EXAMPLE 2

Analysis of the Protective Capacity of Vaccine Preparations Containing Recombinant MSP1a

1. Propagation of *Anaplasma marginale* in tick cell culture and preparation of immunogen. The IDE8 (ATCC CRL 11973) tick cell line derived from embryos of *Ixodes scapularis* was maintained at 31° C. in L-15B medium, pH 7.2, supplemented with 5% heat inactivated fetal bovine serum (FBS; Sigma, USA), 10% tryptose phosphate broth (Difco, USA) and 0.1% bovine lipoprotein concentrate (ICN, USA). Cultures were grown in 25-cm^2 plastic flasks (Nunc, Rosekilde, Denmark) with 5 ml of medium, and the medium was replaced weekly. The cells were subcultured at 1:5 to 1:20, and the cells became tightly adherent to the culture substrate and multiplied with a population doubling time of 3 to 5 days to a density

of about 5×10^6 cells/ml. Nearly confluent monolayers from each passage were collected and stored in liquid nitrogen in medium with 10% DMSO.

Tick cell cultures infected with the Oklahoma isolate of *A. marginale* were propagated. Terminal cell cultures were harvested, the cells centrifuged, and the contents of each T25 flask was resuspended in 1 ml PBS and stored at −70° C. until used as antigen for immunogen doses. The antigen aliquots were thawed, pooled and a sample was taken and tested by indirect ELISA. The cell culture-derived antigen was inactivated with beta propiolactone (BPL) and the volume was adjusted to 5 ml so that each dose contained approximately 2×10^{10} *A. marginale*.

2. Preparation of *A. marginale* antigen from bovine erythrocytes. Two susceptible, splenectomized calves (PA432 and PA433) were each inoculated with 2.5 ml blood stabilate (40% parasitemia) collected from a calf with the Virginia isolate of *A. marginale*. The calves were monitored for infection by examination of stained blood smears. Blood was collected from PA432 at parasitemias of 13.6% and 32.7% and from PA433 at parasitemias of 12.2% and 12.9%. After each collection, the erythrocytes were washed 3 times in PBS, each time removing the buffy coat. The erythrocytes were frozen at −70° C. 1:1 in RPMI 1640 cell culture medium until used as antigen for the immunization studies. The frozen erythrocyte antigen was thawed, washed in PBS, and centrifuged. The resulting pellet was washed to remove the hemoglobin, after which the antigen was pooled and inactivated with β-propiolactone (BPL). An aliquot was tested by ELISA as described previously for the erythrocyte antigen preparation using a known erythrocyte standard. Doses (5 ml) were prepared that contained approximately 2×10^{10} *A. marginale*.
3. Experimental design. Fifty, 16-month month old Angus cattle were randomly assigned into ten groups of five cattle each that were immunized with various antigens as follows. (1) MSP1 complex, (2) MSP1a and MSP1b, (3) MSP1a, (4) MSP1b, (5) cell culture-derived *A. marginale*, (6) cell culture-derived *A. marginale* and MSP1a, (7) cell culture-derived *A. marginale* and MSP1b, (8) erythrocyte-derived *A. marginale*, (9) uninfected IDE8 tick cells and (10) adjuvant only.
4. Immunizations. All cattle were immunized 3 times by subcutaneous injection of the antigen at weeks 1, 4 and 6. Each antigen dose was 5 ml in volume and contained an antigen in the adjuvant, XTEND® III (Grand Laboratories, Larchwood, Iowa). All cattle were challenge-exposed 10 weeks after the last immunization with 1×10^7 *A. marginale* infected erythrocytes collected from a calf experimentally infected with the Oklahoma isolate of *A. marginale*. Blood of the immunized and control cattle was monitored for infection with *A. marginale* by microscopic examination of blood smears and hematology was done daily after the onset of infection. Parameters evaluated in cattle included determination of the peak percent infected erythrocytes (PPE), percent reduction in the packed cell volume (PCV), and the prepatent period (days) determined from the day of challenge-exposure to the onset of infection.
5. Collection of blood and serum samples. Whole blood was collected in vacutainer tubes containing EDTA and used for preparation of stained blood smears for light microscopy and for determination of the PCV. Serum samples were collected from each animal before immunization, weekly until the cattle were challenge-exposed and daily after cattle developed parasitemia as a result of challenge-exposure. Serum samples were stored at −70° C. until tested by competitive ELISA and Western blots.
6. Characterization of the immune response in vaccinated cattle by competitive ELISA and Western blots. Antibody responses of all immunized and control cattle at two weeks after the last immunization to MSP1a, MSP1b and MSP5 were determined using ELISAs specific for detection of antibodies to each of these MSPs. Antibody responses of all immunized and control cattle at two weeks after the last immunization to MSP1a and MSP1b were also analyzed by Western blot. One hundred micrograms of recombinant MSP1a or MSP1b were loaded in an 8% polyacrylamide gel. SDS-PAGE gels were transferred to a nitrocellulose membrane. The membrane was blocked with 5% skim milk for 1 hr at room temperature. Sera from immunized cattle was diluted 1:200 in TBS. Serum from an uninfected bovine was included as a negative control. All sera were incubated with the membrane for 1 hr at room temperature using a Mini-Protean II Multi-screen (BioRad, USA). The membrane was washed 3 times with TBST and incubated for 1 hr at room temperature with goat anti-rabbit IgG alkaline phosphatase conjugate (KPL, USA) diluted 1:10,000. The membrane was washed again and the color developed using Sigma Fast BCIP/NBT alkaline phosphatase substrate tablets. The membrane was then examined for recognition of the bands corresponding to MSP1a and MSP1b.
7. Statistical analysis. For the analysis of results from the immunization experiment, pairwise comparisons (Student's t test) were conducted to compare results between cattle immunized with antigen preparations and the controls. Parameters analyzed included the prepatent period (days), the peak percent parasitized erythrocytes (PPE) and the percent reduction in the packed cell volume (PCV). Mean antibody levels were compared using an ANOVA test.
8. Results. Antibody titers against MSP1a, MSP1b and MSP5 in immunized cattle peaked two weeks after the last immunization. The immune response against MSP1a, MSP1b and MSP5 was analyzed by Western blot. Cattle immunized with recombinant antigen preparations responded to recombinant proteins included on each preparation (FIG. **1**). Cattle immunized with tick cell derived *A. marginale* antigens and with infected erythrocytes-derived antigens recognized primarily MSP1b or MSP1a, respectively (FIG. **1**).

Protection was evaluated using the reduction in PCV, the PPE and the prepatent period. No differences were observed in the prepatent period. The PPE was reduced in cattle immunized with MSP1a, MSP1b, the combination of recombinant antigens with infected tick cells-derived antigens and in animals immunized with infected erythrocytes-derived antigens as shown in Table 1.

TABLE 1

	Peak Parasitemia (%)		
Group	Ave	SD	P
MSP1	5.5	2.8	0.13
1a + 1b	6.0	1.6	0.14
1a	4.8	0.6	0.03
1b	3.9	1.0	0.01
TC	4.1	2.3	0.03
1a + TC	4.7	1.4	0.03
1b + TC	3.9	0.8	0.01

TABLE 1-continued

Group	Peak Parasitemia (%) Ave	SD	P
RBC	2.7	1.1	0.004
Saline	5.5	1.4	0.08
Cells	7.4	2.3	—

The reduction in PCV, associated with clinical signs, was significantly reduced in cattle immunized with MSP1a combined with infected tick cell-derived antigens and in cattle immunized with erythrocyte-derived antigens (See FIG. **2**, wherein Reduction PCV=[(Ave Start PCV-Lowest PCV)/Start PCV]×100).

The results of these experiments demonstrated that:

a. Cattle immunized with infected tick cell-derived antigens had a preferential recognition for MSP1b while cattle immunized with erythrocyte-derived antigens showed a bias toward MSP1a. The bias in the antibody response against MSP1a or MSP1b in cattle immunized with *A. marginale* antigens from IDE8 tick cells or bovine erythrocytes suggests that the MSP1 complex exposure on the surface of parasites may vary during multiplication on the tick and mammalian hosts;

b. The immunization with the MSP1 complex or with MSP1a and MSP1b together did not protect cattle after challenge with *A. marginale* despite that cattle responded to both antigens; and

c. Cattle with a predominant immune response against MSP1a (groups immunized with MSP1a, MSP1a plus infected tick cell-derived antigens and infected erythrocyte-derived antigens) were protected against *A. marginale* infection as demonstrated by the lower reduction in PCV.

It can thus be appreciated that the utilization of recombinant MSP1a in vaccines provides an advantageous mechanism to achieve resistance in cattle against *A. marginale* infection. Whereas erythrocyte-derived *A. marginale* is disadvantaged due to cost, difficulties in purifying antigen from bovine membranes, problems with preventing pathogen contamination and difficulties in standardization, recombinant MSP1a may be readily and cost effectively prepared in a standardized, pure form free of bovine erythrocyte membranes and antigens that might result in formation of an immune response to bovine blood cells.

EXAMPLE 3

Function of MSP1a Tandem Repeats in Adhesion to Host Cell Receptors

1. Construction, expression in *E. coli* and characterization of wild type MSP1a and mutants. A MSP1a (Oklahoma isolate msp1α clone per1 [14]) mutant lacking the tandem repeats was constructed by PCR. Oligonucleotide primers RI0R (5'-CCGAATTCCATGTTAGCGGCTAATTGGCG GCAAGAGATGCG-3') (SEQ. ID NO: 5) and MSP1a3BII (5'-CCAGATCTCTTTACGCCGCCGCC TGCGCC-3') (SEQ. ID NO: 6) were designed to amplify the msp1α gene lacking 6 amino acids preceding the repeats and the tandem repeats in a 50 μl volume PCR (0.2 μM each primer, 1.5 mM $MgSO_4$, 0.2 mM dNTP, 1×AMV/Tfl reaction buffer, 5u Tfl DNA polymerase) employing the Access RT-PCR system (Promega, USA). Reactions were performed in an automated DNA thermal cycler (Eppendorf MASTERCYCLER® personal, USA) for 35 cycles. After an initial denaturation step of 30 sec at 94° C., each cycle consisted of a denaturing step of 30 sec at 94° C. and an annealing-extension step of 2.5 min at 68° C. The program ended by storing the reactions at 4° C. The primers introduced an ATG initiation codon and Eco RI and Bgl II restriction sites for cloning into the pFLAG-CTC expression vector (Sigma). The resulting plasmid pAF0R1 was transformed into *E. coli* JM109 and induced for expression of mutant MSP1a as previously reported for MSP1a [14]. For the expression of MSP1a (Oklahoma isolate msp1α clone per1 [14]) tandem repeats in *E. coli*, this region was amplified using oligonucleotide primers RNOKBS5 (5'-GAGATCTGCT GATGGCTCGTCAGCGGG-3') (SEQ. ID NO: 7) and RNOKBS3 (5'-GGTCGACCCTGATTGAGACGATGT ACTGGCC-3') (SEQ. ID NO: 8). The PCR was conducted as previously described but with amplification cycles consisting of a denaturing step of 30 sec at 94° C., an annealing step of 30 sec at 58° C. and an extension step of 1 min at 68° C. The 5' and 3' amplification primers contained Bgl II and Sal I restriction sites, respectively, for cloning into pFLC1b [14] for expression in *E. coli* as a fusion peptide to the COOH-terminus of MSP1b (locus β1, Oklahoma isolate). The resulting plasmid pF1bRNO4 was transformed into *E. coli* JM109 and induced for expression of mutant MSP1b>MSP1a-repeats protein as previously reported for MSP1b [14]. All constructs were sequenced at the Core Sequencing Facility, Department of Biochemistry and Molecular Biology, Noble Research Center, Oklahoma State University using ABI Prism dye terminator cycle sequencing protocols developed by Applied Biosystems (Perkin-Elmer Corp., Foster City Calif.).

Expression of recombinant mutant proteins was assayed by SDS-PAGE, Western blot or live-cell immunofluorescence assay as previously reported [35]. The hemagglutination of bovine erythrocytes and adhesion to cultured IDE8 tick cells of recombinant *E. coli* expressing the wild type and mutant proteins was evaluated in a microtitre hemagglutination and *E. coli* recovery adhesion assays, respectively, as reported [35].

2. Results. To study the function and structural organization of tandem repeated peptides in MSP1a we selected a recent *A. marginale* field isolate from Oklahoma that is tick-transmissible and for which we have cloned and characterized MSP1a and MSP1b. A recombinant mutant MSP1a was constructed lacking the tandem repeated peptides and expressed in *E. coli*. The recombinant mutant protein was expressed at high levels and was secreted to the *E. coli* membrane as shown by live-cell immunofluorescence. Nevertheless, the adhesion to cultured IDE8 tick cells of recombinant *E. coli* expressing the mutant protein was abolished when compared to the wild type MSP1a (Table 2). To demonstrate that the MSP1a repeats were not only necessary but sufficient to confer adhesion of recombinant *E. coil* to tick cells, we then constructed a chimeric protein containing the MSP1a tandem repeated peptides fused to the COOH-terminus of the MSP1b. MSP1b did not confer an adhesive phenotype when expressed in *E. coli* (Table 3). However, *E. coli* expressing the chimeric protein adhered to cultured IDE8 tick cells at levels comparable to the wild type MSP1a-expressing *E. coli* (Table 3).

The capacity of MSP1a to hemagglutinate bovine erythrocytes was also mediated by the tandem repeats. Recombinant *E. coIi* expressing the MSP1a lacking the tandem

repeats were unable to hemagglutinate bovine erythrocytes (Table 2) while the chimeric MSP1b>MSP1a-repeats protein expressed in *E. coli* conferred to recombinant bacteria a higher hemagglutination capacity (Table 3) when compared to wild type MSPs.

TABLE 2

Hemagglutination of bovine erythrocytes and adhesion to cultured tick IDE8 cells by recombinant *E. coli* expressing *A. marginale* (Oklahoma isolate) MSP1a wild type and mutant protein without repeats

	Plasmid carried by recombinant *E. coli*			
Relevant protein expressed	pFLC1a MSP1a	pAF0R1 MSP1a-no repeats mutant	p33 None	No plasmid None
No. of CFU (mean ± SD) recovered from IDE8 cells (N = 3)	500 ± 141	14 ± 18	231 ± 129	0
Average fold increase over p33 control	2	—	—	—
P (Student's t-Test)	0.05	—	—	—
Average fold decrease over MSP1a (OK)	—	36	—	—
P (Student's t-Test)	—	0.02	—	—
Hemagglutination of bovine erythrocytes (N = 3)[a]	1	0	0	0

[a]0, no hemagglutination; 1, weak hemagglutination; 2, moderate hemagglutination; 3, near maximum hemagglutination; 4, maximum hemagglutination [7].

TABLE 3

Hemagglutination of bovine erythrocytes and adhesion to cultured IDE8 tick cells of *E. coli* expressing wild type MSP1a or MSP1b (Oklahoma isolate) and MSP1b > MSP1a-repeats mutant proteins

	Plasmid carried by recombinant *E. coli*		
Relevant protein expressed	pFLC1a MSP1a	pFLC1b2 MSP1b	PF1bRNO4 MSP1b > MSP1a-repeats
No. of CFU recovered from IDE8 cells (Ave ± SD) (N = 2)	975 ± 742	18 ± 17	530 ± 325
Average fold increase over pFLC1b2 (MSP1b)	54	—	29
Hemagglutination of bovine erythrocytes (N = 2)[a]	1	4	5

[a]Plates were incubated for 2 hours at 40° C. and results scored essentially as reported by McGarey and Allred [7]: 0, no hemagglutination; 1, weak hemagglutination; 2, moderate hemagglutination; 3, near maximum hemagglutination; 4, maximum hemagglutination; 5, maximum hemagglutination in 1 hour.

Accordingly, it can be appreciated that subunits derived from MSP1a are useful as well in the inventive vaccine compositions. The inclusion of MSP1a region(s) effecting MSP1a biological function could enhance the host immune response directed against relevant immunoprotective epitopes.

The preparation of vaccines utilizing as distinct antigenic components MSP1a is easily accomplished using well known methods and techniques. The vaccine and/or antigen preparation is combined into a formulation in an amount effective to provide for a protective immune response against infection with *A. marginale*. A protective immune response against *A. marginale* decreases the clinical signs of anaplasmosis. Clinical symptoms of anaplasmosis include a reduction in packed red cell volume of about 25 to 80% and parasitemia of the red blood cells of about 15 to 70%. A decrease in the symptoms of anaplasmosis includes prevention of the reduction in the packed red cell volume and a decrease in percent parasitemia. Preferably, a protective response includes packed red cell volume change of 25% or less compared with control animals and/or a decrease in parasitemia to about 5 to 25% of the red blood cells or less depending on the conditions. Measurements of packed red cell volume and percent parasitemia are conducted using standard methods. Vaccine preparations are combined with physiologically acceptable carriers to form vaccines. The preferred physiologically acceptable carrier is an oil-based adjuvant.

Preferably, the inventive vaccine formulation is set to contain about 100 micrograms of recombinant antigens associated to *E. coli* membranes in an oil-based adjuvant such as XTEND® III (Grand Laboratories, Larchwood, Iowa).

The vaccines may be administered by a variety of routes including intravenously, intraperitoneally, intramuscularly, and subcutaneously. The preferred route of administration is subcutaneous. The vaccine can be administered in a single dose or multiple doses until a protective effect is achieved.

Recombinant Antigen MSP1a from *Anaplasma marginale* to Reduce Infections in Ticks, Vaccine Compositions and Methods of Use

It has been discovered that the incorporation of recombinant MSP1a in vaccine formulations against *A. marginale* in combination with infected IDE8 cells-derived antigens and/or pathogen and tick-derived antigens would allow the development of vaccines against anaplasmosis with protection effect on the multiplication of *A. marginale* in the bovine host and an effect on the transmission of the pathogen by the tick vector. *A. marginale* Oklahoma isolate major surface protein 1a (msp1α) gene (AY010247) is provided as SEQ. ID NO: 9, and major surface protein 1a (AAG29248) sequence listing is provided as SEQ. ID NO: 10.

1. Cattle Vaccination and Challenge

Twenty Holstein cattle, 12 to 24 months old, were used for this study. These cattle were selected from 55 cattle used for a larger vaccine trial that were randomly assigned to two experimental groups of 20 cattle each and one group of 15 control cattle in which Group 1 cattle were immunized with three isolates of *A. marginale* derived from tick cell culture (Virginia, Oklahoma and Oregon isolates) and 100 μg recombinant MSP1a; Group 2 cattle were immunized with 100 μg of recombinant MSP1a; and cattle in Group 3 were left unvaccinated to serve as controls for natural infection conditions.

It has been demonstrated that *A. marginale* infection levels in ticks fed on cattle immunized with *E. coli* membranes and uninfected cultured IDE8 tick cell-derived antigens were similar to the infection levels in ticks fed on non-immunized cattle.

Ten immunized cattle were selected for this study based on detection of high antibody titers against recombinant MSP1a. Of these ten cattle, 5 were chosen from Group 1 (cattle 226, GT168, 242, 294, 141) and 5 were chosen from Group 3. Cattle GT165, GT155, 219, 248, GT152) for the non-immunized controls, ten cattle (214, 210, 245, 251, 143, 157, 247, 217, 166, 162) were randomly chosen out of the 15 control cattle from the larger study.

A. marginale antigens from infected IDE8 cells were prepared as described previously [20,22]. Recombinant MSP1a was prepared by inducing the expression of the protein in *E. coli* [18]. The *E. coli* cells were then disrupted by sonication followed by centrifugation for separation of

soluble from membrane bound antigens. The resulting pellet that contained the MSP1a in *E. coli* membranes was used for immunization. The total protein concentration was determined and the amount of recombinant MSP1a was estimated from Western blots using affinity purified recombinant MSP1a as standard [22].

Cattle were immunized at weeks 4 and 8 with a 5 ml dose containing the antigen in an oil-based adjuvant (Adjuvant XTEND® III Grand Laboratories, Larchwood, Iowa, USA) [20]. Cattle were challenge-exposed two weeks after the last immunization by intravenous administration of 1.7 ml infected blood containing 10^9 *A. marginale*. The challenge-exposure blood was obtained from a splenectomized calf that was experimentally infected with the Virginia isolate of *A. marginale* (calf PA481, percent infected erythrocytes (PPE) of 10.4%, packed cell volume (PCV) of 31.5%). Parameters used for evaluation of cattle included determination of the PPE and PCV. Whole blood was collected in vacutainer tubes containing EDTA and used for preparation of stained blood smears for light microscopy and for determination of the PCV. Serum samples were collected from each animal upon purchase, at weeks 4 and 8 just prior to immunization, at week 10 and during tick feeding. Serum samples were stored at −70° C. until tested by ELISA and Western blots for determination of MSP1a antibody titers.

2. Identification of Cattle with High Antibody Liters to MSP1a, Tick Feeding Studies and Determination of *A. marginale* Infection Levels in Tick Salivary Glands.

Serum samples collected from cattle two weeks after the last immunization were analyzed as described previously by ELISA and Western blots for recognition of recombinant MSP1a [22]. Ten immunized animals with the highest titers against MSP1a by Western blot were selected from groups 1 and 2 (5 animals from each). Ten control animals from group 3 were randomly selected and sera from these cattle were proven to be negative for MSP1a antibodies by Western blot.

Each of the 20 cattle were infested with 60 male *D. variabilis* that were reared at the Oklahoma State University, Centralized Tick Rearing Facility. The ticks were placed in an orthopedic stockinettes glued to the cow's side when *A. marginale* infection was observed in stained blood smears. The ticks were allowed to feed on cattle for seven days, after which they were removed and held in humidity for 5 days. The ticks were then allowed to feed for 7 days on a sheep to stimulate development of *A. marginale* into tick salivary glands. The ticks were then removed from the sheep and the salivary glands from 20 ticks (40 salivary glands) were dissected and pooled in 500 μl RNALater (Ambion). DNA was extracted from groups of 40 salivary glands and then used in a quantitative msp4 PCR to quantify *A. marginale* infection levels [19, 22].

3. Statistical Analysis

For the analysis of the PPE and percent reduction PCV values between immunized and control cattle, pair wise comparisons (Student's t-test) were conducted. Salivary gland infection levels between ticks fed on vaccinated and control cattle were compared by Student's t-test. A correlation analysis between tick salivary gland infection levels and antibody titers against MSP1a in cattle during tick feeding was performed using Microsoft Excel 2000.

4. Results

Cattle chosen for these studies after vaccination and prior to challenge-exposure and tick feeding were based on high antibody titers to MSP1a Serum samples collected two weeks after the last immunization were analyzed by Western blot for recognition of MSP1a and 10 immunized animals with the highest titers against MSP1a were identified. Ten control animals were confirmed negative for MSP1a antibodies by Western blot. Mean peak PPE (3.6±2.6 and 3.2±1.7 for control and immunized cattle, respectively) and mean percent reduction of PCVs (29.3±7 and 26.8±12.2 for control and immunized cattle, respectively) of immunized and control cattle during tick feeding were not significantly (P>0.05) (Table 4).

TABLE 4

Peak percent parasitized erythrocytes during tick feeding on cattle, anti-MSP1a antibody titers, infection in tick salivary glands and inhibition of infection of *A. marginale* in ticks that acquired infection on immunized and control cattle.

Experimental groups	Cattle number[a]	Immunogen	Peak PPE during tick feeding[b]	Anti-MSP1a antibody titers[c]	Tick infection levels (copies msp4/salivary gland)[d]	Inhibition of tick infection[e]
Vaccinated	GT 152	Recombinant	0.3	1600	0.1	100%
	GT 155	MSP1a	1.6	<100	14	93.5%
	GT 165		5.8	1600	80	62.6%
	219		4.5	200	2	99.1%
	248		3.2	800	14	93.5%
	141	IDE8-	4.1	<100	25	88.3%
	GT 168	derived	1.6	<100	2	99.1%
	226	*A. marginale*	5.0	400	2	99.1%
	242	plus	3.0	400	14	93.5%
	294	recombinant MSP1a	2.8	200	25	88.3%
Control	143	None	4.3	<100	140	—
	157		0.5	100	0.4	—
	162		3.5	100	25	—
	166		2.5	<100	795	—
	210		3.2	100	2	—
	214		1.9	100	80	—
	217		1.6	100	140	—
	245		2.4	100	25	—
	247		6.9	400	140	—
	251		9.2	100	795	—

[a]Cattle were analyzed for antibody response against recombinant MSP1a before challenge-exposure. Ten immunized animals showing the highest titers against MSP1a and 10 controls with sera negative for MSP1a in the Western blot were selected.

[b]The percent infected erythrocytes (PPE) was determined in blood smears of samples collected daily during the 7 days of tick acquisition-feeding.

[c]Values correspond to the maximum dilution that gave an $OD_{450\ nm}$ equal or higher than mean background + 2 SD.

[d]DNA was extracted from 40 salivary glands and used in a quantitative PCR to determine *A. marginale* infection levels. The number of msp4 copies was calculated as $10^{[(\log Ta-0.5)/0.4]}$.

[e]The inhibition of tick infection was determined as [1 − (Infection level/Mean control infection level)] × 100.

Antibody titers against MSP1a were determined by ELISA in sera obtained after tick infestation and compared between immunized and control cattle (Table 1). The average anti-MSP1a antibody titers in immunized cattle (520±153; mean±SE) was higher (P=0.03) than in control cattle (110±34).

A. marginale infection levels in salivary glands from ticks that fed on rickettsemic immunized and control cattle are listed in Table 1. Although infection levels varied among individual ticks, the number of msp4 copies per salivary gland was higher (P=0.04) in ticks fed on control cattle (214±98; mean±SE) when compared to ticks that fed on immunized cattle (18±8). Differences were not observed between ticks fed on cattle immunized with recombinant MSP1a or with IDE8-derived *A. marginale* together with recombinant MSP1a. The average inhibition of tick infection in ticks that fed on the immunized cattle was 91.7% (range 62.69%–100.0%) (Table 4).

Differences in infection rates between ticks that fed on immunized and control cattle did not appear to be affected by the *A. marginale* infections in cattle or the percent reduction PCVs during tick feeding. The PPEs in the cattle were not statistically different among groups and the percent reduction PCVs were not low enough to affect tick feeding.

The results reported herein demonstrated that anti-MSP1a antibodies in vaccinated cattle reduced infection of *A. marginale* for *D. variabilis*. Differences in salivary gland infection levels between ticks fed on immunized and control cattle agreed with statistically significant differences in the anti-MSP1a antibody titers between immunized and control cattle after tick infestation. Difference in the results obtained after vaccination with recombinant MSP1a compared to the antibody response generated after *A. marginale* infection of cattle could be explained by differences in the anti-MSP1a antibody levels and/or by differences in the MSP1a epitopes recognized by the antibodies. The recombinant MSP1a protein is presented separately to the bovine immune system, rather than as a complex with MSP1b, which appears to allow for recognition of all the epitopes in the region containing the tandem repeats involved in adhesion of MSP1a to tick cells [15]. The antibodies against the native MSP1a may not be directed against the neutralizing domain masked by the structure of the MSP1 complex.

Comparison of the data obtained from cattle vaccinated with recombinant MSP1a or with IDE8-derived *A. marginale* together with recombinant MSP1a suggested that the antibody response against IDE8 and IDE8-derived *A. marginale* antigens, other than MSP1a, had little or no inhibitory effect on tick infection. The antibody response against MSP1a inhibited but did not prevent infection of ticks by *A. marginale*. As was reported in previous studies [18, 36], salivary gland infection levels were variable and reflected variation among individual ticks. Although the effect on the transmission of *A. marginale* by ticks fed on vaccinated cattle is unknown, this study suggests that MSP1a may be necessary but not sufficient for infection of ticks by *A. marginale*. Alternatively, over expression of MSP1a in erythrocytic stages of *A. marginale* and/or the native structure of MSP1a may prevent the complete neutralization of the ligand.

A desirable goal for a vaccine for the control of anaplasmosis is to have a protection effect on the multiplication of *A. marginale* in a bovine host and a blocking effect on the transmission of the pathogen by the tick vector. The results reported herein support the role of MSP1a in the transmission of *A. marginale* by ticks and suggest the incorporation of recombinant MSP1a in vaccine formulations against *A. marginale* in combination with infected IDF8 cells-derived antigens and/or as yet unidentified pathogen and tick-derived antigens.

While the invention has been been described with a certain degree of particularity, it is understood that the invention is not limited to the embodiment(s) set for herein for purposes of exemplification, but is to be limited only by the scope of the attached claim or claims, including the full range of equivalency to which each element thereof is entitled.

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What is claimed is:

1. A method for reducing *A. marginale* infection in ticks, said method comprising: administering to a ruminant population susceptible to tick infection a composition comprising recombinant MSP1a and an immunogen derived from *A. marginale*; wherein said immunogen is not MSP1b and said composition further comprises a pharmaceutically acceptable carrier or diluent; and allowing said ticks to feed on said ruminants.

2. The method according to claim **1**, wherein approximately 100 μg of said recombinant MSP1a is administered.

3. The method according to claim **1**, wherein said immunogen is tick cell culture derived *A. marginale*.

4. The method according to claim **3**, wherein said tick cell culture comprises *Ixodes scapularis* tick cell line IDE8.

5. The method according to claim **1**, wherein said recombinant MSP1a is from the Oklahoma isolate of *A. marginale*.

6. The method according to claim **3**, wherein said tick cell culture derived *A. marginale*, is selected from the group consisting of the Oklahoma, Virginia and Oregon isolates of *A. marginale*.

7. The method according to claim **3**, wherein said composition contains approximately 2×10^{10} *A. marginale*.

* * * * *