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Antigens and Alternatives for Control of *Anaplasma marginale* Infection in Cattle

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INTRODUCTION

Bovine Anaplasmosis

Bovine anaplasmosis is an arthropod-borne hemolytic disease of cattle that is caused by the rickettsia *Anaplasma marginale* (*Rickettsiales*: *Anaplasmataceae*) (28, 57, 80, 150). Clinical disease is most notable in cattle, but other ruminants including water buffalo, bison, African antelopes, and mule deer can become persistently infected with *A. marginale* (88). Sir Arnold Theiler first described *A. marginale* infection in erythrocytes of South African cattle as "marginal points" (161). A similar report was published in the United States by Salmon and Smith in 1896, which described the presence of a point-like pathogen in blood smears of cattle as "very minute roundish body which is stained blue to bring it into view. The body as a rule is situated near the edge of the corpuscle" (148). Theiler subsequently described a subspecies of *A. marginale*, *A. centrale*, which appeared to be less pathogenic and for which *Anaplasma* inclusions were more often found in the center of erythrocytes rather than in a marginal location (162).

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Erythrocytes are the only known site of infection of *A. marginale* in cattle (Fig. 1A) Within these cells the membrane-bound inclusions (also called initial bodies) contain four to eight rickettsia (Fig. 1B), and 70% or more of the erythrocytes may become infected during acute infection (137, 140). The incubation period of infection (prepatent period) varies with the number of organisms in the infective dose and ranges from 7 to 60 days, with an average of 28 days. After erythrocytic infection is detected, the number of parasitized erythrocytes increases geometrically. Infected erythrocytes are subsequently phagocytized by bovine reticuloendothelial cells, resulting in the development of mild to severe anemia and icterus without hemoglobinemia and hemoglobinuria. Clinical symptoms may include fever, weight loss, abortion, lethargy, icterus, and often death in animals older than 2 years (138). Cattle that survive acute infection develop persistent infections characterized by cyclic low-level rickettsemia (64, 65, 77) (Fig. 2). Persistently infected or "carrier" cattle have lifelong immunity and are resistant to clinical disease on challenge exposure. However, persistently infected cattle serve as reservoirs of *A. marginale* because they provide a source of infective blood for both mechanical and biological transmission by ticks. *Bos taurus* breeds (i.e., Holstein, Brown Swiss, or Hereford) are more likely to develop acute anaplasmosis than are crossbred Zebu or Creole cattle (2, 3).

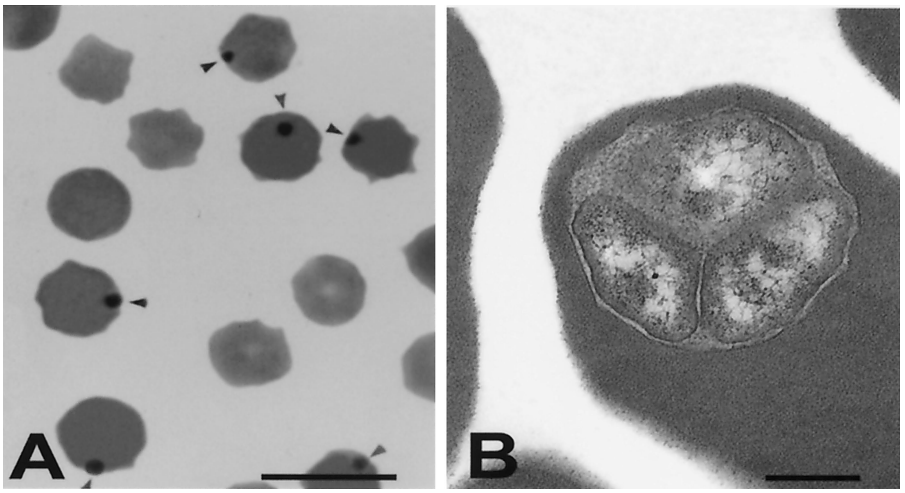

FIG. 1. Bovine erythrocytes infected with *A. marginale*. (A) Inclusion bodies (arrowheads) are located at the periphery of the erythrocyte in a stained blood film. (B) Electron micrograph of an *A. marginale* inclusion that contains three organisms. Bar, 10 μm (A) and 0.5 μm (B).

Calves are less susceptible to infection with *A. marginale* and, when infected, are less susceptible to clinical disease. This phenomenon is not well understood, but removal of the spleen renders calves fully susceptible to infection, and anaplasmosis in splenectomized calves is often more severe than that observed in older cattle. However, once calves become infected, they develop persistent infections and lifelong immunity to anaplasmosis.

Transmission of *A. marginale* can be effected both mechanically by biting flies or blood-contaminated fomites and biologically by ticks (56, 60, 78). Mechanical transmission frequently occurs via blood-contaminated fomites, including needles, dehorning saws, nose tongs, tattooing instruments, ear-tagging devices, and castration instruments. Mechanical transmission by arthropods has been reported for bloodsucking diptera of the genera *Tabanus*, *Stomoxys*, and mosquitoes (60, 63, 132). This form of mechanical transmission is considered to be the major route of dissemination of *A. marginale* in areas of Central and South America and Africa where tick vectors do not occur (60, 63) and where *Boophilus microplus*, the tropical cattle tick, does not appear to be a biological vector of *A. marginale* (42, 61). In areas of the United States where geographic isolates of *A. marginale* are not infective for ticks or where ticks have been eradicated by fire ants, mechanical transmission appears to be the major mode of *A. marginale* transmission (47, 156, 172).

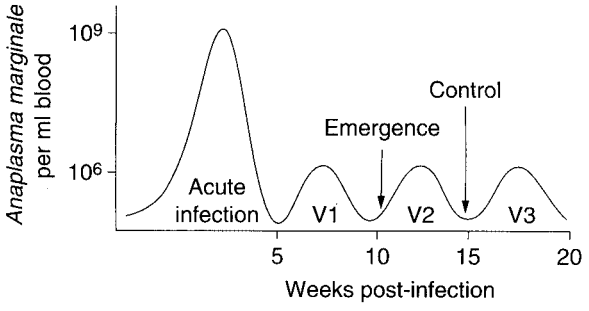

FIG. 2. The high *A. marginale* levels in acute rickettsemia ($>10^9$ ml^{-1}) are resolved after the development of a primary immune response, but the emergence of antigenic variants results in persistent infection. Persistence is characterized by sequential rickettsemic cycles, occurring at approximately 5-week intervals, in which new MSP2 variants replicate to a peak of $>10^6$ ml^{-1} and are then controlled by a variant-specific immune response. Variants arising in three sequential rickettsemic cycles are shown and are designated V1, V2, and V3. The points of variant emergence and variant control are designated for V2. (Reprinted from reference 125 with permission of the publisher.)

In addition to mechanical and biological transmission, *A. marginale* can be transmitted from cow to calf transplacentally during gestation (111, 176, 177). For example, a 15.6% prevalence rate of in utero transmission of *Anaplasma* infections was reported in South Africa (135). Transplacental transmission of anaplasmosis may therefore contribute to the epidemiology of this disease in some regions.

Biological transmission of *A. marginale* is effected by ticks, and approximately 20 species of ticks have been incriminated as vectors worldwide (56, 60). Tick transmission can occur from stage to stage (transstadial) or within a stage (intrastadial), while transovarial transmission from one tick generation to the next does not appear to occur (158). Interstadial transmission of *A. marginale* has been demonstrated by the three-host ticks *Dermacentor andersoni* and *D. variabilis* in the United States (78, 79, 83, 159) and by *Rhipicephalus simus* in South Africa (131, 133, 134). The one-host tick *B. annulatus* transmits *A. marginale* in Israel, Central America, South America, and Mexico (68, 149).

Intrastadial transmission of *A. marginale* is effected by male ticks. Recent studies have demonstrated that male *Dermacentor* ticks may play an important role in the biological transmission of *A. marginale* because they become persistently infected with *A. marginale* and can transmit *A. marginale* repeatedly when they transfer among cattle (82, 85). Male ticks therefore also serve as a reservoir of *A. marginale* along with persistently infected cattle (67, 80, 82, 85). Transmission of *A. marginale* by male ticks may be an important mechanism of transmission of *A. marginale* by one-host ticks, including *Boophilus* spp. and *D. albipictus*. However, it was shown recently that the cofeeding of

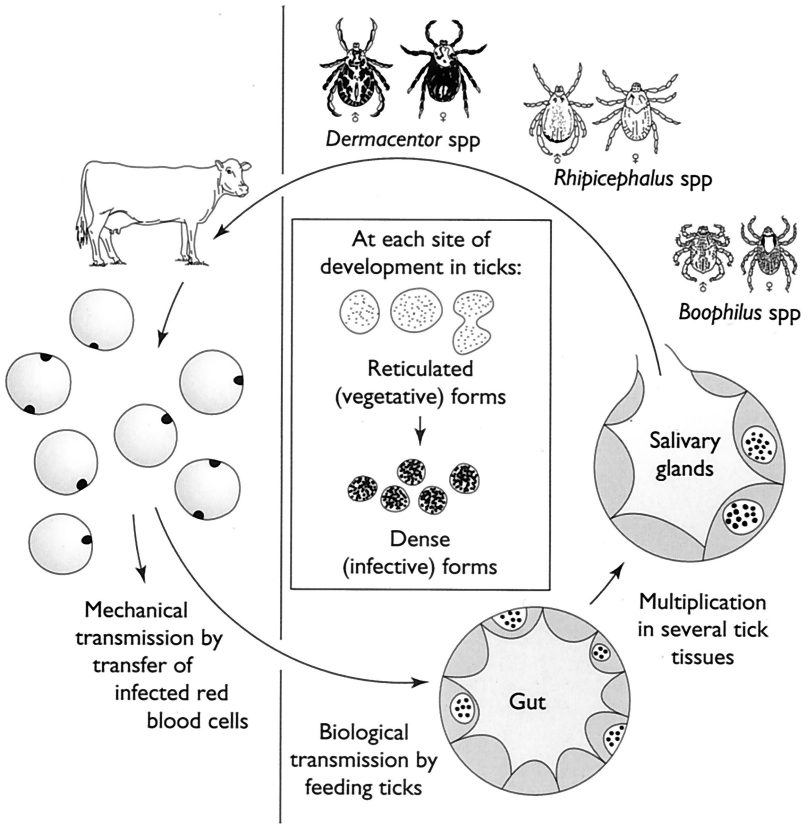

FIG. 3. Schematic of the development cycle of *A. marginale* in cattle and ticks. Infected erythrocytes are ingested by ticks (*Dermacentor* spp., *Rhipicephalus* spp., or *Boophilus* spp.) with the blood meal. The first site of infection of *A. marginale* in ticks is the gut cells. When the ticks feed a second time, many tick tissues become infected, including salivary gland cells, from where the rickettsia is transmitted back to cattle. Two forms of *A. marginale*, reticulated and dense forms, are found in infected tick cells. Reticulated forms appear first and are the vegetative stage that divides by binary fission. The reticulated form changes into the dense form, which is the infective form and can survive extracellularly. (Reprinted from reference 125 with permission from the publisher.)

adult *Dermacentor* spp. does not appear to influence the dynamics of *A. marginale* transmission (81).

The developmental cycle of *A. marginale* in ticks is complex and coordinated with the tick feeding cycle (78, 82, 85) (Fig. 3). Infected erythrocytes taken into ticks with the blood meal provide the source of *A. marginale* infection for tick gut cells (Fig. 4). After development of *A. marginale* in tick gut cells, many other tick tissues become infected, including the salivary glands (Fig. 5), from where the rickettsiae are transmitted to vertebrates during feeding (67, 78, 82, 85). At each site of infection in ticks, *A. marginale* develops within membrane-bound vacuoles or colonies. The first form of *A. marginale* seen within the colony is the reticulated (vegetative) form, which divides by binary fission (Fig. 6A), forming large colonies that may contain hundreds of organisms. The reticulated form then changes into the dense form (Fig. 6B), which is the infective form and can survive outside the host cells. Cattle become infected with *A. marginale* when the dense form is transmitted during tick feeding via the salivary glands.

Geographic Distribution of Bovine Anaplasmosis

Anaplasmosis occurs in tropical and subtropical areas throughout the world and is a major constraint to the cattle production in many countries. In the United States, anaplasmosis is enzootic throughout the southern Atlantic states, Gulf Coast states, and several of the Midwestern and Western states (98). However, anaplasmosis has been reported in almost every state in the United States, and this widening distribution may be due to increased transportation of cattle and hence the opportunity for mechanical transmission from asymptomatic persistently infected cattle.

Bovine anaplasmosis is also endemic in Mexico, Central and South America, and the Caribbean Islands. It is enzootic in most Latin American countries, with the exception of desert areas or mountain ranges such as the Andes (68). The seroprevalence of *A. marginale* varies widely among countries in the Americas (Table 1), and this variability contributes to the development of geographically stable or unstable enzootic regions.

The distribution of anaplasmosis may continue to change due to the trend of global warming, which may influence the movement of the tick hosts (N. N. Jonsson and S. W. J. Reid, Guest Editorial Vet. J. **160:**87–89, 2000). An example of this prediction is the confirmation of anaplasmosis in a bison herd in Saskatchewan, Canada, during the summer of 2000 (136). The first outbreak of anaplasmosis occurred in Canada in 1971 (25), but this outbreak was determined to be due to mechanical

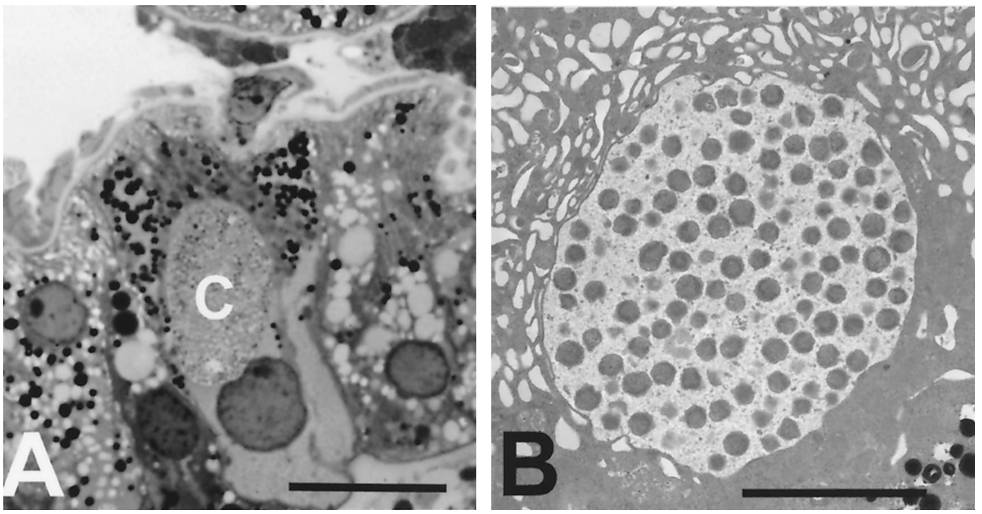

FIG. 4. Micrographs of colonies of *A. marginale* in tick gut cells. (A) Light micrograph of a large colony (C) in a tick gut cell. (B) Electron micrograph of a colony in a tick gut cell. Bar, 10 μm (A) and 5 μm (B).

transmission from imported carrier cattle. Protocols proposed in the Sanitary and Phytosanitary Measures and the Risk Assessment Methodology may be important to control the spread of diseases such as anaplasmosis in the future global trading market (152).

Economic Impact of Bovine Anaplasmosis

Bovine anaplasmosis causes important economic loss in most countries, mainly due to the high morbidity and mortality in susceptible cattle herds. The losses due to anaplasmosis are measured through several parameters: low weight gain, reduction in milk production, abortion, the cost of anaplasmosis treatments, and mortality. However, few controlled studies have been carried out to determine the exact annual loss caused by anaplasmosis in a country, since in general loss is reported as high, tremendous, or enormous. Nonetheless, the current annual losses in beef cattle in the United States as a result of anaplasmosis morbidity and mortality are estimated to be over $300 million per year (98), whereas in Latin America those losses were calculated to be approximately $800 million (95). More recently, it was reported that bovine anaplasmosis and babesiosis were responsible for causing an economic loss of $875 millions in Latin American nations (33). However, the most important economic constraint of anaplasmosis to cattle production in the tropics is on public or private programs for genetic improvement of cattle. Imported *Bos taurus* cattle brought from temperate nations to the tropics for breed improvement are highly susceptible to tick-borne diseases and often do not survive to become part of planned reproduction

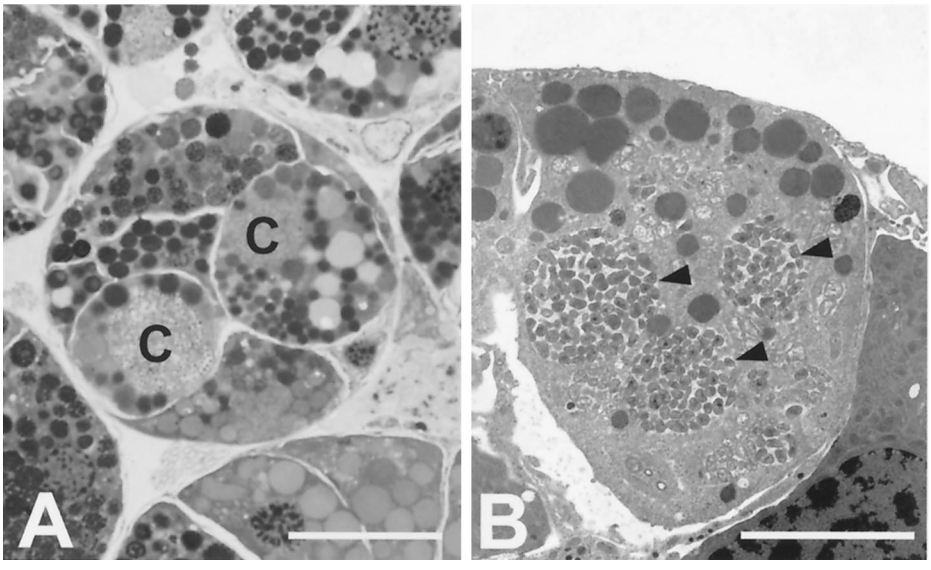

FIG. 5. Micrographs of colonies of *A. marginale* in tick salivary gland cells. (A) Light micrograph of two colonies (C) in salivary gland cells. (B) Electron micrograph of a tick salivary gland cell that contains several *A. marginale* colonies (arrowheads). Bar, 10 μm (A) and 5 μm (B).

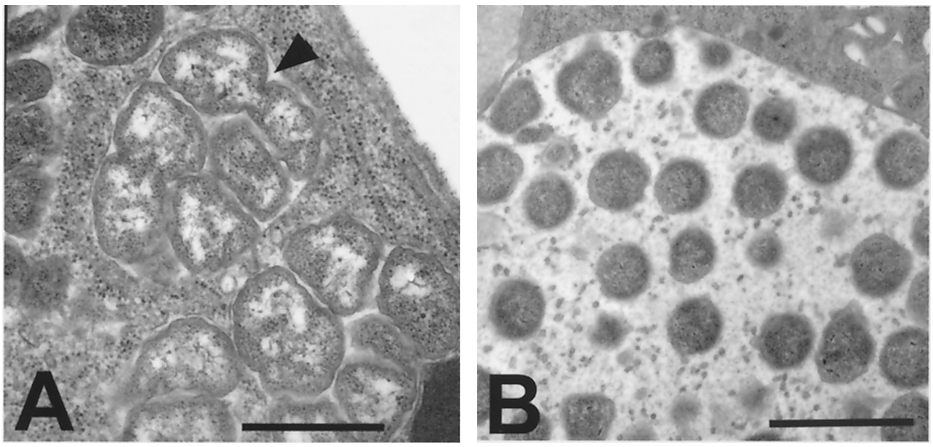

FIG. 6. Electron micrographs of the two developmental stages of *A. marginale* within colonies in tick cells. (A) Reticulated forms within a colony, dividing by binary fission (arrowhead). (B) Dense forms within a colony in an infected tick cell. Bars, 1 μm.

programs. This constraint is a notable reality for programs for the improvement of cattle in most Latin American countries (105).

TAXONOMY

Present Classification and Phylogenetic Relationships of *A. marginale* with Respect to the Organisms in the Family Anaplasmataceae

The organisms in the order *Rickettsiales* were recently reclassified based on biological characteristics and genetic analyses of 16S rRNA genes, *groESL*, and surface protein genes (57). These phylogenetic analyses consistently supported the formation of four distinct genera within the family *Anaplasmataceae*: (i) *Anaplasma*, with a 96.1% minimum similarity; (ii) *Ehrlichia*, with a 97.7% similarity; (iii) *Wolbachia*, with a 95.6% similarity; and (iv) *Neorickettsia*, with a 94.9% similarity (57) (Table 2). Organisms classified within the family *Rickettsiaceae* (genera *Rickettsia* and *Orientia*) are all obligate intracellular bacteria that grow freely within the cytoplasm of eukaryotic cells. While organisms placed in the family *Anaplasmataceae* are also obligate intracellular organisms, they are found exclusively within membrane-bound vacuoles in the host cell cytoplasm. Furthermore, most all organisms in the family *Anaplasmataceae* multiply in both vertebrates and invertebrates (primarily ticks and trematodes).

The genus of interest in this review, *Anaplasma*, includes three species that infect ruminants: *A. marginale* (the type

TABLE 1. Geographic distribution and seroprevalence of anaplasmosis in countries of the Americas

Country	Prevalence (%)	Technique[a]	Reference(s)
United States (Louisiana)	5.6	CT	73
United States (Oklahoma)	4.7–17.6	CF[b]	143
Costa Rica	61–90	cELISA, PCR	72
Venezuela	57.7	IFA	74, 106
Colombia	64–100	IFA	117
Brazil	67.3	IFA	167
Paraguay	92	CT	128
Argentina	7–61	Blood smears	93
Jamaica	69.9	CT	102
Lesser Antilles	18–71	Dot ELISA	41

[a] CF, complement fixation test; CT, card test; cELISA, competitive enzyme-linked immunosorbent assay; IFA, immunofluorescence assay.

[b] The results of the complement fixation test provide minimum estimates of seropositive cattle and are likely to underreport the prevalence of *A. marginale* (27).

TABLE 2. Current classification of the order *Rickettsiales*

Family *Rickettsiaceae* (obligate intracellular bacteria that grow freely in the cytoplasm of their eukaryotic host cells)
- Genus *Rickettsia*
- Genus *Orientia*

Family *Anaplasmataceae* (obligate intracellular bacteria that replicate with membrane-derived vacuoles in the cytoplasm of eukaryotic host cells)
- Genus *Anaplasma*
  - *Anaplasma marginale* (type species)
  - *Anaplasma centrale*
  - *Anaplasma ovis*
  - *Anaplasma bovis* (formerly *Ehrlichia bovis*)
  - *Anaplasma phagocytophilum* (formerly *Ehrlichia phagocytophilum*, *E. equi*, HGE agent)
  - *Anaplasma platys* (formerly *Ehrlichia platys*)
  - *Aegyptianella* (genus incertae sedis due to lack of sequence information)
- Genus *Ehrlichia*
  - *Ehrlichia chaffeensis*
  - *Ehrlichia ruminantium* (formerly *Cowdria ruminantium*)
  - *Ehrlichia ewingii*
  - *Ehrlichia ovis*
  - *Ehrlichia canis*
  - *Ehrlichia muris*
- Genus *Neorickettsia*
  - *Neorickettsia helminthoeca*
  - *Neorickettsia risticii* (formerly *Ehrlichia risticii*)
  - *Neorickettsia sennetsu* (formerly *Ehrlichia sennetsu*)
- Genus *Wolbachia*
  - *Wolbachia pipientis*

species). *A. marginale* subsp. *centrale* (referred to in this review as *A. centrale*), and *A. ovis* (57). Bovine anaplasmosis is caused primarily by *A. marginale*. While *A. centrale* is less pathogenic for cattle and has been used as a live vaccine in Israel, Australia, Africa, and South America, infection with this organism can, on occasion, cause clinical disease. *A. ovis* is a pathogen of sheep and does not establish persistent infection in cattle.

The genus *Anaplasma* also includes *A. phagocytophilum* (formerly *Ehrlichia equi*, *E. phagocytophila*, and the agent of human granulocytic ehrlichiosis [HGE], now recognized as synonymous), *A. bovis* (formerly *E. bovis*), and *A. platys* (formerly *E. platys*). *Aegyptianella* was retained in this genus because of phenotypic similarities to the species of *Anaplasma* and was designated a genus incertae sedis due to lack of sequence information.

The organisms classified within the other three genera of the family *Anaplasmataceae* (*Ehrlichia*, *Neorickettsia*, and *Wolbachia*) are listed in Table 2 (57). Note that *E. ruminantium* (formerly *Cowdria ruminantium*), the tick-borne pathogen that causes heartwater disease in cattle, was found to be more closely related to the organisms in the genus *Ehrlichia*, which includes organisms that infect a variety of vertebrate hosts ranging from humans to rodents.

A. marginale Genome and Major Surface Protein Genes

The small genome of *A. marginale* is circular, and the size is estimated at 1.2 to 1.6 Mb (6, 104). Research during the past 20 years has focused on identification of the major surface proteins (MSPs) of *A. marginale*. Six MSPs, MSP1a, MSP1b, MSP2, MSP3, MSP4, and MSP5, have been identified on erythrocyte-derived organisms, and information about the gene sequences, recombinant protein, monospecific and monoclonal antibodies, isolate variability, and potential value in diagnostic assays and vaccines is available. MSP1a, MSP4, and MSP5 are encoded by single genes, while MSP1b, MSP2, and MSP3 are encoded by multigene families.

MSP1a and MSP1b form the MSP1 complex. MSP1a is variable in molecular weight among geographic isolates because of different numbers of tandem 28- or 29-amino-acid repeats located in the amino-terminal portion of the protein (8, 46, 52, 53, 54). Because of the variation in the repeated portion of the MSP1a gene, it has been used as a stable genetic marker for identification of *A. marginale* geographic isolates (8, 14, 17, 53). The gene, *msp1a*, that encodes MSP1a is conserved during the multiplication of the rickettsia in cattle and ticks (26, 126). A neutralization-sensitive epitope was demonstrated on the MSP1a tandem repeats (127) and was found to be conserved among *A. marginale* isolates (46, 47, 52, 53, 114, 127). MSP1a was shown to be an adhesin for bovine erythrocytes and both native and cultured tick cells by using recombinant *Escherichia coli* expressing MSP1a in microtiter hemagglutination and adhesion recovery assays and by microscopy (45, 46, 100, 101). The portion of MSP1a with the tandem repeats was found to be necessary and sufficient to effect adhesion to bovine erythrocytes and tick cells (46). MSP1a was shown to be involved in infection and transmission of *A. marginale* by *Dermacentor* ticks (44) and to contribute to immunity to *A. marginale* infection in cattle (34, 35, 118).

MSP1b, encoded by at least two genes, *msp1*β*1* and *msp1*β*2*, is polymorphic among geographic isolates of *A. marginale* (17, 26, 40, 169). Although MSP1b is encoded by a multigene family, only small variations in protein sequences of $MSP1b_1$ and $MSP1b_2$ were observed during the life cycle of the rickettsia in cattle and ticks (26). This protein, which forms a complex with MSP1a, is an adhesin for bovine erythrocytes (100, 101). However, MSP1b was recently demonstrated to be an adhesin only for bovine erythrocytes and did not prove to be an adhesin for tick cells (45).

MSP2 and MSP3 are both encoded by large polymorphic, multigene families (7, 123). The MSP2 sequence and antigenic composition varies during cyclic rickettsemia in cattle (15, 64, 65) and in persistently infected ticks (49). MSP2 is encoded on a polycistronic mRNA. The *msp2* gene within the expression site is polymorphic. *msp2* encodes numerous amino acid sequence variants selected in bovine erythrocytic and tick salivary gland populations of *A. marginale* (16, 29, 49, 64, 65, 104). MSP3 also varies in antigenic properties and structure among geographic isolates (5). MSP2 and MSP3 are involved in the induction of a protective bovine immune response to *A. marginale* (125). MSP4 and MSP5 are encoded by single-copy genes. Although MSP4 is highly conserved (52, 53, 54, 112), information about its function is not available. MSP5 is also a highly conserved surface protein that has been proven effective as a diagnostic antigen and used in a competitive enzyme-linked immunosorbent assay (ELISA) commercially available in the United States (163). The function of MSP5 is also unknown. The *msp2* operon-associated genes OpAG1, OpAG2, and OpAG3, have been identified recently in *A. marginale* and may encode for surface proteins (94).

Despite the advances in characterizing major MSPs in *A. marginale*, our knowledge of these proteins is limited and will be greatly enhanced by the completion of the genome sequence presently under way at Washington State University (http://www.vetmed.wsu.edu/research_vmp/anagenome/index.html), which will further facilitate the analysis of sequence information.

Gene Regulation in *A. marginale* Multigene Families

At least two transcriptionally active copies of *msp1*β have been identified in the genome of *A. marginale* (26). However, although small variations were observed in the $MSP1b_1$ and $MSP1b_2$ protein sequences, recombination does not seem to be an important mechanism in *msp1*β regulation and the expression from different loci appears to play the major role (26). The *msp2* gene is estimated to have 10 or more copies in the genome of *A. marginale* (30). However, all but one of the identified *msp2* copies are pseudogenes, and the operon containing the expressed *msp2* is a single copy (30). The *msp2* transcripts are polycistronic and linked to the MSP2-encoding open reading frame (16). The pseudogenes recombine into the *msp2* gene to generate new hypervariable sequences and new antigenic variants during the multiplication of the bacterium (30). Partial pseudogene cassettes are also present for the *msp3* gene family, and the pseudogenes for the two gene families often appear close together (29, 104). There is increasing evidence that for pathogenic microbial species, loss of gene function or genome decay increases with adaptation to the host (174). The *msp2* and *msp3* pseudogenes may be remnants of

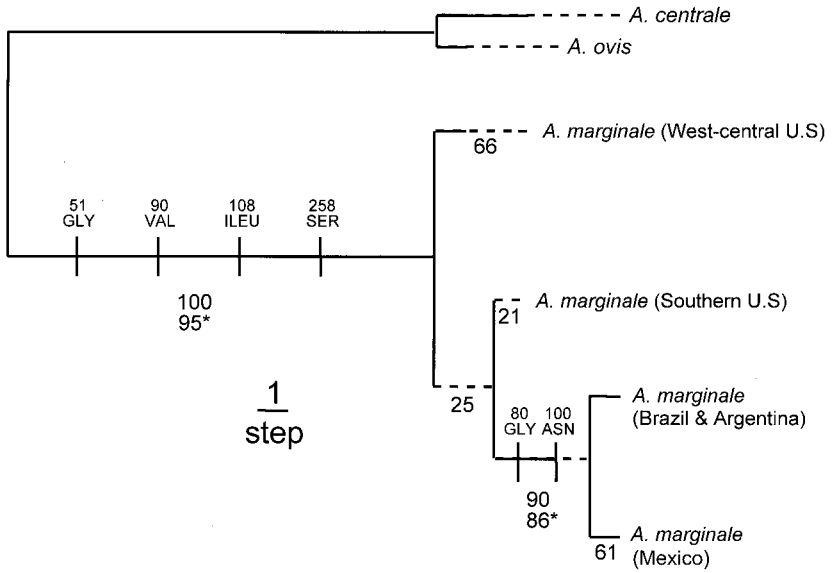

FIG. 7. Maximum-parsimony analysis of MSP4 sequences. The topology of 1 of 11 equally most-parsimonious trees based on DNA sequence variation in the *msp4* gene is shown. The tree was rooted with *A. centrale* and *A. ovis*. The solid portion of each branch represents the minimum branch length; the dashed portion of each branch represents the maximum branch length as determined by PAUP*4.0b4a. Numbers below the branches represent the percentage of 500 bootstrap iterations in which each clade was detected, and numbers with asterisks indicate bootstrap support based on a phylogenetic analysis of deduced amino acid residues. Vertical lines show synapomorphic amino acid changes documenting the monophyly of *A. marginale* and the Latin American clade of *A. marginale* isolates along with the amino acid residue and its position in the sequence. (Adapted from reference 52 with permission of the publisher.)

functional genes from a host-adapted pathogen in the process of down-sizing its genome content with a more efficient use of genome information: a process of reductive convergent evolution caused by prolonged intracellular life (119, 146). The two gene families have the same 5′ sequence, suggesting that they could use similar mechanisms to regulate recombination into the expression site; this specificity is guaranteed by the respective 3′ recombinatorial site in the coding region of each gene (29, 104). The coordinated control of the recombination of these genes contributes to the evasion of the host immune response by the pathogen. However, variation must arise spontaneously and frequently to allow selection of variants that escape the host immune system (30). Genome decay and variation of gene expression have been reported for other pathogenic bacteria including *Rickettsia prowazekii* (174).

Phylogenetic Relationships of Geographic Isolates of *A. marginale*

Phylogenetic analysis of *A. marginale* geographic isolates from the United States was performed using the single-copy genes *msp1*α and *msp4* (53). The results of these analyses strongly support a southeastern clade of *A. marginale* composed of isolates from Virginia and Florida. Analysis of 16S ribosomal DNA fragment sequences from the tick vector of *A. marginale*, *D. variabilis*, from various areas of the United States was performed and suggested coevolution of the vector and pathogen (53).

Phylogenetic studies were also done using New World isolates of *A. marginale* from the United States, Mexico, Brazil, and Argentina. Seventeen isolates of *A. marginale* plus two outgroup taxa (*A. centrale* and *A. ovis*) were included for the analysis of MSP4 sequences (52) (Fig. 7). Maximum-parsimony analysis of MSP4 sequences provided phylogenetic information about the evolution of *A. marginale* isolates. Strong bootstrap support was detected for a Latin American clade of *A. marginale* isolates. Moreover, within this Latin American clade, strong bootstrap support was detected for Mexican and South American clades. Isolates of *A. marginale* from the United States also grouped into two clades, a southern clade consisting of isolates from Florida, Mississippi, and Virginia, and a west-central clade consisting of isolates from California, Idaho, Illinois, Oklahoma, and Texas. Although little phylogeographic resolution was detected within any of these higher clades, *msp4* sequences appear to be a good genetic marker for inferring phylogeographic patterns of isolates of *A. marginale* on a broad geographic scale. In contrast to the phylogeographic resolution provided by MSP4, DNA and protein sequence variation from MSP1a representing 20 New World isolates of *A. marginale* failed to provide phylogeographic resolution (52). Most variation in MSP1a sequences appeared unique to a given isolate. In fact, similar DNA sequence variation in MSP1a was detected within isolates from Idaho and Florida and from Idaho and Argentina. These results suggest that the MSP1a sequence may be rapidly evolving and that the *msp1*α gene may provide phylogeographic information only when numerous MSP1a sequences from a given area are included in the analysis.

Eleven *A. marginale* isolates isolated from cattle with anaplasmosis in Oklahoma during 2001, plus two previous isolates from Wetumka (Oklahoma isolate [52, 53]) and Pawhuska identified in 1997 and the 1960s, respectively, were analyzed for the *msp1*α and *msp4* gene and protein sequences (54). Only the phylogenetic analysis with *msp4* sequences provided phy-

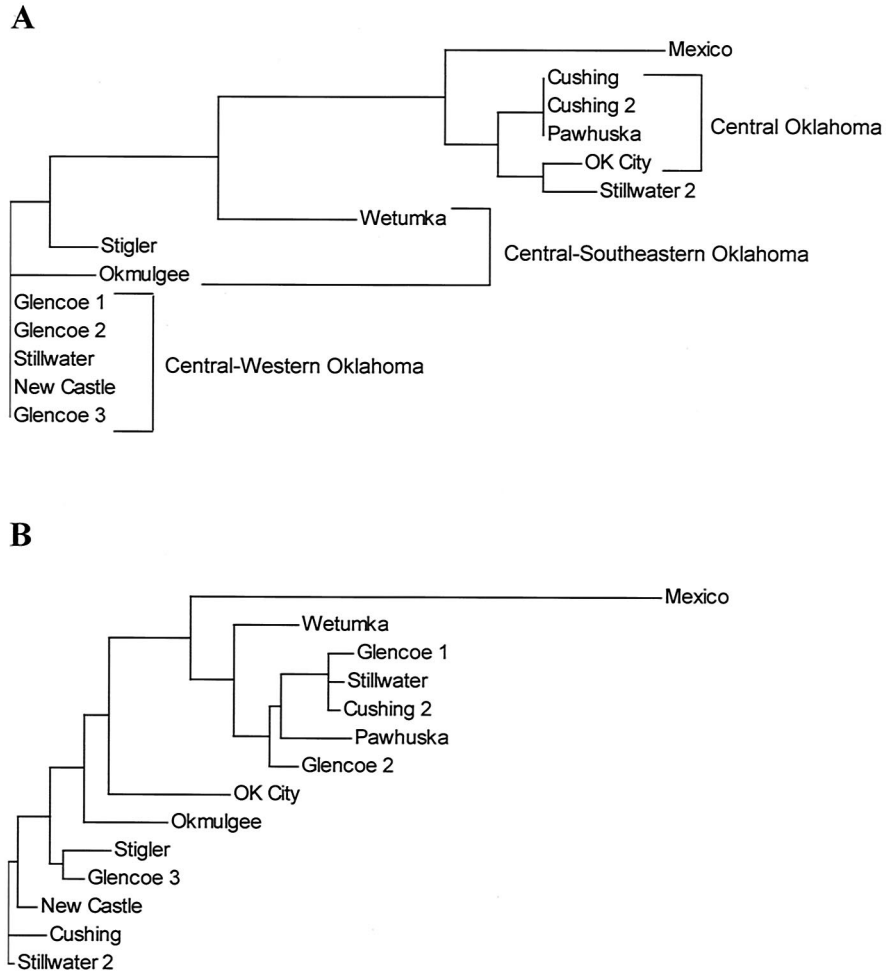

FIG. 8. Phylogenetic tree constructed from analysis of the *msp4* (A) and *msp1*α (B) coding sequences based on a sequence distance method utilizing the neighbor-joining algorithm (147). Sequences derived from the Mexico isolate of *A. marginale* (52) were used as outgroup. The geographic distribution of the Oklahoma isolates of *A. marginale* is shown for the *msp4* analysis in panel A.

logeographic information within Oklahoma (Fig. 8A). On a broader geographic scale, including other isolates from the United States and Latin America with *A. centrale* and *A. ovis* as outgroups, phylogenetic analysis with *msp4* sequences gave essentially the same results as reported previously, identifying two main clades composed of isolates from the United States and Latin America (52) (Fig. 7). The analysis of MSP1a DNA and protein sequences demonstrated extensive genotypic variation among Oklahoma isolates of *A. marginale* and failed to provide phylogeographic resolution within Oklahoma or on a broader scale, including isolates from other U.S. states and Latin America (Fig. 8B). Furthermore, analysis of codon and amino acid changes over the *msp1*α and *msp4* phylogenies provided evidence that *msp1*α, but not *msp4*, is under positive selection pressure (54).

These results suggest that even if MSP1a sequences are rapidly evolving, *msp1*α genotypes reflect the history of cattle movement more than the geographic distribution of *A. marginale* isolates. Recent results suggest that different *A. marginale* genotypes are maintained within a herd in an area of endemic infection by independent transmission events and that infection with more than one genotype per host is prevented, a phenomenon described as infection exclusion (43, 48, 126). The mechanism by which infection exclusion of *A. marginale* isolates occurs is unknown, but it has also been documented in *Rickettsia* spp. (38). Therefore, if cattle movement imports a new *A. marginale* genotype, it could be established by mechanical and/or biological transmission to susceptible cattle. In regions with few cattle introductions, like Australia, little genotypic variation is found within *A. marginale* isolates (24). In regions with extensive cattle movement, like Oklahoma, a highly heterogeneous *A. marginale* population would be expected. *msp4* sequences appear to be a good genetic marker for evolutionary studies within the genus *Anaplasma* and for inferring phylogeographic patterns of *A. marginale* isolates (54). Heat shock protein 60 (Hsp60) (*groEL*) gene sequences have been also used for phylogenetic analysis of *Anaplasma* spp. (92). However, phylogenies are working hypotheses, and more isolates should be included in future analysis.

ANAPLASMOSIS VACCINES

Control measures for anaplasmosis have not changed markedly over the past 60 years. They vary with geographic location and include arthropod control by application of acaricides, by administration of antibiotics, and by vaccination (as reviewed in reference 80). Arthropod control is not practical in many areas and may only partially prevent against *A. marginale* transmission, which occurs both by mechanical transmission of infected blood via insects and fomites and by biological transmission via ticks. Chemotherapy, probably used more often for prevention of anaplasmosis in the United States than in other areas of the world, is expensive and often not applicable to range cattle, and the intensive use of antibiotics bears the risk of causing selection of resistant strains.

Vaccination has been an economical and effective way to control bovine anaplasmosis worldwide. Vaccines for the control of anaplasmosis can be divided into two major types: live and killed vaccines. Both types of vaccines rely on the use of *A. marginale* from infected bovine erythrocytes. Both types induce protective immunity that reduces or prevents clinical disease, but these vaccines do not prevent cattle from becoming persistently infected with *A. marginale*. Persistent infections in cattle contribute to the further spread of *A. marginale* because these cattle serve as a reservoir of infection for mechanical transmission or as a source of infection for ticks.

Research carried out in the last two decades has contributed greatly to our knowledge of the antigenic composition of *A. marginale* and the role of MSPs in the development of protective immunity to *A. marginale* infection. However, at present, novel vaccines using molecular technologies have not been developed and marketed.

Live Vaccines

Use of live vaccines for control of anaplasmosis was initiated by Sir Arnold Theiler in the early 1900s and continues to be the vaccine of choice in many parts of the world (162). Live vaccines involve the infection of cattle via inoculation with erythrocytes infected with less pathogenic isolates of *A. marginale* or *A. centrale*. For vaccine production, splenectomized calves maintained under quarantine conditions are experimentally inoculated with defined strains and serve as a source of infective blood. Vaccinated cattle develop persistent infections, which induce lifelong protective immunity in cattle, and revaccination is usually not required (1, 139, 160, 175). Vaccination strategies using live organisms include (i) infection and treatment, (ii) live vaccines containing attenuated strains of *A. marginale*, and (iii) live vaccines containing the less pathogenic *A. centrale*.

Infection-treatment method. Infection of cattle with *A. marginale* followed by treatment with antibiotics has been used in the past for prevention of clinical anaplasmosis. This procedure involves inoculation of cattle with *A. marginale*-infected erythrocytes followed by treatment with low doses of tetracycline drugs during the initial appearance of patent infection. The cattle then become persistently infected without experiencing acute anaplasmosis and are subsequently immune to challenge exposure with the same or different isolates (9, 175). However, even with timely treatment with tetracycline, control of postinoculation reactions was often unsuccessful in preventing acute disease (89). Use of this type of immunization requires supervision by a veterinarian, which increases expense. The difficulty of closely monitoring cattle for effective and timely treatment often renders this approach impractical, especially for large herds of cattle.

Vaccination with attenuated strains of *A. marginale*. Attenuated strains have been considered for use in commercial live vaccines (87, 171). Attenuation of *A. marginale* was attempted by passage of the organism in sheep or deer (90, 93), while other workers claimed success after ^{60}Co irradiation of pathogenic strains (58, 140, 151). Investigators have used both techniques (irradiation or sheep and deer passage) to develop an attenuated *A. marginale* strain; however, in Australia a similar method was used without success (145). The attenuated vaccine developed by Ristic and coworkers was tested in several trials and found to be effective (76, 139), and the authors claimed that this vaccine was safe for cattle of any age, sex, and breed (115, 116). Nevertheless, other authors observed postvaccination reactions after the use of this vaccine (18, 71). In Argentina it was reported that dairy cows inoculated with the attenuated strain suffered reduction of milk production, fever, anorexia, adynamia, icterus, and death of the most severely affected cows (10). The use of this vaccine was not recommended in cattle older than 12 months. Recently, live trivalent vaccine for babesiosis and anaplasmosis, which contained a less pathogenic strain of *A. marginale*, was tested in Colombia; it failed to induce protective immunity against anaplasmosis and resulted in clinical disease in some cattle (19).

Live *A. centrale* vaccine. *A. centrale*, isolated by Sir Arnold Theiler in the early 1900s, is the most widely used live vaccine strain for control of bovine anaplasmosis (162). Theiler observed that *A. centrale* was less pathogenic for cattle than *A. marginale* was and that cattle infected with *A. centrale* developed protective immunity against *A. marginale* infection. This *A. centrale* strain continues to be used for vaccine production in several areas of the world including Africa, Australia, Israel, and Latin America.

A. centrale and *A. marginale* share immunodominant epitopes that may play a role in the protection induced by *A. centrale* (155). Recent studies have demonstrated that antigenic variation of MSP2 occurs during persistent *A. centrale* infections in a manner similar to that described for *A. marginale* (153). In addition, $CD4^+$ T-cell epitopes were conserved between the two species, which may contribute to the cross-protection afforded by the *A. centrale* live vaccine (154).

A. centrale infection induced by the live vaccine in cattle may also prevent subsequent *Anaplasma* infections on challenge exposure. Recent studies suggest that cattle can become infected with only one genotype of *A. marginale* (48, 126). Using the *msp1*α genotype as a stable isolate marker, only one genotype was found per animal in herds of cattle from endemic areas where many genotypes were detected (54, 126). An infection exclusion phenomenon was demonstrated in another study in which cattle simultaneously inoculated with two *A. marginale* isolates became infected with only one isolate, apparently excluding the other (48). The same phenomenon was demonstrated in an *Anaplasma*-tick cell culture system (48) and naturally infected ticks (43). Inoculation of cultured cells with two *A. marginale* isolates resulted in the establishment of

only one of the isolates. In addition, infection of the cultured cells with a second species, *A. ovis*, prevented the establishment of *A. marginale* in cell culture. However, recent results by Shkap et al. (154) show that cattle vaccinated with *A. centrale* later became infected with *A. marginale*, suggesting that the phenomenon of infection exclusion may not operate for all *Anaplasma* spp. or occurs in cattle infected by isolates of the same species (i.e., *A. marginale*) only. As discussed above, the mechanism of infection exclusion of *A. marginale* isolates is unknown and may be different in infected cattle and ticks. Therefore, more research is needed to study the phenomenon of infection exclusion and to determine whether *A. centrale* infection, established in cattle via live vaccines, prevents cattle from subsequently becoming infected with *A. marginale*. If so, protection of cattle against subsequent challenge exposure with other *A. marginale* isolates would be an important advantage of live vaccines.

Field and laboratory failures of *A. centrale* vaccines have been reported and are not uncommon (31, 69, 173), and the vaccine strain has been reported to cause severe anaplasmosis in splenectomized and adult cattle (86, 130). High-performance milking cows appear to be most severely affected after *A. centrale* infection (129), and the vaccine has been most successfully used in young cattle (130). When live vaccines for *A. centrale* and *Babesia bovis* were administered together, the growth rate of calves was not affected (4, 157). In a recent study conducted in Australia (24), use of the live *A. centrale* vaccine appeared to be justified because although the bovine response was variable, protection against challenge exposure was adequate to prevent disease in most cases. However, the authors cautioned that this vaccine may not provide protection against antigenically diverse and highly virulent stocks of *A. marginale* in other parts of the world. Nevertheless, *A. centrale* vaccines have been used for almost a century, and it is apparent that herdsmen and veterinarians promote the use of this live vaccine for prevention of anaplasmosis outbreaks under field conditions. This is especially evident in countries such as Argentina, Australia, Brazil, South Africa, and Uruguay, where several hundreds of thousands doses are sold yearly, and use of the vaccine will most probably continue until more effective vaccines become available.

Possible side effects. The repeated usage of live vaccines in cows may result in the production of erythrocytic isoantibodies which, when ingested by calves in colostrum, may cause hemolytic anemia (55). Live vaccines, if administered correctly, should induce persistent infection in cattle. Therefore, subsequent vaccinations should not be required, and the use of only one inoculation would minimize the development of erythrocytic antibodies and the associated risk of hemolytic anemia in calves (39).

Another drawback of live, blood-derived vaccines is the risk of transmitting other pathogens that persistently infect cattle. The spread of bovine leukosis virus by live vaccines has been reported (144). Emerging infectious agents may infect and cause disease in cattle, which may increase the risk of introducing contaminating pathogens via live vaccines. It is recommended that the use of these blood-derived live vaccines be restricted to the area where they were produced.

Killed Vaccines

Killed vaccines developed in the United States in the 1960s were marketed until 1999, when they were withdrawn from the marketplace due to company restructuring. Killed vaccines continue to be tested (141, 142) and may still be used in some areas. They have several advantages over to live vaccines. The risk of contamination with undesirable infectious agents is low, storage is inexpensive, and postinoculation reactions are of minimal clinical relevance. Disadvantages of killed vaccines include the need for yearly boosters, the higher cost of purification of *A. marginale* from erythrocytes, and the lack of cross-protection among isolates from widely separated geographic areas. In addition, the protective immunity afforded by killed vaccines is usually lower than that of live vaccines. However, despite the advantages of killed vaccines, these vaccines are not used worldwide as frequently as live vaccines.

The first commercial killed vaccine for the control of anaplasmosis used *A. marginale* from hemolyzed erythrocytes as an antigen that was lyophilized and combined with an oil-based adjuvant at the time of vaccination (32). Two vaccine doses administered 4 weeks apart were required during the first year, followed by one booster immunization per year. This original vaccine was heavily contaminated with erythrocyte stroma, which resulted in the development of erythrocytic isoantibodies in vaccinated cattle. Hemolytic anemia occurred in calves after they ingested colostrum from cows with high antibody titers (55). This problem was subsequently overcome by purification of *A. marginale* from erythrocytes (70). In addition, vaccination was not recommended for cows in the latter part of pregnancy, thus ensuring that calves would not be exposed to high levels of erythrocytic isoantibodies. A technique for large-scale production of *A. marginale* antigen from infected bovine blood was developed (99). This vaccine was used effectively until it was removed from the market in 1999 (96).

Some killed vaccines were effective for the prevention of anaplasmosis (32, 107), while others showed protection failures (3, 62, 91). It was demonstrated that some *A. marginale* isolates were not cross-protective, and it appears the vaccines are most effective when made from local isolates (91). A killed erythrocyte-derived vaccine which was tested in Mexico contained three isolates which provided complete protection against one isolate and partial protection against a second; protection could not be evaluated for the third isolate (141).

Prospects for Development of New and More Effective Vaccines

Development of a cell culture-derived killed vaccine. Recently, a cell culture system was developed for *A. marginale* in which the rickettsia was propagated in a continuous culture in a cell line, IDE8, derived from embryos of the tick *Ixodes scapularis* (108, 109). The developmental cycle of *A. marginale* in cultured tick cells was similar to that described previously in naturally infected ticks (22). *A. marginale* isolates harvested from cell culture were infective for both cattle and ticks (20, 108). The six MSPs characterized on *A. marginale* from bovine erythrocytes were found to be conserved on the cell culture-derived organisms, and the antigenic composition of *A. margi-*

nale remained the same after successive passage in cell culture (14) or after passage through ticks (15). The antigenic identity of the *A. marginale* isolate, as determined by the molecular weight of the MSP1a, was retained in culture (20, 26, 47, 108). *A. marginale* derived from the cultured tick cell was tested as an immunogen for cattle. In two trials, cattle immunized with the cell culture-derived *A. marginale* isolate developed protective immunity and did not develop clinical signs of anaplasmosis after challenge exposure by infected blood or by feeding infected ticks (50, 84). Nevertheless, the protection was partial and the disease was not prevented. The main effect of the vaccine was similar to the effect observed with erythrocyte-derived *A. marginale*, resulting predominantly in a less pronounced reduction in the levels of packed cell volume, which directly correlate with the anemia produced by *A. marginale* infection. These studies were conducted by the group at Oklahoma State University and the vaccine licensee, Grand Laboratories (Larchwood, Iowa), now part of Novartis Animal Vaccines Inc. (Larchwood, Iowa).

A differential immune response to MSP1a and MSP1b was observed in cattle immunized with erythrocyte- or cell culture-derived *A. marginale* (50, 84; J. C. Garcia-Garcia, J. de la Fuente, E. F. Blouin, and K. M. Kocan, Conf. Research Workers Anim. Dis., abstr. 199, 2002); Cattle immunized with erythrocyte-derived organisms had a preferential antibody response to MSP1a, while cattle immunized with cell culture-derived organisms produced antibodies predominantly to MSP1b. These findings suggest that the expression of MSP1 may vary during multiplication of the rickettsia in the tick and cattle hosts. These differences appear to correlate with the differential function of MSP1a and MSP1b in bovine erythrocytes and tick cells. MSP1a is an *A. marginale* adhesin for both bovine erythrocytes and tick cells, while MSP1b is an adhesin only for bovine erythrocytes (45, 100, 101).

Recent phylogenetic studies of U.S. geographic isolates of *A. marginale* demonstrated two clades: one from the southeastern United States and the other from the central and western United States (53). The inclusion of *A. marginale* isolates from geographic regions of the United States into a cell culture-derived vaccine may enhance the efficacy of the vaccine. To date, three isolates of *A. marginale* (from Virginia, Oklahoma, and Oregon) have been propagated in the cell culture system (20, 21, 108). Use of the cell culture-derived vaccine would avoid problems associated with previous erythrocyte-derived vaccines. This vaccine would be easily standardized, would be free of bovine erythrocyte stroma and contaminating pathogens, and, importantly, would not require the use of cattle for antigen production. The cell culture-derived antigen is being used for the development of a new killed vaccine in the United States and is projected to be marketed within the next 2 years. This cell culture-derived vaccine should fill a void in the United States, where vaccines for anaplasmosis are currently not available. The same approach could be used for other countries as well, using local isolates.

Nevertheless, despite the advances in vaccine development and the improvements introduced by use of the cell culture-derived vaccine, this preparation requires further research to evaluate the effect of combinations with recombinant antigens in order to improve the efficacy of the vaccine, to confer protection to *A. marginale* infection, and to block the biological transmission of the pathogen.

Development of novel vaccines. The success of novel vaccines for anaplasmosis by using molecular technologies will depend on their ability to either mimic or redirect the host response during natural infections or block infection of host cells. Recent research, as reviewed by Palmer (118) and Palmer et al. (125), has provided much information about the nature of the immune response of cattle to *A. marginale* infection, as well as the definition of key *A. marginale* antigens that appear to play a role in the immune response (120). A model for vaccine-induced immunity to *A. marginale* was proposed in which pathogen clearance is effected by antibody against surface epitopes in combination with macrophage activation for enhanced phagocytosis and killing. The centerpiece of this model is the $CD4^+$ T lymphocyte expressing gamma interferon, which enhances the synthesis of the predominant opsonizing bovine immunoglobulin G (IgG) subclass, IgG2, and concomitantly activates macrophages to increase receptor expression, phagocytosis, phagolysomosal fusion, and release of rickettsiacidal nitric oxide. Brown et al. (36) demonstrated that induction of these responses using purified outer membrane proteins prevented *A. marginale* rickettsemia on challenge exposure. T-lymphocyte clones from protectively immunized cattle were found to be diverse, and several clones responded to MSP2 and MSP3 (37). Interleukin-12, when used as an adjuvant, promoted IgG and type 1 cytokine recall responses to MSP2 (164). Highly conserved regions of MSP2 were found to be rich in naturally derived $CD4^+$ T-lymphocyte epitopes. These immunodominant peptides induced the high levels of gamma interferon required for rapid generation of variant-specific IgG2 (34). MSP1a was also recognized by $CD4^+$ T lymphocytes. The carboxyl terminus of MSP1a, which is conserved among *A. marginale* isolates, was preferentially recognized by these immune cells (35). However, in a recent study, thymectomized calves were able to control acute anaplasmosis after their $CD4^+$ T lymphocytes were selectively depleted by treatment with an anti-CD4 monoclonal antibody (165). Therefore, although $CD4^+$ T lymphocytes may play a role in controlling *A. marginale* infection, the antibody response appears to be essential.

Limited vaccine trials have been conducted with recombinant MSPs (as reviewed in references 40, 118, and 124) and recombinant vaccinia virus expressing *A. marginale* antigens (103) or with naked DNA (11). Thus far, only partial protection has been obtained with recombinant antigens used for vaccination, indicating that a combination of several antigens will probably be required to attain a strong protective immune response. DNA vaccines, as reviewed in reference 166, show promise for vaccine development because they may produce long-lived immunity and a broad spectrum of immune responses (both humoral and cell-mediated) and may be used for simultaneous vaccination against multiple pathogens (11). In addition, the magnitude and direction of the immune response by coadministration of plasmid-encoded cytokines and antigens may be modulated. These novel vaccine approaches show promise, but considerable research and development are required before new vaccines using DNA as a delivery system are developed and marketed.

The ideal vaccine for anaplasmosis would be one that prevents infection, as well as inducing protective immunity. Current vaccines do not prevent infection, and persistently infected cattle are a major reservoir of *A. marginale*, serving as a source of infection for mechanical transmission and biological transmission by ticks. At present, development of a vaccine for induction of protective immunity appears to be a realistic goal. Additionally, the possibility of blocking the biological transmission of *A. marginale* is an important goal of vaccines for anaplasmosis. Although no transmission-blocking antigens have been identified from the tick vector or the pathogen, recent results suggest that antibodies to recombinant MSP1a reduce infectivity for *D. variabilis* (J. de la Fuente, K. M. Kocan, J. C. Garcia-Garcia, E. F. Blouin, T. Halbur, and V. Onet, submitted for publication), in accordance with results obtained in neutralization studies in vitro (21, 23). However, further research is needed to more fully understand the development cycle of *A. marginale* in cattle and ticks in order to design a vaccine that will prevent the infection of both hosts.

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