CHARACTERIZATION OF THE ANTIGENIC DETERMINANTS, GLYCOSYLATION AND EXPRESSION OF THE MAJOR SURFACE PROTEIN 1A OF *ANAPLASMA MARGINALE*

by

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CHARACTERIZATION OF THE ANTIGENIC DETERMINANTS, GLYCOSYLATION AND EXPRESSION OF THE MAJOR SURFACE PROTEIN 1A OF *ANAPLASMA MARGINALE*

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PREFACE

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ABBREVIATIONS

AMV, avian myeloblastosis virus

BPL, β-propiolactone

dNTP, deoxynucleoside triphosphate

GALase III, β1-4 galactosidase

HEXase I, β1-2,3,4,6 *N*-acetylhexosaminidase

mAb, mouse monoclonal antibody

MALDI-TOF, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight

MHC, major histocompatibility complex

MSP, major surface protein

NANase II, β2–3,6 neuraminidase

PBS, phosphate-buffered saline

PCV, packed cell volume

PNGase F, *N*-glycosidase F

PPE, percent parasitized erythrocytes

RT, reverse transcriptase

SDS-PAGE, SDS-polyacrylamide gel electrophoresis

TBS, Tris-buffered saline

TFMS, trifluoromethanesulfonic acid

Chapter 1

INTRODUCTION

Bovine Anaplasmosis

Bovine anaplasmosis, also known as gall sickness, is a tick-transmitted disease of cattle caused by the rickettsia *Anaplasma marginale* (Rickettsiales: Anaplasmatacea). *A. marginale* was first described by Sir Arnold Theiler in 1910 in erythrocytes of African cattle suffering acute anemia (Theiler, 1910). Theiler named the small punctiform organism on the basis of staining characteristics. The term "anaplasma" indicates an apparent lack of cytoplasm in what was thought to be a protozoan, and the term "marginale" indicates the peripheral location of the marginal body within erythrocytes.

Anaplasmosis is the most prevalent hemoparasitic disease of cattle and is enzootic to nearly half of the world's livestock (National Research Council, 1982). *A. marginale* is endemic in many tropical and subtropical regions of the world, and is found on all six continents (Kreier et al., 1992). Since *A. marginale* was identified in South Africa (Theiler, 1910), its presence has been confirmed in North America, South and Central America (Guglielmone, 1995), the Middle East (El-Metenawy, 2000), Asia (Jorgensen et al., 1992), Australia (Kudamba et al., 1982), and southern Europe (Baumgartner et al., 1992). Annual losses due to anaplasmosis in the United States alone have been estimated to be over $300 million a year (McCallon, 1973; National Research Council, 1982).

A. marginale has been reported in 40 of the 50 United States (Fig. 1), but is more prevalent in the Gulf Coast, lower plains and western states (Siegmund, 1979). *A. marginale* isolates have been classified based on differences in tick transmissibility, molecular size of surface proteins

and DNA restriction fragments, reactivity to monoclonal antibodies and geographic origin (Table 1).

Anaplasmosis is caused by the infection of bovine erythrocytes by *A. marginale*, which results in high rickettsemia levels that reach 10^9 infected erythrocytes per ml. Infected erythrocytes are then removed by the bovine reticuloendothelial system. Animals that survive infection remain persistently infected with low levels of parasitemia ($<10^7$ infected erythrocytes per ml), and are resistant to clinical disease if they are challenged with the homologous isolate (Dikmans, 1950; reviewed by Palmer et al., 1999).

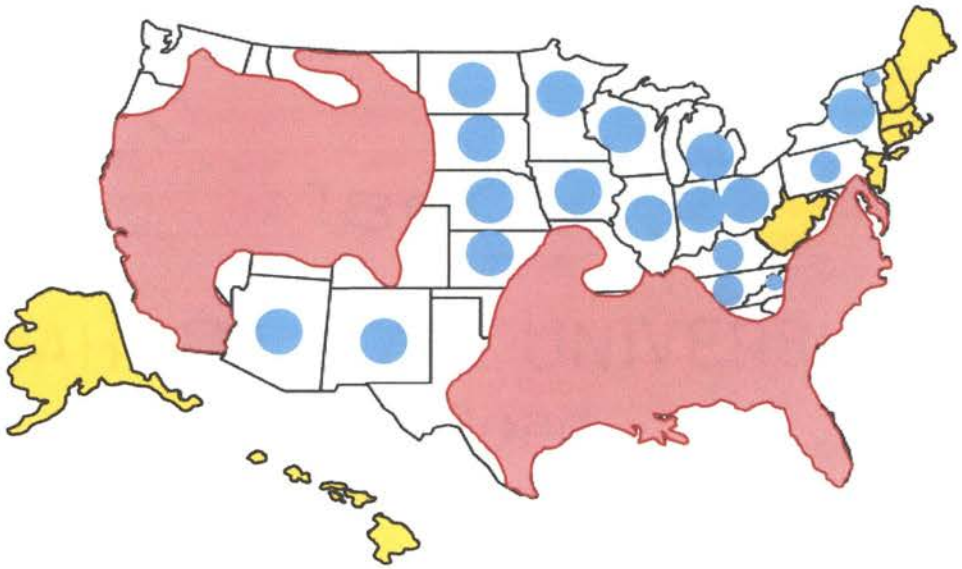

Figure 1. Distribution of *Anaplasma marginale* in the United States.
Pink denote areas where *A. marginale* is endemic. Blue spots over white background indicate states where *A. marginale* has been reported and yellow no occurrence.

TABLE 1. Examples of North American isolates of *Anaplasma marginale*.

Isolate (Year Isolated)	Number of MSP1a Repeats	Tick Transmission	Reference
Florida (1955)	8	No	Ristic & Carson, 1977
Southern Idaho (1983)	6	Yes	McGuire et al., 1984
Okeechobee, FL (1999)	5	No	de la Fuente et al., 2001b
Mississippi	5	Yes	Hidalgo et al., 1989
Illinois	5	No	Wickwire et al., 1987
Washington-Okanogan (1981)	4	Yes	Barbet et al., 1982
Northern Texas (1977)	4	NR	McGuire et al., 1984
Missouri	4	NR	McGuire et al., 1991
South Dakota (1999)	4	NR	Palmer et al., 2001
Oklahoma (1997)	3	Yes	Blouin et al., 2000
St. Maries, ID (1994)	3	Yes	Eriks et al., 1994
California	3	No	de la Fuente et al., 2001c
Rasmussen, OR (1999)	3	Yes	Palmer et al., 2001
Virginia (1972)	2	Yes	Kuttler & Winward, 1984
Washington-Clarkston (1982)	NR	NR	McGuire et al., 1984

The incubation period of anaplasmosis is typically 21 days, but may range from 4 to 65 days. The acute phase of the disease is characterized by severe anemia, fever (40-41°C), icterus (jaundice), weight loss, weakness, abortion, lower milk production and, occasionally, death (Kuttler, 1984). In acute cases the fever may rise to 42°C (107°F) and is followed by moderate to severe anemia. The susceptibility to anaplasmosis varies with age. Calves generally do not develop clinical symptoms, while the mortality rate in older cattle (2-3 years or older) can be 20-50%.

Classification

Anaplasma spp. were originally regarded as protozoan parasites, but were later shown to be gram-negative bacteria (Amerault et al., 1973). *Anaplasma* spp. have been classified in superkingdom Bacteria, phylum Proteobacteria, class Alphaproteobacteria, order Rickettsiales,

family Anaplasmataceae, genus *Anaplasma* (Skerman et al., 1980; Dumler et al., 2001). *A. marginale* (Theiler, 1910) is the type species of the genus *Anaplasma.* Other names historically given to *Anaplasma marginale* include *A. argentium*, *A. rossicum*, *A. theileri*, *A. argentium* Lignieres 1914, *A. rossicum* Yakimoff and Belawine 1927, *A. theileri* Neitz 1957 and *Paranaplasma caudatum* (Ristic, 1977). Recently, the family Anaplasmataceae was reorganized based on the genetic analyses of 16S rRNA genes (Fig. 2), *groESL* and surface protein genes (Dumler et al., 2001). According to this most recent classification, the genus *Anaplasma* now includes not only *A. centrale*, *A. marginale* and *A. ovis*, but also *A. bovis*, *A. platys*, and the *A. phagocytophilum* group that encompasses the microorganisms formerly known as *Ehrlichia phagocytophila* (HGE agent) and *E. equi* (reviewed by Kocan et al., 2003a).

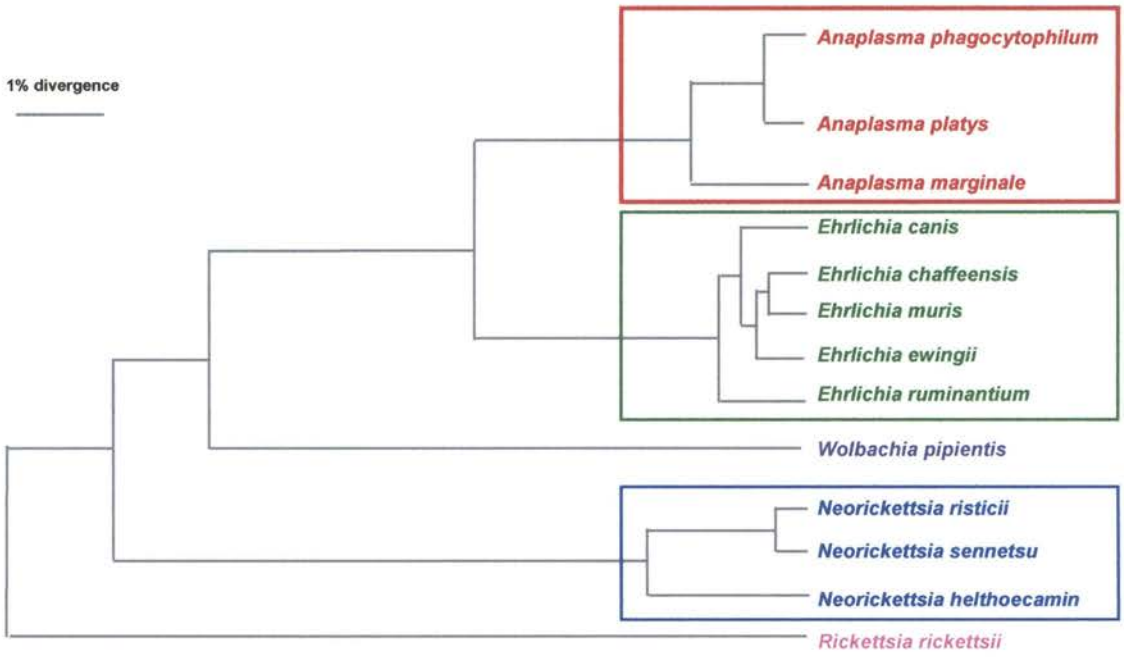

Figure 2. Phylogram tree of family Anaplasmataceae based on 16S rRNA similarity.
Boxes indicate the clades formed by *Anaplasma* spp., *Ehrlichia* spp. and *Neorickettsia* spp. (Dumler et al., 2001)

Anaplasmosis is included in the List B of the Office International des Epizooties, among other transmissible diseases that are considered to be of socio-economic and/or public health importance and that impact the international trade of cattle (World Organization for Animal Health, 2000).

Diagnosis and control of anaplasmosis

Diagnosis of anaplasmosis can be done by demonstrating the presence of either *Anaplasma* organisms or *Anaplasma*-specific antibodies in samples of infected animals. However, definitive diagnosis can only be achieved by detecting the organism.

Detection of *A. marginale* inclusion bodies has commonly been based on microscopic examination of stained erythrocyte smears. *A. marginale* inclusion bodies are small, round, basophilic bodies located near the margin of the erythrocytes that range from 0.3 to 1.0 μm in diameter. Microscopic examination can only detect levels of approximately $>10^6$ infected erythrocytes per ml, but rickettsemia levels are often lower, particularly in carrier cattle, in which infection levels range from $10^{2.5}$ to 10^7 infected erythrocytes per ml (Kieser et al., 1990). A more sensitive approach is based on direct fluorescent antibody staining (Johnston et al., 1980), but non-specific staining and cross-reactive antibodies have hindered the use of this technique. Subinoculation of *A. marginale*-infected erythrocytes into susceptible, splenectomized calves remains the "gold standard" for detection of persistently infected cattle, but this procedure is expensive and impractical for routine testing (Luther et al., 1980).

Serological tests have been the most commonly used method for the detection of *A. marginale*-infected cattle in the field (Wilson et al., 1978). Complement fixation (CF) and card agglutination assays have been used since the 1960's and were accepted by the World Organization for Animal Health until recently as the basis for the identification of cattle

infected with *A. marginale* prior to interstate or international movement of animals (World Organization for Animal Health, 2000). The sensitivity of the CF is low and therefore this test is not adequate for regulatory and surveillance programs (Bradway et al., 2001). An indirect fluorescence antibody test has also been used (de Kroon et al., 1990), but its use has been restricted by the limited number of tests that can be performed and the specificity of the assay.

ELISA assays have been developed for the detection of *A. marginale* infection. Trueblood et al. (1991) developed an antigen capture ELISA with monoclonal antibodies against conserved epitopes of *A. marginale* major surface protein (MSP) 1a. This assay detected *A. marginale* prior to the onset of clinical signs (Trueblood et al., 1991). A competitive ELISA for detection of *A. marginale*-specific antibodies based on erythrocyte or tick cell culture-derived *A. marginale* has also been developed (Knowles et al., 1996; Saliki et al., 1998). This assay, using a monoclonal antibody against the *A. marginale* MSP5, proved to be more sensitive than the CF test. Similar assays using erythrocyte-derived *A. marginale* and recombinant *Anaplasma* antigens have been reported (Nielsen et al., 1996). All these ELISA tests are reportedly more specific and sensitive than the traditionally used CF and card agglutination assays (Molloy et al., 1999). A competitive ELISA assay is being approved for use in the United States and Canada.

Nucleic acid-based techniques, more sensitive and specific, have been developed recently (Eriks et al., 1989; Goff et al., 1988; Stich et al., 1993; Ge et al., 1997). These tests, based on the use of nucleic acid probes or the polymerase chain reaction (PCR), have been used to detect *A. marginale* infection in tick cells and erythrocytes. Detection of parasitemia levels as low as 0.00025% (percent of infected erythrocytes) using a radioactive DNA probe has been described (Eriks et al., 1989). *A. marginale* in infected ticks has also been identified

using a cloned DNA probe (Goff et al., 1988). PCR-based methods detect rickettsemia levels of 0.0001%, and the recent use of a nested-PCR has increased the specificity and sensitivity of the assay to 0.000001% (Torioni De Echaide et al., 1998). The complexity of these techniques limits their use only for research, but nucleic acid-based techniques hold promise for use in future diagnostic and epidemiological studies.

Control of anaplasmosis can be achieved primarily by antibiotic therapy, vaccination or maintenance of an *Anaplasma*-free herd (Peregrine, 1994; as reviewed by Kocan et al., 2000; 2003a). Although protection of animals from exposure to vectors can also help control anaplasmosis (Kocan et al., 2000), only a decrease in incidence of the disease has been achieved using this approach.

Vaccination for control of anaplasmosis dates back to the early 1910's (Theiler, 1912a), soon after *A. marginale* was first described (Theiler, 1910). Two main types of vaccines have been commonly used for preventing clinical anaplasmosis in the last decades (reviewed by Kocan et al., 2003a). Premunization occurs when a live vaccine based on *A. centrale* or on attenuated *A. marginale* is used to infect cattle (Vizcaino et al., 1978; Palmer, 1989; Pipano, 1995). Killed vaccines involve vaccination of cattle with inactivated *A. marginale* derived from infected bovine erythrocytes or infected cultured tick cells (Brock et al., 1965; reviewed by Kocan et al., 2000).

Live vaccines of *A. centrale* are widely used to protect cattle against *A. marginale* infection in Israel and Africa (Pipano, 1995; as reviewed by Kocan et al., 2003a). The *A. centrale* vaccine produces low parasitemia and provides partial protection against challenge with virulent *A. marginale* (Anziani et al., 1987). *A. centrale*-based vaccines have been ineffective in some areas and do not provide adequate protection against some *A. marginale* isolates (Turton

et al., 1998). Blood-derived vaccines are not entirely safe because they can be contaminated with other blood-borne pathogens. Live vaccines are also expensive to produce and require strict conditions for storage and transportation (World Organization for Animal Health, 2000). Live vaccines have not been approved for use in North America because of the risk of infecting cattle with other hemoparasites.

Vaccination with killed vaccines stimulates an immune response that is adequate to protect against anemia and illness, and is the most efficient and economical method for control of anaplasmosis in the United States (as reviewed by Palmer, 1989 and Kocan et al., 2000; 2003a). Killed vaccines marketed previously in the United States used *A. marginale* antigen that was partially purified from infected bovine erythrocytes. Killed vaccines protected cattle against homologous challenge (same isolate used for vaccine preparation), but were only partially successful in preventing clinical anaplasmosis in geographic regions where the endemic *A. marginale* was different from the vaccine isolate (Brock et al., 1965; Hart et al., 1990; Montenegro-James et al., 1991). In addition, these killed vaccines were expensive, difficult to standardize and were at risk of being contaminated with bovine cells and pathogens that commonly infect cattle.

Current strategies for the development of anaplasmosis vaccines are directed toward a subunit vaccine using surface-exposed epitopes that induce protective immunity (reviwed by Palmer et al., 1999 and Kocan et al., 2003). Some of these proteins are conserved among *A. marginale* isolates (McGuire et al., 1984; Palmer et al., 1986a; Visser et al., 1992; Oberle et al., 1993), and in both the intraerythrocytic and tick stages of *A. marginale* (Palmer et al., 1985; Barbet et al., 1999). Immunization of cattle with these surface proteins induced partial

protective immunity against homologous and heterologous *A. marginale* challenge (Palmer et al., 1986b, 1988, 1989).

A new killed vaccine based on *A. marginale* grown in a culture cell line is also being developed (Kocan et al., 2000, 2001). This vaccine may overcome the problems associated with the use of blood-derived vaccines and should be a safer and less expensive vaccine, easily standardized and free of contaminating bovine cells and pathogens. Other new strategies for the control of anaplasmosis are targeted at both *A. marginale* and the tick vector (Kocan, 1994; Kocan et al., 1996a,b). Host immunoglobulins have been shown to cross the tick midgut and reach the hemolymph without proteolytic cleavage (Vaz Junior Ida et al., 1996; Jasinskas et al., 2000). These antibodies, directed either to *A. marginale* or to tick molecules involved in pathogen transmission, could block the biological transmission of *A. marginale* (Blouin et al., 2003a; de la Fuente et al., 2003a,b), and decrease the incidence of anaplasmosis in endemic areas. Anti-tick vaccines have also been suggested to reduce the incidence of some tick-borne hemoparasites in vaccinated cattle (de la Fuente et al., 1998).

Transmission

A. marginale develops persistent infections in mammalian and tick hosts, both of which serve as reservoirs for infection of susceptible hosts. *A. marginale* is pathogenic for both *Bos indicus* and *B. taurus* cattle (Wilson et al., 1980) and has been shown, at least experimentally, to infect other ruminant species (Table 2). Some of the wildlife ruminant species shown in Table 2 become infected but do not develop clinical disease, although they may play a role as reservoirs of infection for susceptible cattle at enzootic sites. For instance, unusually high prevalence rates (69%) of *A. marginale* infection have been detected in white-tailed deer populations in Mexico (Martinez et al., 1999), although the role of white-tailed deer in the

epizootiology of anaplasmosis has been questioned by others (Keel et al., 1995). Non-ruminant mammals have been suggested to serve as *A. marginale* hosts (Akinboade et al., 1981), but this observation has not been confirmed by others.

Cattle that recover from acute infection remain persistently infected and serve as reservoir of infection for mechanical and biological transmission (Ewing, 1981). *A. marginale* can be transmitted mechanically when infected blood is transferred to susceptible animals by biting insects, needles or veterinary instruments such as those used for dehorning, castration and attachment of eartags. Although *A. marginale* does not establish infection in insect vectors (Roberts & Love, 1977), some biting flies, such as a number of species of *Tabanus* (horseflies) and *Psorophora* (mosquitoes), carry infected blood in the mouthparts and transmit the rickettsia to susceptible cattle (Potgieter et al., 1981). The role of mechanical transmission of *A. marginale* is not well documented and may have been historically underrated. Although it appears to vary from region to region, biting insects are probably the primary means of transmission of *Anaplasma* in certain regions, such as Florida, where *A. marginale* isolates appear to be non-infective for ticks (Ewing, 1981; as reviewed by Kocan et al., 2003a).

Biological transmission of *A. marginale* is effected primarily by feeding ixodid ticks. A wide range of tick species become infected by *A. marginale* and have been identified as vectors (Table 3; Ewing, 1981; Kocan et al., 2003b), although some *A. marginale* isolates have proved not to be transmissible by certain tick species (Table 1).

TABLE 2. Natural and experimental ruminant hosts of *Anaplasma marginale* infection.

Reservoir Host	Common Name	Location	Evidence	Reference
Bison bison	American bison	US	serology, experimental infection	Zaugg & Kuttler, 1985 Taylor et al., 1997
Odocoileus hemionus hemionus	mule deer	US	serology, experimental infection	Renshaw et al., 1977
Odocoileus virginianus	white-tailed deer	US, Mexico	serology, experimental infection	Smith et al., 1982
Odocoileus hemionus columbianus	black-tailed deer	US	serology	Chomel et al., 1994
Cervus elaphus	Rocky Mountain elk	US	experimental infection	Zaugg et al., 1996
Cervus canadensis	elk	US	serology, experimental infection	Renshaw et al., 1979
Taurotragus oryx	eland	Kenya, Africa	molecular	Ngeranwa et al., 1998
Antilocapra americana	pronghorn antelopes	US	serology	Stauber et al., 1980
Syncerus caffer	African buffalo	Africa	serology, experimental infection	Schreuder et al., 1977; Reddy et al., 1988
Bubalus bubalis	water buffalo	Africa	cytology,	Carmichael & Hobday, 1975
Cephamophys rufulatus	red-flanked duiker	Africa	cytology	Dipeolu & Akinboade, 1984
Ovis canadensis	bighorn sheep		subinoculation serology	Kuttler, 1984

Intrastadial transmission of *A. marginale* has been shown to be effected by male ticks (Kocan et al., 1992a,b). Numerous studies have demonstrated that male *Dermacentor* ticks may play an important role in the biological transmission of *A. marginale* because they become persistently infected and can transmit *A. marginale* repeatedly when they transfer among cattle (Kocan et al., 1992a,b; Eriks et al., 1993). Therefore, *Dermacentor* males serve as both reservoirs and vectors of *A. marginale* (Kocan et al., 1992a,b). Interstadial transmission occurs when nymph or adult ticks infected in a previous stage transmit the rickettsia (Kocan et al., 1992a,b). Transovarial transmission has been suggested to occur in some ixodid ticks (Howell et al., 1941b), but this finding has not been confirmed by others (Stich et al., 1989). *Anaplasma* infection can be transmitted from an infected cow to her unborn calf (transplacental transmission) (Potgieter & van Rensburg, 1987). Transplacental transmission may not contribute greatly to the epizootiology of anaplasmosis.

Tick vectors

Ticks are biological vectors of *A. marginale*, and at least 14 tick species are capable of transmitting infection under natural or experimental conditions (Table 3). However, experimental demonstration of vector competence does not necessarily imply a role in *A. marginale* transmission in the field. Some of these tick species serve as reservoirs of *A. marginale* and different stages can transmit *A. marginale* to cattle (Stiller & Coan, 1995).

Different tick species serve as vectors of *A. marginale* infection in different regions of the world. *Dermacentor* ticks are the most common vector in the United States. The cattle tick, *Boophilus microplus*, is the major vector of anaplasmosis in Australia and in Central and South America (Nari, 1995), while other *Boophilus* spp. are important vectors in Africa.

TABLE 3. Studies in which *Anaplasma* transmission has been attempted with Ixodid and Argasid ticks.[a]

Tick species	Author(s)	Year	Tick Transmission
IXODIDS			
Amblyomma americanum	Rees	1934	-
A. americanum	Sandborn & Moe	1934	-
A. americanum	Piercy & Schmidt	1941	-
Amblyomma cajennense	Rees	1934	-
A. cajennense	Sanborn & Moe	1934	-
Amblyomma maculatum	Rees	1934	-
A. maculatum	Piercy	1938	-
A. maculatum	Piercy & Schmidt	1941	-
Boophilus decoloratus	Theiler	1912b	+
Boophilus microplus	Quevedo	1916	+
B. microplus	Rosenbuch & Gonzalez	1927	+
B. microplus	Brumpt	1931	+
B. annulatus	Rees	1934	+/-
Boophilus calcaratus	Sergent et al.	1945	+/-
Dermacentor albipictus	Boynton et al.	1936	+
D. albipictus	Stiller et al.	1981	+
D. albipictus	Sanborn and Moe	1934	-
D. albipictus	Ewing et al.	1997	+
Dermacentor andersoni	Rees	1933	+
D. andersoni (larvae–nymph)	Rees	1934	+
D. andersoni (nymph–adult)	Rees	1962	+
D. andersoni	Boynton et al.	1936	+
D. andersoni	Sanborn et al.	1938	+
D. andersoni (transovarial)	Howell et al.	1941b	+
D. andersoni	Rees & Avery	1939	-
D. andersoni	Rozeboom et al.	1940	+/-
D. andersoni (delayed feeding)	Anthony & Roby	1962	+
D. andersoni	Kocan et al.	1981	+
D. andersoni (transovarian)	Anthony & Roby	1962	-
Dermacentor nitens	Sanborn & Moe	1934	-
D. nitens	Rees & Avery	1939	-

Dermacentor occidentalis	Boyton et al.	1936	+
D. occidentalis	Howarth & Roby	1972	+
D. occidentalis (transstadial)	Howarth & Hokama	1973	+
D. occidentalis (transovarian)	Howarth & Hokama	1973	-
Dermacentor parumapertus	Sanborn & Moe	1934	
Dermacentor variabilis	Rees	1932	+
D. variabilis (transstadial)	Rees	1934	+
D. variabilis (transovarian)	Rees	1934	-
D. variabilis	Sanders	1933	+
D. variabilis	Sanborn & Moe	1934	-
D. variabilis (transovarian)	Rees & Avery	1939	-
D. variabilis (carrier animals)	Schmidt & Piercy	1937	-
D. variabilis	Piercy	1938	-
D. variabilis (transstadial)	Anthony & Roby	1962	+
D. variabilis (transovarian)	Anthony & Roby	1962	-
D. variabilis (transovarian)	Stich et al.	1989	-
D. variabilis	Stich et al.	1989	+
D. variabilis	Kocan et al.	1981	+
D. venustus	Sanborn & Moe	1934	-
Haemaphysalis leporis-palustris	Sanborn & Moe	1934	-
Hyalomma lusitanicum	Sergent et al.	1945	-
Hyalomma mauritanicum	Sergent et al.	1945	-
Ixodes pacificus	Howarth & Hokama	1973	-
Ixodes ricinus	Zeller & Helm	1923	+
I. ricinus	Helm	1924	+
I. ricinus	Sanborn & Moe	1934	-
I. ricinus	Piercy	1938	-
Ixodes scapularis	Rees	1934	+/-
I. scapularis	Sanborn & Moe	1934	-
Ixodes sculptus	Rees	1934	-
I. sculptus	Sanborn & Moe	1934	-
Rhipicephalus bursa	Brumpt	1931	+
R. bursa	Sergent et al.	1945	+/-
Rhipicephalus sanguineus	Rees	1930	+
R. sanguineus	Rees	1934	+/-
R. sanguineus	Sanborn & Moe	1934	-

R. *sanguineus* (transovarian)	Rees & Avery	1939	-
Rhipicephalus simus (transovarian)	Theiler	1912b	+
R. *simus*	Potgieter et al.	1983	+
ARGASIDS			
Argas persicus	Howell et al.,	1941a	
Ornithodoros coriaceus	Howell et al.	1943	+/-
Ornithodoros megnini	Howarth & Hokama	1973	-
O. megnini	Sanborn & Moe	1934	-
Ornithodoros turicata	Howell et al.	1943	-
O. turicata	Sanborn & Moe	1934	-

[a] Reprinted from Kocan et al., 2003b.

Developmental cycle of A. marginale in cattle and ticks

A. marginale is an obligate intracellular parasite that multiplies within membrane-bound inclusions in the cytoplasm of the host cells. In cattle, the only known site of development of *A. marginale* is within erythrocytes (Ristic & Watrach, 1963). However, within ticks *A. marginale* undergoes a complex developmental cycle that involves several tissues and is coordinated with the tick feeding cycle (Fig. 3; Kocan, 1986; Kocan et al., 1992a,b). Infected erythrocytes taken into ticks with the bloodmeal provide the source of *A. marginale* infection for tick gut cells. After development of *A. marginale* in tick gut cells, many other tick tissues become infected, including the salivary glands, from where the rickettsiae are transmitted to vertebrates during feeding (Kocan, 1986; Kocan et al., 1992a,b; Ge et al., 1996). At each site of infection in ticks, *A. marginale* develops within membrane-bound vacuoles or colonies (Fig. 4). The first form of *A. marginale* seen within the colony is the reticulated (vegetative) form that divides by binary fission, forming large colonies that may contain hundreds of organisms. The reticulated form then changes into the dense form (Fig. 4), which is the infective form and can survive outside

of cells. Cattle become infected with *A. marginale* when the dense form is transmitted during tick feeding via the salivary glands.

Upon *A. marginale* infection in cattle, the number of infected erythrocytes increases logarithmically and removal of these infected cells by phagocytosis results in development of anemia and icterus without hemoglobinemia and hemoglobinuria. Cattle that recover from acute infection remain persistently infected and are protected from clinical disease, serving as reservoirs for mechanical and biological transmission (Dikmans, 1950; Ewing, 1981).

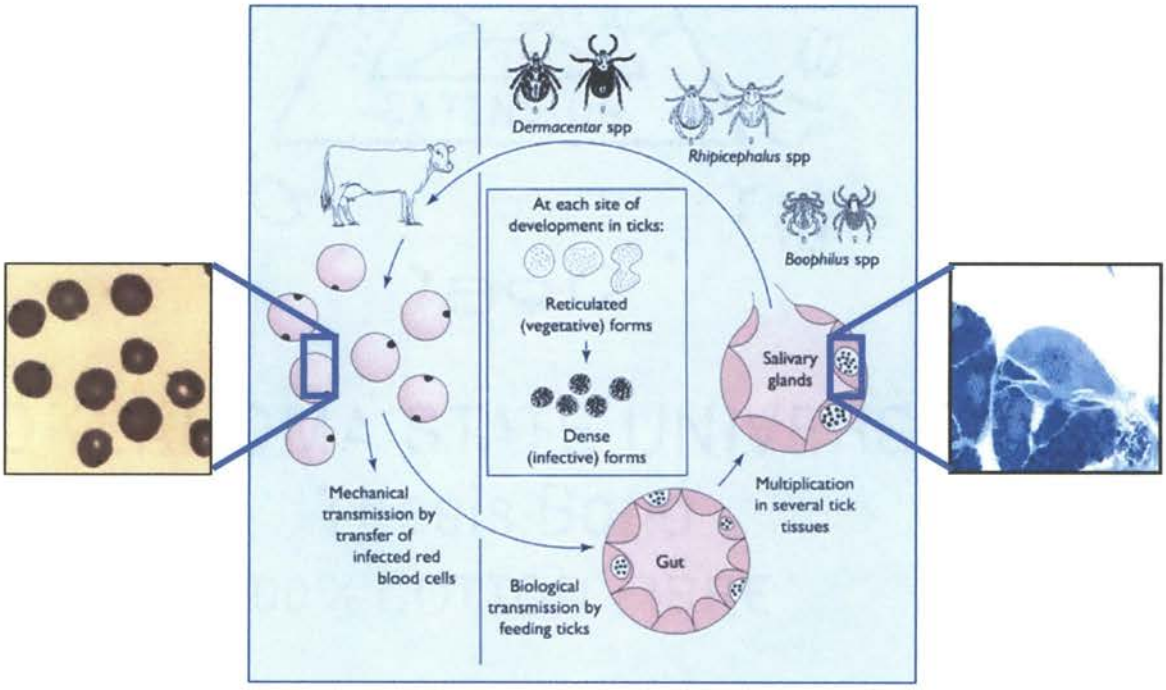

Figure 3. Life cycle of *Anaplasma marginale* in the bovine and tick hosts.
Adapted from Poster inside Parasitology Today, Vol. 15 (169).

Within the erythrocytes, membrane-bound inclusion bodies contain from 4-8 rickettsiae. The percentage of infected erythrocytes varies with the stage and severity of the disease, but maximum parasitemias in excess of 70% may occur during acute infection. Multiple infections of individual erythrocytes are common during periods of high rickettsemia.

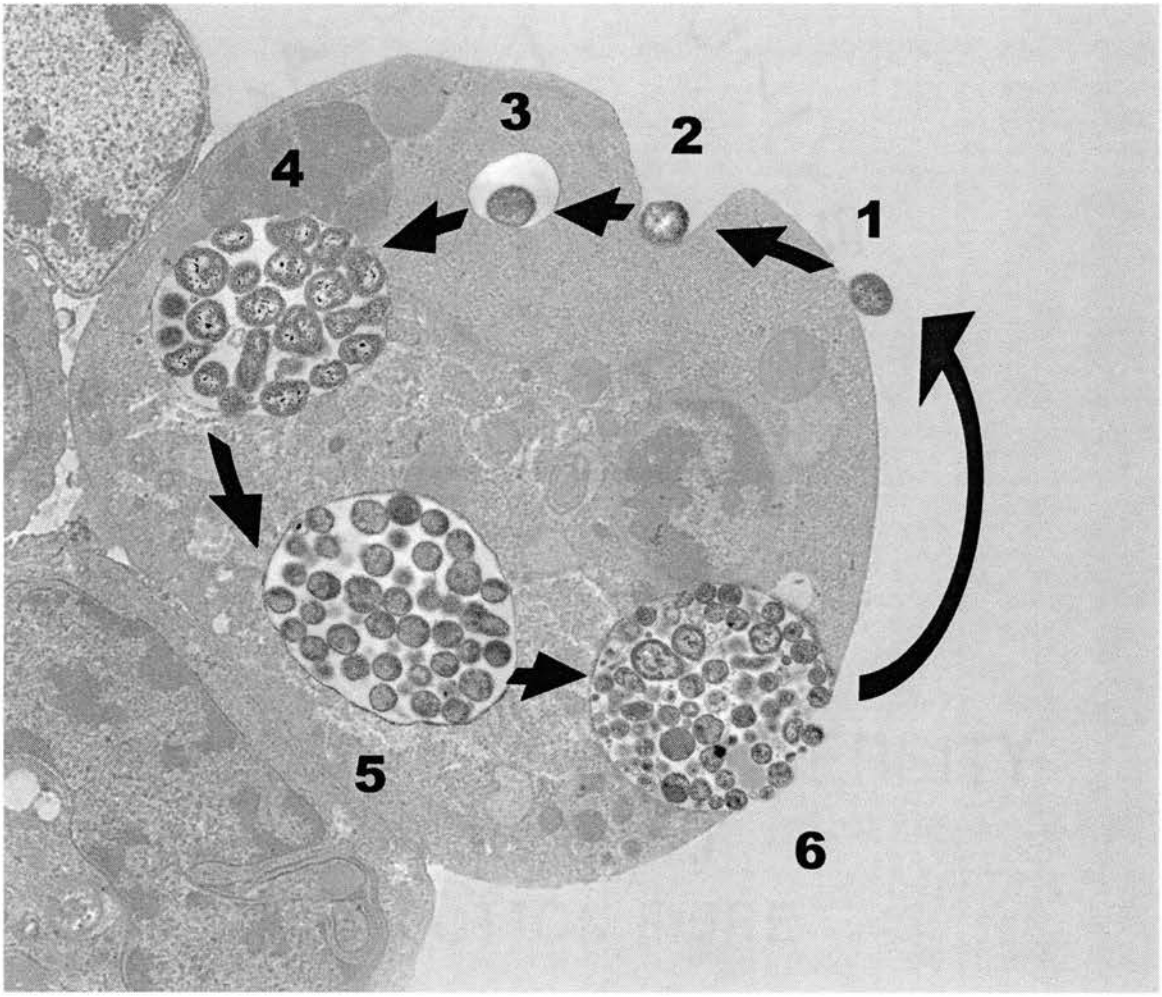

Figure 4. Developmental cycle of *Anaplasma marginale* in tick cells.
A. marginale adheres to the membrane of the tick cell (1) and a depression forms in the cell membrane (2). The rickettsia is internalized (3), and remains within a vacuole. *A. marginale* then divides by binary fission and forms a colony of reticulated forms (4), which later become dense forms (5). The rickettsial colony fuses with the host cell membrane and infective dense forms of *A. marginale* are released from the cells (6). Free rickettsiae are then able to infect other host cells and restart the cycle of development (Blouin & Kocan, 1998).

Tick Cell Culture System for *A. marginale*

Recently, *A. marginale* was propagated in continuous culture in a cell line, IDE8, derived from embryos of *Ixodes scapularis* ticks (Munderloh et al., 1996). The IDE8-*A. marginale* culture system has been shown to be a valuable model for the study of pathogen-tick cell interactions (Barbet et al., 1999; de la Fuente 2001a,b; 2002a; Blouin et al., 2003a,b).

The developmental cycle of *A. marginale* in culture cells is similar to the cycle in naturally infected tick cells (Fig. 4) (Blouin & Kocan, 1998). Development of *A. marginale* in the cultured tick cells was documented using light and electron microscopy (Blouin & Kocan, 1998). Host cell invasion is initiated by the adhesion of the dense form of *A. marginale* to the host cell membrane (Fig. 4). The adhesion between the rickettsiae and tick cell membrane increases along a continuous section forming a depression in the host cell membrane (Fig. 4). *A. marginale* is subsequently enclosed by the host cell membrane and internalized within a vacuole (Fig. 4). *A. marginale* transforms into the reticulated (vegetative) form that divides by binary fission. Repeated division results in the formation of colonies that contain hundreds of rickettsiae (Fig. 4). The reticulated forms of *A. marginale* subsequently transform into the infective or dense forms. Colony membranes then fuse with the host cell plasmalemma, followed by rupture of the membrane complex (Fig. 4). A flap opened in the fused cell membranes allows for the release of the dense forms from the parasitophorous vacuole without loss of host cell cytoplasm. The released rickettsiae then initiate a new series of infections resulting in host cells containing 5 or more colonies per cell (Blouin & Kocan, 1998). Tick cell death occurs after most of the cells become infected, resulting in detachment of tick cell monolayers and cytopathic effect. The mechanism of *A. marginale* exit involves the fusion of the colony and host cell membranes, and appears to be controlled by the host cell

and the pathogen (Blouin & Kocan, 1998). The adherence of rickettsiae to the tick cell membrane prior to infection has suggested the presence of adhesion molecules on the surface of *A. marginale* that are recognized by tick cell receptors. One of these *A. marginale* surface molecules have been recently identified (de la Fuente et al., 2001a).

A. marginale propagated in culture has been shown to be infective for both cattle and ticks (Munderloh et al., 1996; Blouin et al., 2000). In addition, cell culture derived-*A. marginale* antigen conferred partial protection to immunized cattle in preliminary studies (Kocan et al., 2001; de la Fuente et al., 2002b). Immunity generated in cattle by *A. marginale* antigens purified from infected culture tick cells was found to be similar to the protection elicited by erythrocyte-derived *A. marginale* antigens (Kocan et al., 2001).

Cell culture-derived *A. marginale* has also been compared with erythrocyte-derived *A. marginale* in immunized and control cattle that were challenge-exposed with infected *D. variabilis* ticks (de la Fuente et al., 2002b). These challenge conditions more closely resemble those occurring in nature where male ticks act as reservoirs and effect biological transmission of *A. marginale*. Under these experimental conditions, the cell culture-derived *A. marginale* antigen provided partial protection in cattle in a manner similar to erythrocyte-derived antigens (de la Fuente et al., 2002b).

Bovine Immune Response to *A. marginale* Infection

The clearance of *A. marginale* infection by the bovine immune system is mediated by the concomitant development of a high titer humoral immune response and a CD4+ T-cell-mediated response (Palmer & McElwain, 1995; Palmer et al., 1999). The possible role of antibodies directed against *A. marginale* surface molecules was demonstrated by Palmer & McGuire (1984), who were able to neutralize *A. marginale* infection of susceptible,

splenectomized calves by using antiserum against initial bodies. Later reports challenged the antibody mediated model for protective immunity (Gale et al., 1992), and proved that antibodies alone are not sufficient for protection. More recent studies confirmed involvement of antibodies in three main mechanisms of protection against *A. marginale* infections (Cantor et al., 1993; reviewed by Palmer et al., 1999), and demonstrated that the level of antibodies against *A. marginale* major surface proteins, in contrast to the overall antibody levels, correlates with protection (Tebele et al., 1991). One mechanism involves the direct action of antibodies and complement, which results in killing of the rickettsia and neutralization of its ability to attach to and invade host cells (Palmer et al., 1999; Blouin et al., 2003a). A second mechanism requires the antibody-dependent cellular cytotoxicity by major histocompatibility complex non-restricted lymphocytes (Brown et al., 2001, 2002). The third mechanism involves antibodies conferring specificity to macrophage phagocytosis for opsonization. These mechanisms are involved in the protective immune response against *A. marginale* infections, and are stimulated by vaccination with live or killed organisms, initial body membranes, purified native or recombinant outer membrane proteins, or DNA encoding for *A. marginale* surface proteins (Palmer et al., 1989; Montenegro-James et al., 1991; Tebele et al., 1991; Arulkanthan et al., 1999; Kocan et al., 2001; de la Fuente et al., 2002b).

Recent studies demonstrated that cattle immunized with erythrocyte or cell culture-derived *A. marginale* developed a differential antibody response to *A. marginale* major surface proteins 1a and 1b (Kocan et al., 2001; de la Fuente et al., 2002b). Cattle immunized with tick cell-derived antigens elicited a preferential response against MSP1b while cattle immunized with erythrocyte-derived antigens developed a preferential response against MSP1a (Kocan et al., 2001; de la Fuente et al., 2002b). The molecular basis for this difference will be explored in

this proposed research and may be due to differences in the expression or conformation of MSP1a and MSP1b proteins in the tick and erythrocytic stages of *A. marginale*.

Molecular Biology of *A. marginale*

Genome size and composition

A. marginale has a circular genome of 1,197,701 bp, as determined from its genome sequence (http://www.vetmed.wsu.edu/research_vmp/anagenome/). The G+C content of the *A. marginale* genome had been estimated at 33-50% (Ellender & Dimopoullos, 1967; Senitzer et al., 1972; Ambrosio & Potgieter, 1987), but was later determined to be 56 mol% using spectral analysis, a more accurate approach (Alleman et al., 1993).

The genome of *A. marginale* appears to have undergone reductive evolution (Palmer, 2002), a process in which initial mutation events accumulate, resulting in loss of function and eventual gene deletion (Andersson & Kurland, 1998). Reductive evolution, the result of gene degradation (Andersson & Andersson, 1999), is a common and ongoing process in obligate intracellular pathogens, which have retained only the functions necessary for survival and propagation within the host cells (Palmer, 2002). As a result, *A. marginale* has one of the smallest genomes and is considered a small genome pathogen (Fig. 5) (organisms that have a genome $\leq$1.5 Mb, or 1/3 of the size of the *E. coli* genome). In the process of genomic reduction, *A. marginale* became an obligate intracellular parasite for bovine erythrocytes and tick cells, due to the loss of gene functions not necessary for survival within the predictable intracellular environment.

The primary deletion events during gene degradation are associated with the deletion of redundant, overlapping and duplicated genes, as illustrated by the unique gene arrangement of the rRNA genes in rickettsial organisms (Andersson et al., 1995, 1999; Massung et al., 2002).

A. marginale retained only one of the seven rRNA copies usually found in bacteria (Rurangirwa et al., 2002).

Remarkably, small genome pathogens dedicate a large part of their genome for encoding surface molecules that are essential for infecting host cells. *A. marginale*, for example, has retained numerous copies of genes and pseudogenes encoding for membrane molecules (Brayton et al., 2001). These surface molecules are under selective pressure and are required by the pathogen for survival, either because of their function or because they are necessary in order to overcome the host's mechanisms of defense. Selected major surface proteins of *A. marginale* have been characterized, and some, such as MSP1a and MSP1b, are involved in the interaction of the rickettsia with the host cells, while others, such as MSP2 and MSP3, appear to be necessary for development of persistent infection within the host by generating antigenic variation required for overcoming the immune response (Brayton et al., 2001, 2002).

As of November 24, 2003, 144 microbial genomes have been sequenced, 127 of which correspond to bacterial genomes, including rickettsial organisms and other tick-borne pathogens such as the Lyme borrelia, *Borrelia burgdorferi*, *Rickettsia prowazekii* and *Rickettsia conorii* (http://www.ncbi.nlm.nih.gov/genomes/MICROBES/Complete.html). The first genome of an organism of the genus *Anaplasma* to be completely sequenced was the genome of *A. phagocytophilum*, which was completed and is being annotated by the Ehrlichia Research Laboratory, Ohio State University, in collaboration with The Institute for Genome Research (TIGR) (http://riki-lb1.vet.ohio-state.edu/ehrlichia/index.php). The genome of the St. Maries isolate of *A. marginale* has also been completed and is subject to final editing (http://www.vetmed.wsu.edu/research_vmp/anagenome).

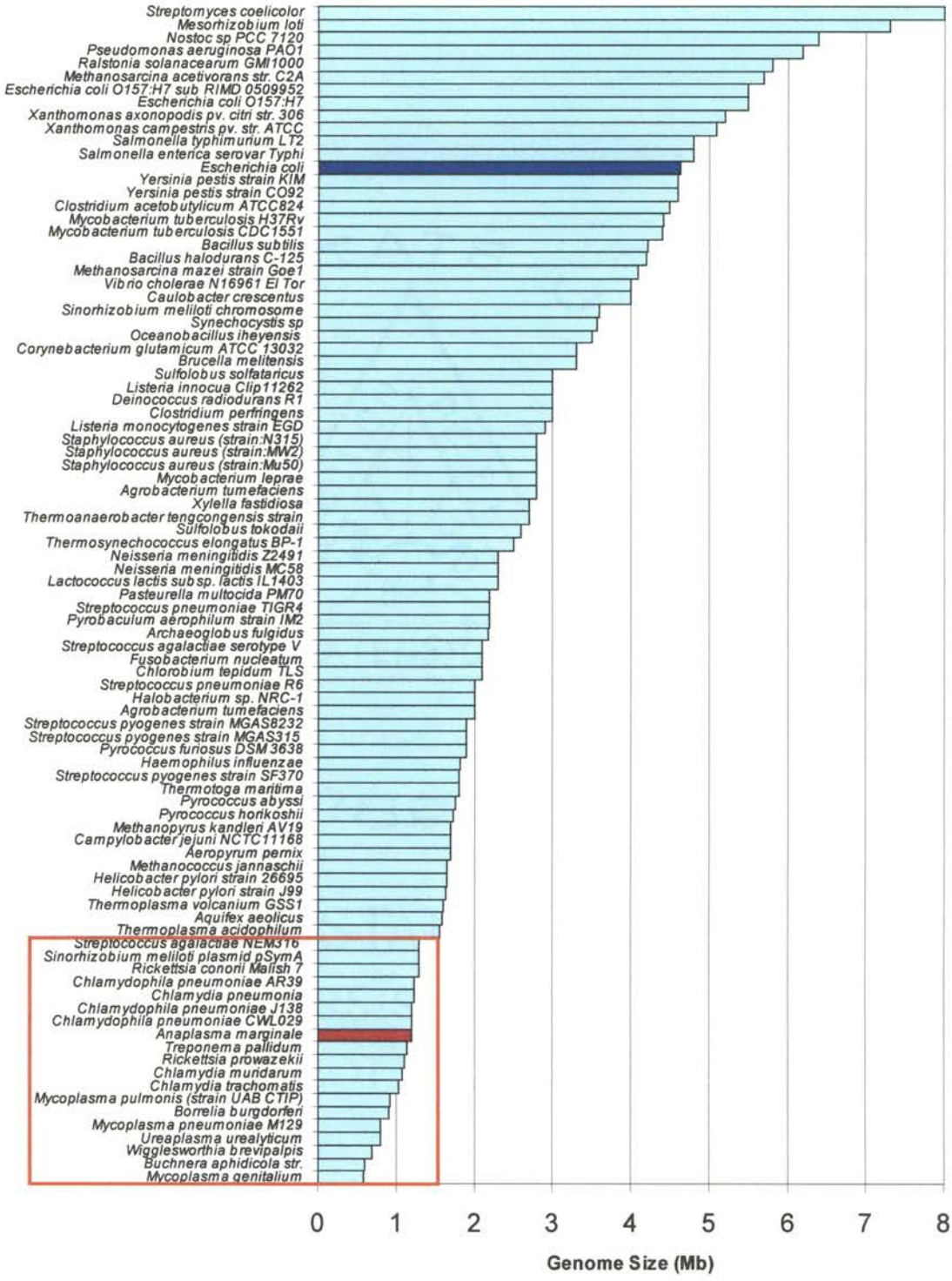

Figure 5. Prokaryotic genomes.
Anaplasma marginale (red bar) contains one of the smallest genomes among bacterial organisms. Organisms with a genome ≤1/3 the size of the *E. coli* genome (blue bar), such as the organisms within the box, lower left corner, are considered small genome pathogens.

Anaplasma marginale major surface proteins

Six major surface proteins have been identified on *A. marginale* derived from bovine erythrocytes and were found to be conserved on tick- and cell culture-derived organisms (Barbet et al., 1999). Three of these MSPs, namely MSP1a, MSP4 and MSP5, are encoded by single genes and do not vary antigenically during the multiplication of the bacterium (Barbet et al., 1987; Allred et al., 1990; Visser et al., 1992; Oberle et al., 1993), while the other three, MSP1b, MSP2 and MSP3, are from multigene families and may vary antigenically, most notably in persistently infected cattle (Barbet & Allred, 1991; Palmer et al., 1994; Alleman et al., 1997; Kocan et al., 2000; Barbet et al., 2001).

MSP1b, a 100 kDa protein, is encoded by two genes, *msp1β1* and *msp1β2* (Barbet et al., 1987; Barbet & Allred, 1991; Camacho-Nuez et al., 2000; Viseshakul et al., 2000; Bowie et al., 2002) and has been suggested to be an adhesin for bovine erythrocytes but not for tick cells (McGarey & Allred, 1994; McGarey et al., 1994; de la Fuente et al., 2001b). Because MSP1a is the focus of this research, this protein will be described in a separate section.

MSP2 is a membrane protein of approximately 36 kDa encoded by a polymorphic multigene family (Palmer et al., 1994). MSP2 is present in different *A. marginale* stages (Palmer et al., 1985), including the tick and intraerythrocytic stages (Barbet et al., 1999), although new antigenic variants are generated in both vertebrate and tick hosts during the life cycle of the pathogen (Barbet et al., 2001; de la Fuente & Kocan, 2001). MSP2 is also conserved between *A. marginale* and *A. centrale* (Shkap et al., 1991), and *msp2* orthologs have been found in other rickettsial organisms (Palmer et al., 1994).

MSP3 is an immunodominant 86 kDa membrane polypeptide. It is also encoded by a polymorphic multigene family, and contains regions with amino acid sequence homology to

MSP2 (Alleman et al., 1997). MSP3 is also suspected to be involved in antigenic variation that contributes to the development of persistent infections.

The 31 kDa MSP4 protein does not vary in molecular size among isolates (Visser et al., 1992). The *msp4* gene is highly conserved among *A. marginale* isolates and has been used to infer phylogenetic and biogeographic relationship among isolates (de la Fuente et al., 2002c). The function of MSP4 is unknown.

MSP5 is a 19 kDa protein conserved among all *A. marginale* isolates (Visser et al., 1992), and in tick and erythrocytic stages of *A. marginale* (Knowles et al., 1996). MSP5 is also conserved among several *Anaplasma* species, namely *A. marginale*, *A. centrale* and *A. ovis* (Visser et al., 1992). MSP5 has been shown to form intramolecular and intermolecular disulfide-bonded multimers (Vidotto et al., 1994). The biological function of MSP5 is presently not known.

Major surface protein 1a of A. marginale

The MSP1 complex is a heterodimer composed of MSP1a and MSP1b, two structurally unrelated polypeptides (Vidotto et al., 1994). *msp1α* has been found to be a stable genetic marker for identification of *A. marginale* strains in individual animals during acute and chronic phases of infection and before, during and after tick transmission (Palmer et al., 2001; Bowie et al., 2002). MSP1a contains a neutralization sensitive epitope (Palmer et al., 1987), and was shown to be involved in adhesion of *A. marginale* to bovine erythrocytes and tick cells in experiments using recombinant *E. coli* expressing MSP1a, microtiter hemagglutination, adhesion recovery assays and microscopy (McGarey & Allred, 1994; McGarey et al., 1994; de la Fuente et al. 2001a,b). MSP1a size polymorphism exists among *A. marginale* isolates because

of a different number of tandemly repeated 28-29 amino acid peptides in the N-terminal of the protein (Allred et al., 1990).

MSP1a is recognized by the bovine immune response after *A. marginale* infection (Barbet et al., 1987) and is involved in immunity to *A. marginale* infection in cattle (Palmer et al., 1987, 1989; Brown et al., 2001). Immunization of cattle with affinity-purified native MSP1 complex induced partial protective immunity in cattle (Palmer et al., 1989). Furthermore, MSP1a has been shown to affect *Dermacentor* spp. infection and transmission of *A. marginale* (de la Fuente et al., 2001a).

Discrepancy between the observed and the deduced molecular mass of A. marginale MSP1a

The molecular weight of *A. marginale* MSP1a varies among isolates with the number of tandemly repeated peptides (Allred et al., 1990; de la Fuente et al., 2001d). However, the observed molecular mass of MSP1a estimated from its electrophoretic mobility is greater than predicted from the primary sequence of the MSP1a proteins from all the *A. marginale* isolates studied so far (Table 4; Oberle et al., 1988). This apparent contradiction has been attributed to the primary sequence of the protein and to the presence of repeated sequences that could affect the electrophoretic migration of MSP1a (Barbet et al., 1987; Palmer et al., 1987; Oberlé et al., 1988). The presence of post-translational modifications in MSP1a has been suggested (Brown et al., 2001), particularly after other ehrlichial proteins were shown to be post-translationally modified (McBride et al., 2000), although it has been disregarded by others (Barbet et al., 1987; Palmer et al., 1987).

TABLE 4. Observed and predicted molecular mass of MSP1a protein from different *A. marginale* isolates.

Isolate	No. Repeats	Observed MW[a]	Predicted MW[b]
Virginia	2	70	61
Washington	4	86	63
North Texas	4	89	63
South Idaho	6	95	63
Florida	8	105	66

[a] Molecular mass estimated from the electrophoretic mobility by Oberle et al., 1988.

[b] Molecular mass predicted from the amino acid sequence.

Functional characterization of A. marginale MSP1a

A. marginale MSP1a has been shown to mediate adhesion, infection and transmission of the organism, as well as to contribute to protective immunity in cattle (McGarey & Allred, 1994; McGarey et al., 1994; de la Fuente et al., 2001b). Using a direct hemagglutination assay, McGarey & Allred (1994) demonstrated that the interaction of *A. marginale* with bovine erythrocytes is inhibited by anti-MSP1a antibodies. The ability of recombinant *E. coli* expressing MSP1a on its surface to hemagglutinate erythrocytes was later reported (McGarey et al., 1994), and confirmed by de la Fuente et al., (2001b). Recombinant MSP1a was also shown to mediate adhesion to native and culture tick cells and transmission by *Dermacentor* spp. ticks (de la Fuente et al., 2001b). However, MSP1a from a non tick-transmissible isolate did not adhere to tick cells (de la Fuente et al., 2001a). Since the only region of MSP1a that varies among isolates is the N-terminal region containing the tandem repeats, different MSP1a mutants including and lacking the tandem repeats were assayed for their ability to adhere to bovine erythrocytes and tick cells (de la Fuente et al., 2003a). The repeated peptides of MSP1a proved to be necessary and sufficient to mediate adhesion of MSP1a to host cells (de la Fuente et al., 2003a). Studies using synthetic peptides and tick cell extract showed that MSP1a repeat

peptides containing acidic amino acids (aspartic or glutamic acid) at position 20 are able to bind to tick cells, while peptides with a glycine as the 20th amino acid are not adhesive (de la Fuente et al., 2003a).

Analysis of tandemly repeated MSP1a peptides of several geographic isolates of *A. marginale* revealed a complex relationship between the *msp1a* genotype and the tick-transmissible phenotype of the isolate and suggested that the sequence and conformation of the repeated peptides influences the adhesive properties (de la Fuente et al., 2003a).

Gene Regulation in Tick-Borne Bacteria

Bacterial genomes are commonly small and generally devoid of unnecessary information, which is particularly important in obligate intracellular bacteria that have undergone reductive evolution and loss of duplicated and redundant genes (Andersson & Kurland, 1998). Intracellular bacteria take advantage of the predictable and stable environment within the host cell. However, these bacteria have to exit the host cell and invade uninfected host cells, and eventually exit the host organism to be transmitted to another susceptible host. This process of invasion and spreading often requires the exposure of the pathogen to hostile and variable conditions to which the bacterium must have adapted in order to survive. Therefore, pathogens have evolved mechanisms in order to respond to environmental changes and escape the host's antimicrobial response.

Since the bacterial cell membrane is the interface between the pathogen and its environment, a significant fraction of the bacterial genome is devoted to surface molecules and the generation of antigenic variation that ensures the persistence and adaptability of the pathogen. The expression of many of these surface molecules is highly regulated in most bacteria, which are able to respond to numerous factors including temperature (Konkel &

Tilly, 2000), pH (Foster, 1999), osmolarity (Sleator & Hill, 2002), ion levels (Litwin & Calderwood, 1993), growth phase (Phillips & Strauch, 2002), population density (Miller & Bassler, 2001), and presence of host cells (Obonyo et al., 1999).

Tick-borne pathogens are not the exception since they have to alternate between the tick and vertebrate hosts. A major difference between both hosts is the temperature, a factor that is known to regulate the expression of surface molecules in a number of pathogens (Konkel & Tilly, 2000). Regulation of the expression of Lyme borrelia spirochete outer surface proteins is probably the best studied gene regulation mechanism among tick-borne pathogens (Indest et al., 2000). For example, during tick feeding, *B. burgdorferi* downregulates the expression of OspA and upregulates OspC (Obonyo et al., 1999). The switch in OspA and OspC expression is regulated by the temperature and the contact with the tick host cells (Obonyo et al., 1999). The expression of OspA was higher in spirochetes cultivated at 31°C, while OspC expression was enhanced in *B. burgdorferi* grown at 37°C (Obonyo et al., 1999). OspC production was also increased when the spirochete was co-cultivated with cultured tick cells or tick hemolymph (Obonyo et al., 1999; Johns et al., 2000). Similar results have been obtained in *B. burgdorferi*-infected ticks. OspA is expressed in unfed ticks, but its expression is downregulated upon tick feeding (Schwan & Piesman, 2000). The temporal analysis of the expression of OspA and OspC suggested that OspC is involved in transmission from tick to mammal but not from mammal to tick (Schwan & Piesman, 2000), while OspA has an important function in the vector (Pal et al., 2000). Understanding the temporal profile of expression of surface proteins will facilitate the identification of the function of these proteins. The regulation of the expression of surface molecules in other tick-borne bacteria suggests that some of these proteins determine the capacity for survival and adaptation of the pathogen.

Immunodominant membrane proteins in several ehrlichial organisms are differentially expressed. For instance, the *E. canis* P30 proteins, which are encoded by a polymorphic multigene family, are differentially expressed in infected dogs and *R. sanguineus* ticks (Unver et al., 2001). Of the 14 paralogs analyzed by Unver and colleagues, 11 were transcribed at higher levels in infected dogs. The expression of only one of the *p30* paralogs was detected in *R. sanguineus* ticks. The same paralog was expressed in nymphs, adult males and adult females, suggesting that either this paralog is predominantly expressed in the tick stages of *E. canis*, or that the expression of the other paralogs is downregulated in ticks. Experiments using *E. canis* cultivated in a dog monocyte cell line indicate that temperature is at least one of the factors that regulate the expression of the *p30* paralogs (Unver et al., 2001).

E. canis P30 proteins are highly cross-reactive with 28-kDa antigens (OMP-1s) of *E. chaffeeensis* (Rikihisa et al., 1994). The OMP-1s are encoded by at least 22 paralogs of a single polymorphic multigene family (Ohashi et al., 2001). Most of the *p28* paralogs are active genes (Long et al., 2002), but are differentially transcribed in infected dogs and *A. americanum* ticks (Unver et al., 2002). Sixteen of the *p28* paralogs are transcribed in infected dog monocytes, but only one is expressed in nymph and adult *A. americanum* ticks (Unver et al., 2002).

A 120-kDa *E. chaffeensis* antigen (P120) has been shown to be differentially expressed in different stages of the development of the pathogen (Popov et al., 2000). Expression of P120 was detected in dense forms of *E. chaffeensis*, while P120 was not detected in the cell wall of the reticulated forms. This observation is consistent with the putative role of P120 in *E. chaffeensis* adhesion and invasion (Popov et al., 2000).

The major antigenic protein 1 (MAP1) of *E. ruminantium* is closely related to the P28 and P30 proteins of *E. chaffeeensis* and *E. canis*, respectively. MAP1 proteins are encoded by the

map1 multigene family. One out of the three *msp1* paralogs is expressed in both infected bovine endothelial cells and *A. variegatum* ticks (Bekker et al., 2002). The *map1-1* gene transcript was detected in infected ticks but not in infected bovine cells, while expression of the *map1-2* paralog was not detected under any condition (Bekker et al., 2002).

A. phagocytophilum is another ehrlichial organism closely related to *A. marginale*. This pathogen expresses P44s, a family of immunodominant 44-kDa proteins encoded by a multigene family (IJdo et al., 1998; Murphy et al., 1998). Among the 20 different *p44* paralogs detected in infected mammals, ticks and cell cultures, the *p44-18* transcript was preferentially expressed in mice and horses, but not in ticks (Zhi et al., 2002). Other transcripts were detected in infected ticks but were not detected in mammals. Notably, some *p44* transcripts are present in ticks during transmission feeding but not in non-feeding ticks (IJdo et al., 2002).

In *A. marginale*, MSP2s are encoded by a multigene family orthologous to *E. ruminantium map1*, *E. canis p28*, *E. chaffeensis p30*, and *A. phagocytophilum p44* (Palmer et al., 1994). It has been suggested that, similar to its orthologs, only some *msp2* transcripts are expressed in *A. marginale*-infected ticks (Rurangirwa et al., 1999). Moreover, it has been stated that the restriction of *msp2* transcript variants occurs in the midgut as an early event during acquisition feeding (Löhr et al., 2002). However, only one *msp2* expression site has been identified, and it encodes for a polycistronic mRNA (Barbet et al., 2000). Other studies have shown that more *msp2* variants are expressed within infected ticks (de la Fuente & Kocan, 2001), and that not all *A. marginale* strains undergo restriction to tick-specific *msp2* variants (Barbet et al., 2001). In any case, the expression of transcript variants in *Anaplasma* spp. do not seem to be transcriptionally regulated since no significant changes in transcript or protein amounts has been observed (Löhr et al., 2002), in contrast to the transcriptional regulation of

the expression of *msp2* orthologs in *Ehrlichia* spp. The generation of new MSP2 antigenic variants appears to be mediated by the recombination of pseudogenes into the *msp2* expression site (Brayton et al., 2002). Sequential recombination of small segments in the hypervariable region of *msp2* results in a combinatorial number of antigenic variants generated by gene conversion (Brayton et al., 2002). Variability of MSP3 has also been found to be mediated by the generation of new antigenic variants by gene conversion (Brayton et al., 2001). Although other surface proteins have been shown to be conserved between tick- and erythrocyte-derived *A. marginale* (Palmer et al., 1985; Barbet et al., 1999), these studies were based on a qualitative approach intended to identify rather than quantify the surface proteins. It has been suggested that differential expression of certain outer membrane proteins accounts for the different biological properties of the pathogen in different life stages of *A. marginale* (Löhr et al., 2002).

Protein Glycosylation in Pathogenic Bacteria

Protein glycosylation was thought to be restricted to eukaryotic organisms for a long time, but numerous examples of glycosylation in prokaryotes have been found in the last decades. Surface layer (S-layer) proteins were the first bacterial proteins to be shown to be glycosylated (Mescher et al., 1974). In recent years, non-S-layer glycoproteins have also been found in a growing number of bacterial species, and the belief that protein glycosylation only occurs in eukaryotes has been disproved. Most of the proteins glycosylated in pathogenic bacteria are surface proteins, many of which are involved in the process of adhesion to and invasion of the host organism (Table 5).

TABLE 5. Protein glycosylation in gram-negative pathogenic bacteria.

Organism	Protein	Linkage	Function	Reference
Anaplasma phagocytophilum	100 kDa	N/R	unknown	de la Fuente et al., 2003c
	130 kDa	N/R	unknown	de la Fuente et al., 2003c
Borrelia burgdorferi	OspA	-Asn	unknown	Sambri et al., 1992
	OspB	-Asn	unknown	Sambri et al., 1993
	FlaA (flagellin)	N/R	motility	Ge et al., 1998
Campylobacter jejuni	Peb3	-Asn	unknown	Young et al., 2002
	CgpA	-Asn	unknown	Linton et al., 2002
	Flagellin	-Ser/Thr	motility	Thibault et al., 2001
Campylobacter coli	Flagellin	-Ser/Thr	motility	Doig et al., 1996
Chlamydia trachomatis	40 kDa MOMP	-Asn	adhesion	Swanson & Kuo, 1991
Mycobacterium tuberculosis	45/47 kDa (Apa)	-Thr	immunomodulation	Dobos et al., 1996
	19 kDa	-Thr	unknown	Herrmann et al., 1996
Ehrlichia chaffeensis	P120	-Ser/Thr	unknown	McBride et al., 2000
Ehrlichia canis	P140	-Ser/Thr	unknown	McBride et al., 2000
Neisseria gonorrhoea	Pilin	-Ser	adhesion	Marceau et al., 1998
Neisseria meningitidis	Pilin	-Ser	adhesion	Marceau et al., 1998
Escherichia coli	TibA	-Ser/Thr	invasion	Lindenthal & Elsinghorst, 1999
	AIDA-I	-Ser/Thr	adhesion	Benz & Schmidt, 2001

Protein glycosylation is involved in the functional properties of many of these glycoproteins. For instance, *M. tuberculosis* Apa glycoproteins (45/47 kDa antigen) elicit different kinds of immune response depending on the extent of glycosylation (Horn et al., 1999). The carbohydrate moieties in several bacterial adhesion molecules have been shown to affect the adhesion to host cells (Marceau & Nassif, 1999; Szymanski et al., 2002). Glycosylation of the host cell receptor for the bacterial adhesin has also been shown to be involved in bacterial adhesion to host cells (Yago et al., 2003). Glycosylation can also regulate the sensitivity of glycoproteins to proteolysis. The 19-kDa antigen of *M. tuberculosis*, for example, is more sensitive to proteolytic cleavage when it is not glycosylated (Herrmann et al.,

1996). The solubility of the glycoprotein can also be affected by the extent of glycosylation (Marceau & Nassif, 1999). In addition, glycosylation can also contribute to antigenic variation (Marceau & Nassif, 1999). The number of functionally important bacterial glycoproteins has increased in recent years, as has the roles, implications and importance of glycosylation of surface molecules in pathogenic bacteria.

Recently, the genes encoding for antigenic high molecular weight membrane proteins from several ehrlichial organisms were cloned and completely sequenced (Yu et al., 1997; Storey et al., 1998). Some of these proteins, namely P120 (120-kDa protein from *E. chaffeensis*), P140 (140-kDa protein from *E. canis*), and P100 and P130 (100- and 130-kDa proteins from *A. phagocytophilum*), exhibited molecular masses higher than predicted from their primary sequences (Yu et al., 1997; Storey et al., 1998). The *p120* and *p140* genes encode for proteins of predicted molecular masses of 61 and 73 kDa, respectively, two times smaller than the observed molecular masses (Yu et al., 1997). These proteins contain a number of tandem repeat units with conserved Ser/Thr-rich motifs. The anomalous SDS-PAGE electrophoretic mobility and differences between the expected and observed molecular masses were initially attributed to the presence of repeated regions in these proteins (Yu et al., 1997). However, post-translational modifications of the native and recombinant P120 and P140 proteins have been shown to account for this apparent contradiction (McBride et al., 2000). Using periodate oxidation, McBride and colleagues detected carbohydrates on the recombinant P120 and P140 proteins. Although the glycosidase and lectin-binding analysis produced negative results (no enzymatic deglycosylation or lectin binding) due to the absence of the specific motifs recognized by these molecules, the monosaccharide compositional analysis using gas chromatography indicated that the recombinant P120 and P140 proteins expressed in *E. coli*

contain three carbohydrate residues, i.e., glucose, galactose and xylose. The glycosylation of these proteins is unusual since they do not contain the core sugars *N*-acetylglucosamine and *N*-acetylgalactosamine usually found in *N*- and *O*-linked sugar moieties from eukaryotic origin (McBride et al., 2000).

BIBLIOGRAPHY

Akinboade OA, Dipeolu OO, Ogunji FO, Adegoke GO. 1981. The parasites obtained and bacteria isolated from house rats (Rattus rattus Linnaeus, 1758) caught in human habitations in Ibadan, Nigeria. Int. J. Zoonoses 8:26-32.

Alleman AR, Kamper SM, Viseshakul N, Barbet AF. 1993. Analysis of the *Anaplasma marginale* genome by pulsed-field electrophoresis. J. Gen. Microbiol. 139:2439-44.

Alleman AR, Palmer GH, McGuire TC, McElwain TF, Perryman LE, Barbet AF. 1997. *Anaplasma marginale* major surface protein 3 is encoded by a polymorphic, multigene family. Infect. Immun. 65:156-63.

Allred DR, McGuire TC, Palmer GH, Leib SR, Harkins TM, McElwain TF, Barbet AF. 1990. Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*. Proc. Natl. Acad. Sci. 87:3220-4.

Ambrosio RE, Potgieter FT. 1987. The genome of *Anaplasma*: DNA base composition and DNA/DNA hybridization. Onderstepoort J. Vet. Res. 54:63-5.

Amerault TE, Mazzola V, Roby TO. 1973. Gram-staining characteristics of *Anaplasma marginale*. Am. J. Vet. Res. 34:552-5.

Andersson JO, Andersson SG. 1999. Genome degradation is an ongoing process in *Rickettsia*. Mol. Biol. Evol. 16:1178-91.

Andersson SG, Kurland CG. 1998. Reductive evolution of resident genomes. Trends Microbiol. 6:263-8.

Andersson SG, Zomorodipour A, Winkler HH, Kurland CG. 1995. Unusual organization of the rRNA genes in *Rickettsia prowazekii*. J. Bacteriol. 177:4171-5.

Andersson SG, Stothard DR, Fuerst P, Kurland CG. 1999. Molecular phylogeny and rearrangement of rRNA genes in *Rickettsia* species. Mol. Biol. Evol. 16:987-95.

Anthony DW, Roby TO. 1962. Anaplasmosis transmission studies with *Dermacentor variabilis* (Say) and *Dermacentor andersoni* (Stiles) (=*D. venustus* Marx) as experimental vectors. In: Proceedings of the 4th National Anaplasmosis Research Conference, Reno, Nevada, pp. 78-81.

Anziani OS, Tarabla HD, Ford CA, Galleto C. 1987. Vaccination with *Anaplasma centrale*: response after an experimental challenge with *Anaplasma marginale*. Trop. Anim. Health. Prod. 19:83-7.

Arulkanthan A, Brown WC, McGuire TC, Knowles DP. 1999. Biased immunoglobulin G1 isotype responses induced in cattle with DNA expressing

msp1a of *Anaplasma marginale*. Infect. Immun. 67:3481-7.

Barbet AF, Allred DR. 1991. The *msp1β* multigene family of *Anaplasma marginale*: nucleotide sequence analysis of an expressed copy. Infect. Immun. 59:971-6.

Barbet AF, Anderson LW, Palmer GH, McGuire TC. 1982. Comparison of proteins synthesized by two different isolates of *Anaplasma marginale*. Infect. Immun. 40:1068-74.

Barbet AF, Palmer GH, Myler PJ, McGuire TC. 1987. Characterization of an immunoprotective protein complex of *Anaplasma marginale* by cloning and expression of the gene coding for polypeptide AM 105L. Infect. Immun. 55:2428-35.

Barbet AF, Blentlinger R, Lundgren AM, Blouin EF, Kocan KM. 1999. Comparison of surface proteins of *Anaplasma marginale* grown in tick cell culture, tick salivary glands and cattle. Infect. Immun. 67:102-7.

Barbet AF, Lundgren A, Yi J, Rurangirwa FR, Palmer GH. 2000. Antigenic variation of *Anaplasma marginale* by expression of MSP2 mosaics. Infect. Immun. 68:6133-8.

Barbet AS, Jooyoung Yi, Lundgren AM, McEwen BR, Blouin EF, Kocan KM. 2001. Antigenic variation of *Anaplasma marginale*: MSP2 diversity during cyclic transmission between ticks and cattle. Infect. Immun. 69:3057-66.

Baumgartner W, Schlerka G, Fumicz M, Stoger J, Awad-Masalmeh M, Schuller W, Weber P. 1992. Seroprevalence survey for *Anaplasma marginale*-infection of Austrian cattle. Zentralbl Veterinarmed [B] 39:97-104.

Bekker CP, Bell-Sakyi L, Paxton EA, Martinez D, Bensaid A, Jongejan F. 2002. Transcriptional analysis of the major antigenic protein 1 multigene family of *Cowdria ruminantium*. Gene 285:193-201.

Benz I, Schmidt MA. 2001. Glycosylation with heptose residues mediated by the *aah* gene product is essential for adherence of the AIDA-I adhesin. Mol. Microbiol. 40:1403-13.

Blouin EF, Kocan KM. 1998. Morphology and development of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in cultured *Ixodes scapularis* (Acari: Ixodidae) cells. J. Med. Entomol. 35:788-97.

Blouin EF, Barbet AF, Jooyoung Yi, Kocan KM, Saliki JT. 2000. Establishment and characterization of an Oklahoma isolate of *Anaplasma marginale* in cultured *Ixodes scapularis* cells. Vet. Parasitol. 87:301-13.

Blouin EF, Saliki JT, de la Fuente J, Garcia-Garcia JC, Kocan KM. 2003a. Antibodies to *Anaplasma marginale* major surface proteins 1a and 1b inhibit infectivity for cultured tick cells. Vet. Parasitol. 111:247-60.

Blouin EF, de la Fuente J, Garcia-Garcia JC, Sauer JR, Saliki JT, Kocan KM. 2003b. Use of a cell culture system for studying the interaction of *Anaplasma marginale* with tick cells. Anim. Health Res. Rev. 3:57-68.

Bowie MV, de la Fuente J, Kocan KM, Blouin EF, Barbet AF. 2002. Conservation of major surface protein 1

genes of the ehrlichial pathogen *Anaplasma marginale* during cyclic transmission between ticks and cattle. Gene 282:95-102.

Boynton WH, Hermes WB, Howell DE, Woods GM. 1936. Anaplasmosis transmission by three species of ticks in California. J. Am. Vet. Med. Assoc. 88:500-2.

Bradway DS, Torioni de Echaide S, Knowles DP, Hennager SG, McElwain TF. 2001. Sensitivity and specificity of the complement fixation test for detection of cattle persistently infected with *Anaplasma marginale*. J. Vet. Diagn. Invest. 13:79-81.

Brayton KA, Knowles DP, McGuire TC, Palmer GH. 2001. Efficient use of a small genome to generate antigenic diversity in tick-borne ehrlichial pathogens. Proc. Natl. Acad. Sci. U.S.A. 98:4130-5.

Brayton KA, Palmer GH, Lundgren A, Yi J, Barbet AF. 2002. Antigenic variation of *Anaplasma marginale msp2* occurs by combinatorial gene conversion. Mol. Microbiol. 43:1151-9.

Brendel V, Bucher P, Nourbakhsh I, Blaisdell BE, Karlin S. 1992. Methods and algorithms for statistical analysis of protein sequences. Proc. Natl. Acad. Sci. U. S. A. 89:2002-6.

Brock WE, Kliewer IO, Pearson CC. 1965. A vaccine for anaplasmosis. J. Am. Vet. Med. Assoc. 147:943-51.

Brown WC, Palmer GH, Lewin HA, McGuire TC. 2001. CD4(+) T lymphocytes from calves immunized with *Anaplasma marginale* major surface protein 1 (MSP1), a heteromeric complex of MSP1a and MSP1b, preferentially recognize the MSP1a carboxyl terminus that is conserved among strains. Infect. Immun. 69:6853-62.

Brown WC, McGuire TC, Mwangi W, Kegerreis KA, Macmillan H, Lewin HA, Palmer GH. 2002. Major histocompatibility complex class II DR-restricted memory CD4(+) T lymphocytes recognize conserved immunodominant epitopes of *Anaplasma marginale* major surface protein 1a. Infect. Immun. 70:5521-32.

Brumpt E. 1931. Transmission *d'Anaplasma marginale* par *Rhipicephalus bursa* et par margraopus. Annuals de Parasitologie 9:4-9.

Camacho-Nuez M, de Lourdes Munoz M, Suarez CE, McGuire TC, Brown WC, Palmer GH. 2000. Expression of polymorphic *msp1β* genes during acute *Anaplasma marginale* rickettsemia. Infect. Immun. 68:1946-52.

Cantor GH, Pontzer CH, Palmer GH. 1993. Opsonization of *Anaplasma marginale* mediated by bovine antibody against surface protein MSP-1. Vet. Immunol. Immunopathol. 37:343-50.

Carmichael IH, Hobday E. 1975. Blood parasites of some wild bovidae in Botswana. Onderstepoort J. Vet. Res. 42:55-62.

Chomel BB, Carniciu ML, Kasten RW, Castelli PM, Work TM, Jessup DA. 1994. Antibody prevalence of eight ruminant infectious diseases in California mule and black-tailed deer

(*Odocoileus hemionus*). J. Wildl. Dis. 30:51-9.

de Kroon JF, Perie NM, Franssen FF, Uilenberg G. 1990. The indirect fluorescent antibody test for bovine anaplasmosis. Vet. Q 12:124-8.

de la Fuente J, Kocan KM. 2001. Expression of *Anaplasma marginale* major surface protein 2 variants in persistently infected ticks. Infect. Immun. 69:5151-6.

de la Fuente J, Rodriguez M, Redondo M, Montero C, Garcia-Garcia JC, Mendez L, Serrano E, Valdes M, Enriquez A, Canales M, Ramos E, Boue O, Machado H, Lleonart R, de Armas CA, Rey S, Rodriguez JL, Artiles M, Garcia L. 1998. Field studies and cost-effectiveness analysis of vaccination with Gavac against the cattle tick *Boophilus microplus*. Vaccine 16:366-73.

de la Fuente J, Garcia-Garcia JC, Blouin EF, Kocan KM. 2001a. Major surface protein 1a effects tick infection and transmission of the ehrlichial pathogen *Anaplasma marginale*. Int. J. Parasitol. 31:1705-14.

de la Fuente J, Garcia-Garcia JC, Blouin EF, Kocan KM. 2001b. Differential adhesion of major surface proteins 1a and 1b of the ehrlichial cattle pathogen *Anaplasma marginale* to bovine erythrocytes and tick cells. Int. J. Parasitol. 31:145-53.

de la Fuente J, van den Bussche RA, Kocan KM. 2001c. Molecular phylogeny and biogeography of North American isolates of *Anaplasma marginale* (Rickettsiaceae: Ehrlichieae). Vet. Parasitol. 97:65-76.

de la Fuente J, Garcia-Garcia JC, Blouin EF, Rodríguez SD, García MA, Kocan KM. 2001d. Evolution and function of tandem repeats in the major surface protein 1a of the ehrlichial pathogen *Anaplasma marginale*. Anim. Health Res. Rev. 2:163-73.

de la Fuente J, Garcia-Garcia JC, Blouin EF, Saliki JT, Kocan KM. 2002a. Infection of tick cells and bovine erythrocytes with one genotype of the intracellular ehrlichia *Anaplasma marginale* excludes infection with other genotypes. Clin. Diagn. Lab. Immunol. 9:658-68.

de la Fuente J, Kocan KM, Garcia-Garcia JC, Blouin EF, Claypool PL, Saliki JT. 2002b. Vaccination of cattle with *Anaplasma marginale* derived from tick cell culture and bovine erythrocytes followed by challenge-exposure with infected ticks. Vet. Microbiol. 89:239-51.

de la Fuente J, Van Den Bussche RA, Garcia-Garcia JC, Rodriguez SD, Garcia MA, Guglielmone AA, Mangold AJ, Friche Passos LM, Barbosa Ribeiro MF, Blouin EF, Kocan KM. 2002c. Phylogeography of New World isolates of *Anaplasma marginale* based on major surface protein sequences. Vet. Microbiol. 88:275-85.

de la Fuente J, Garcia-Garcia JC, Blouin EF, Kocan KM. 2003a. Characterization of the functional domain of major surface protein 1a involved in adhesion of the rickettsia *Anaplasma marginale* to host cells. Vet. Microbiol. 91:265-83.

de la Fuente J, Kocan KM, Garcia-Garcia JC, Blouin EF, Halbur T, Onet V. 2003b. Antibodies to *Anaplasma marginale* major surface protein 1a reduce

infectivity for ticks. Acta Tropica, In press.

de la Fuente J, Garcia-Garcia JC, Barbet AF, Blouin EF, Kocan KM. 2003c. Adhesion of outer membrane proteins containing tandem repeats of *Anaplasma* and *Ehrlichia* species (Rickettsiales: Anaplasmataceae) to tick cells. Vet. Microbiol., In press.

Dikmans G. 1950. The transmission of anaplasmosis. Am. J. Vet. Res. 11:5-16.

Dipeolu OO, Akinboade OA. 1984. Studies on ticks of veterinary importance in Nigeria. XI. Observations on the biology of ticks detached from the red-flanked duiker (*Cephamophys rufulatus*) and parasites encountered in their blood. Vet. Parasitol. 14:87-93.

Dobos KM, Khoo KH, Swiderek KM, Brennan PJ, Belisle JT. 1996. Definition of the full extent of glycosylation of the 45-kilodalton glycoprotein of *Mycobacterium tuberculosis*. J. Bacteriol. 178:2498-506.

Doig P, Kinsella N, Guerry P, Trust TJ. 1996. Characterization of a post-translational modification of *Campylobacter* flagellin: identification of a sero-specific glycosyl moiety. Mol. Microbiol. 19:379-87.

Dumler JS, Barbet AF, Bekker CPJ, Dasch GA, Palmer GH, Ray SC, Rikihisa Y, Rurangirwa FR. 2001. Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, description of six new species combinations and designation of *Ehrlichia equi* and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. Int. J. Syst. Evol. Microbiol. 51:2145-65.

Ellender RD Jr, Dimopoullos GT. 1967. Base composition and thermal denaturation of DNA isolated from *Anaplasma marginale*. Proc. Soc. Exp. Biol. Med. 125:82-5.

El-Metenawy TM. 2000. Prevalence of blood parasites among cattle at the central area of Saudi Arabia. Vet. Parasitol. 87:231-6.

Eriks IS, Palmer GH, McGuire TC, Allred DR, Barbet AF. 1989. Detection and quantitation of *Anaplasma marginale* in carrier cattle by using a nucleic acid probe. J. Clin. Microbiol. 27:279-84.

Eriks IS, Stiller D, Palmer GH. 1993. Impact of persistent *Anaplasma marginale* rickettsemia on tick infection and transmission. J. Clin. Microbiol. 31:2091-6.

Eriks IS, Stiller D, Goff WL, Panton M, Parish SM, McElwain TF, Palmer GH. 1994. Molecular and biological characterization of a newly isolated *Anaplasma marginale* strain. J. Vet. Diagn. Invest. 6:435-41.

Ewing SA. 1981. Transmission of *Anaplasma marginale* by arthropods. In: Proceedings of the 7th National Anaplasmosis Conference, Mississippi State University, MS, USA, pp. 395-423.

Ewing SA, Panciera RJ, Kocan KM, Ge NL, Welsh RD, Olson RW, Barker R, Rice LE. 1997. A winter outbreak of anaplasmosis in a non-endemic area of Oklahoma: a possible role for

Dermacentor albipictus. J. Vet. Diag. Investig. 9:206-8.

Foster JW. 1999. When protons attack: microbial strategies of acid adaptation. Curr. Opin. Microbiol. 2:170-4.

Frank R. 1992. SPOT synthesis: an easy technique for the positionally addressable, parallel chemical synthesis on a membrane support. Tetrahedron 48:9217-32.

Gale KR, Leatch G, Gartside M, Dimmock CM. 1992. *Anaplasma marginale*: failure of sera from immune cattle to confer protection in passive-transfer experiments. Parasitol. Res. 78:410-5.

Ge NK, Kocan KM, Blouin EF, Murphy GL. 1996. Developmental studies of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in male *Dermacentor andersoni* (Acari:Ixodidae) infected as adults using nonradioactive in situ hybridization. J. Med. Entomol. 33:911-20.

Ge NL, Kocan KM, Ewing SA, Blouin EF, Edwards WW, Murphy GL, Dawson LJ. 1997. Use of a non-radioactive DNA probe for detection of *Anaplasma marginale* infection in field cattle: comparison with complement fixation serology and microscopic examination. J. Vet. Diagn. Invest. 9:39-43.

Ge Y, Li C, Corum L, Slaughter CA, Charon NW. 1998. Structure and expression of the FlaA periplasmic flagellar protein of *Borrelia burgdorferi*. J. Bacteriol. 180:2418-25.

Goff W, Barbet A, Stiller D, Palmer G, Knowles D, Kocan KM, Gorham J, McGuire T. 1988. Detection of *Anaplasma marginale*-infected tick vectors by using a cloned DNA probe. Proc. Natl. Acad. Sci. USA, 85:919-23.

Guglielmone AA. 1995. Epidemiology of babesiosis and anaplasmosis in South and Central America. Vet. Parasitol. 57:109-19.

Hansen JE, Lund O, Tolstrup N, Gooley AA, Williams KL, Brunak S. 1998. NetOglyc: Prediction of mucin type *O*-glycosylation sites based on sequence context and surface accessibility. Glycoconj. J. 15:115-30.

Hart LT, Todd WJ, Luther DG. 1990. *Anaplasma marginale* antigen, antigen composition, vaccine and process for production of said antigen, antigen composition and vaccine. US Patent 4,956,278.

Helm R. 1924. Beitrag zum Anaplasmen-Problem. Ztschr f Infektionskr 25:199-226.

Herrmann JL, O'Gaora P, Gallagher A, Thole JE, Young DB. 1996. Bacterial glycoproteins: a link between glycosylation and proteolytic cleavage of a 19 kDa antigen from *Mycobacterium tuberculosis*. EMBO J. 15:3547-54.

Hidalgo RJ, Palmer GH, Jones EW, Brown JE, Ainsworth AJ. 1989. Infectivity and antigenicity of *Anaplasma marginale* from tick cell culture. Am. J. Vet. Res. 50:2033-6.

Horn C, Namane A, Pescher P, Riviere M, Romain F, Puzo G, Barzu O, Marchal G. 1999. Decreased capacity of recombinant 45/47-kDa molecules (Apa) of *Mycobacterium tuberculosis* to stimulate T lymphocyte responses

related to changes in their mannosylation pattern. J. Biol. Chem. 274:32023-30.

Howarth JA, Roby TO. 1972. Transmission of anaplasmosis by field collections of *Dermacentor occidentalis* Marx (Acarina: Ixodidae). In: 76th Meeting of the United States Animal Health Association, pp. 98-102.

Howarth JA, Hokama Y. 1973. Tick transmission of anaplasmosis under laboratory conditions. In: Proceedings of the 6th National Anaplasmosis Research Conference, Las Vegas, Nevada, pp. 117-20.

Howell DE, Stiles GW, Moe LH. 1941a. The fowl tick (*Argas persicus*), a new vector of anaplasmosis. Am. J. Vet. Res. 4:73-5.

Howell DE, Stiles GW, Moe LH. 1941b. The hereditary transmission of anaplasmosis by *Dermacentor andersoni* Stiles. Am. J. Vet. Res. 2:165-6.

IJdo JW, Sun W, Zhang Y, Magnarelli LA, Fikrig E. 1998. Cloning of the gene encoding the 44-kilodalton antigen of the agent of human granulocytic ehrlichiosis and characterization of the humoral response. Infect. Immun. 66:3264-9.

IJdo JW, Wu C, Telford SR 3rd, Fikrig E. 2002. Differential expression of the *p44* gene family in the agent of human granulocytic ehrlichiosis. Infect. Immun. 70:5295-8.

Indest KJ, Ramamoorthy R, Philipp MT. 2000. Transcriptional regulation in spirochetes. J. Mol. Microbiol. Biotechnol. 2:473-81.

Jasinskas A, Jaworski DC, Barbour AG. 2000. *Amblyomma americanum*: specific uptake of immunoglobulins into tick hemolymph during feeding. Exp. Parasitol. 96:213-21.

Johns RH, Sonenshine DE, Hynes WL. 2000. Enhancement of OspC expression by *Borrelia burgdorferi* in the presence of tick hemolymph. FEMS Microbiol. Lett. 193:137-41.

Johnston LA, Trueman KF, Leatch G, Wilson AJ. 1980. A comparison of direct fluorescent antibody and Giemsa staining for the post-mortem diagnosis of anaplasmosis. Aust. Vet. J. 56:116-18.

Jorgensen WK, Weilgama DJ, Navaratne M, Dalgliesh RJ. 1992. Prevalence of *Babesia bovis* and *Anaplasma marginale* at selected localities in Sri Lanka. Trop. Anim. Health Prod. 24:9-14

Keel MK, Goff WL, Davidson WR. 1995. An assessment of the role of white-tailed deer in the epizootiology of anaplasmosis in the southeastern United States. J. Wildl. Dis. 31:378-85.

Kieser ST, Eriks IS, Palmer GH. 1990. Cyclic rickettsemia during persistent *Anaplasma marginale* infection of cattle. Infect. Immun. 58:1117-9.

Knowles D, Torioni de Echaide S, Palmer G, McGuire T, Stiller D, McElwain T. 1996. Antibody against an *Anaplasma marginale* MSP5 epitope common to tick and erythrocyte stages identifies persistently infected cattle. J. Clin. Microbiol. 34:2225-30.

Kocan KM. 1986. Development of *Anaplasma marginale*: coordinated

development of a rickettsial organism and its tick host. In: JR Sauer and JA Hair (eds.), Morphology, Physiology and Behavioral Ecology of Ticks. Chichester, Horwood, pp. 472-505.

Kocan KM. 1994. Strategies for control of *Anaplasma marginale* focused on the tick vector. Curr. Top. Vet. Res. 1:43-51.

Kocan KM, Hair JA, Ewing SA, Stratton JG. 1981. Transmission of *Anaplasma marginale* Theiler by *Dermacentor andersoni* Stiles and *Dermacentor variabilis* (Say). Am. J. Vet. Res. 42:15-8.

Kocan KM, Stiller D, Goff WL, Claypool PL, Edwards W, Ewing SA, McGuire TC, Hair JA, Barron SJ. 1992a. Development of *Anaplasma marginale* in male *Dermacentor andersoni* transferred from infected to susceptible cattle. Am. J. Vet. Res. 53:499-507.

Kocan KM, Goff WL, Stiller D, Claypool PL, Edwards W, Ewing SA, Hair JA, Barron SJ. 1992b. Persistence of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in male *Dermacentor andersoni* (Acari: Ixodidae) transferred successively from infected to susceptible cattle. J. Med. Ent. 29:657-68.

Kocan KM, Blouin EF, Palmer GH, Eriks IS, Edwards WL. 1996a. Strategies to interrupt the development of *Anaplasma marginale* in its tick vector. The effect of bovine-derived antibodies. Ann. N. Y. Acad. Sci. 791:157-65.

Kocan KM, Blouin EF, Palmer GH, Eriks IS, Edwards WL, Claypool PL. 1996b. Preliminary studies on the effect of *Anaplasma marginale* antibodies ingested by *Dermacentor andersoni* ticks (Acari:Ixodidae) with their blood meal on infections in salivary glands. Exp. Appl. Acarol. 20:297-311.

Kocan KM, Blouin EF, Barbet AF. 2000. Anaplasmosis control: past, present and future. Ann. N.Y. Acad. Sci. 916:501-9.

Kocan KM, Halbur T, Blouin EF, Onet V, de la Fuente J, Garcia-Garcia JC, Saliki JT. 2001. Immunization of cattle with *Anaplasma marginale* derived from tick cell culture. Vet. Parasitol. 102:151-61.

Kocan KM, de la Fuente J, Guglielmone AA, Meléndez RD. 2003a. Antigens and alternatives for control of Anaplasma marginale infection in cattle. Clin. Microbiol. Rev. 16:698-712.

Kocan KM, de la Fuente J, Blouin EF, Garcia-Garcia JC. 2003b. *Anaplasma marginale* (Rickettsiales: Anaplasmataceae): Recent advances in defining host-pathogen adaptations of a tick-borne rickettsiae. In: AS Bowman and P Nuttal (eds.), Ticks, Disease and Control. Submitted.

Komalavilas P, Mort AJ. 1989. The acetylation at O-3 of galacturonic acid in the rhamnose-rich region of pectins. Carbohydr. Res. 189:261-72.

Konkel ME, Tilly K. 2000. Temperature-regulated expression of bacterial virulence genes. Microbes Infect. 2:157-66.

Kreir JP, Gothe R, Ihler GM, Krampitz HE, Mernaugh G, Palmer GH. 1992. The hemotrophic bacteria: The families Bartonellaceae and Anaplasmataceae. In: A Balows et al. (eds.), The Prokaryotes: A Handbook on the Biology of Bacteria, 2nd edition, Springer-Verlag, New York.

Kudamba C, Campbell RS, Paull NI, Holroyd RG. 1982. Serological studies of babesiosis and anaplasmosis of cattle. Aust. Vet. J. 59:101-4.

Kuttler KL. 1984. *Anaplasma* infections in wild and domestic ruminants: a review. J. Wildl. Dis. 20:12-20.

Kuttler KL, Winward LD. 1984. Serologic comparisons of 4 *Anaplasma* isolates as measured by the complement-fixation test. Vet. Microbiol. 9:181-6.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-5.

Lindenthal C, Elsinghorst EA. 1999. Identification of a glycoprotein produced by enterotoxigenic *Escherichia coli*. Infect. Immun. 67:4084-91.

Linton D, Allan E, Karlyshev AV, Cronshaw AD, Wren BW. 2002. Identification of N-acetylgalactosamine-containing glycoproteins PEB3 and CgpA in *Campylobacter jejuni*. Mol. Microbiol. 43:497-508.

Litwin CM, Calderwood SB. 1993. Role of iron in regulation of virulence genes. Clin. Microbiol. Rev. 6:137-49.

Long SW, Zhang XF, Qi H, Standaert S, Walker DH, Yu XJ. 2002. Antigenic variation of *Ehrlichia chaffeensis* resulting from differential expression of the 28-kilodalton protein gene family. Infect. Immun. 70:1824-31.

Löhr CV, Rurangirwa FR, McElwain TF, Stiller D, Palmer GH. 2002. Specific expression of *Anaplasma marginale* major surface protein 2 salivary gland variants occurs in the midgut and is an early event during tick transmission. Infect. Immun. 70:114-20.

Luther DG, Cox HU, Nelson WO. 1980. Comparisons of serotests with calf inoculations for detection of carriers in anaplasmosis- vaccinated cattle. Am. J. Vet. Res. 41:2085-6.

Marceau M, Nassif X. 1999. Role of glycosylation at Ser63 in production of soluble pilin in pathogenic *Neisseria*. J. Bacteriol. 181:656-61.

Marceau M, Forest K, Beretti JL, Tainer J, Nassif X. 1998. Consequences of the loss of O-linked glycosylation of meningococcal type IV pilin on piliation and pilus-mediated adhesion. Mol. Microbiol. 27:705-15.

Martinez A, Salinas A, Martinez F, Cantu A, Miller DK. 1999. Serosurvey for selected disease agents in white-tailed deer from Mexico. J. Wildl. Dis. 35:799-803.

Massung RF, Lee K, Mauel M, Gusa A. 2002. Characterization of the rRNA genes of *Ehrlichia chaffeensis* and *Anaplasma phagocytophila*. DNA Cell Biol. 21:587-96.

McBride JW, Yu XJ, Walker DH. 2000. Glycosylation of homologous immunodominant proteins of *Ehrlichia chaffeensis* and *Ehrlichia canis*. Infect. Immun. 68:13-8.

McCallon BR. 1973. Prevalence and economic aspects of anaplasmosis. In: EW Jones (ed.), Proceedings of the 6th National Anaplasmosis Conference. Heritage Press, Stillwater, OK, pp. 1-3.

McGarey DJ, Allred DR. 1994. Characterization of hemagglutinating

components on the *Anaplasma marginale* initial body surface and identification of possible adhesins. Infect. Immun. 62:4587-93.

McGarey DJ, Barbet AF, Palmer GH, McGuire TC, Allred DR. 1994. Putative adhesins of *Anaplasma marginale*: major surface polypeptides 1a and 1b. Infect. Immun. 62:4594-601.

McGuire TC, Palmer GH, Goff WL, Johnson MI, Davis WC. 1984. Common and isolate-restricted antigens of *Anaplasma marginale* detected with monoclonal antibodies. Infect. Immun. 45:697-700.

McGuire TC, Davis WC, Brassfield AL, McElwain TF, Palmer GH. 1991. Identification of *Anaplasma marginale* long-term carrier cattle by detection of serum antibody to isolated MSP-3. J. Clin. Microbiol. 29:788-93.

Mescher MF, Strominger JL, Watson SW. 1974. Protein and carbohydrate composition of the cell envelope of *Halobacterium salinarium*. J. Bacteriol. 120:945-54.

Miller MB, Bassler BL. 2001. Quorum sensing in bacteria. Annu. Rev. Microbiol. 55:165-99.

Molloy JB, Bowles PM, Knowles DP, McElwain TF, Bock RE, Kingston TG, Blight GW, Dalgliesh RJ. 1999. Comparison of a competitive inhibition ELISA and the card agglutination test for detection of antibodies to *Anaplasma marginale* and *Anaplasma centrale* in cattle. Aust. Vet. J. 77:245-9.

Montenegro-James S, James MA, Toro Benitez M, Leon E, Baek BK, Guillen AT. 1991. Efficacy of purified *Anaplasma marginale* initial bodies as a vaccine against anaplasmosis. Parasitol. Res. 77: 93-101.

Munderloh UG, Blouin EF, Kocan KM, Ge NL, Edwards WL, Kurtti TJ. 1996. Establishment of the tick (Acari: Ixodidae)-borne cattle pathogen *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in tick cell culture. J. Med. Entomol. 33:656-64.

Murphy CI, Storey JR, Recchia J, Doros-Richert LA, Gingrich-Baker C, Munroe K, Bakken JS, Coughlin RT, Beltz GA. 1998. Major antigenic proteins of the agent of human granulocytic ehrlichiosis are encoded by members of a multigene family. Infect. Immun. 66:3711-8.

Nari A. 1995. Strategies for the control of one-host ticks and relationship with tick-borne diseases in South America. Vet. Parasitol. 57:153-65.

National Research Council. 1982. Priorities in biotechnology research for international development - Proceedings of a workshop. Directed by the Board on Science and Technique for International Development, Office of International Affairs, National Academy Press, Washington, DC, pp. 1.

Ngeranwa JJ, Venter EH, Penzhorn BL, Soi RK, Mwanzia J, Nyongesa. 1998. Characterization of *Anaplasma* isolates from eland (*Taurotragus oryx*). Pathogenicity in cattle and sheep and DNA profiles analysis. Vet. Parasitol. 74:109-22.

Nielsen K, Smith P, Gall D, de Eshaide ST, Wagner G, Dajer A. 1996. Development and validation of an indirect enzyme

immunoassay for detection of antibody to *Anaplasma marginale* in bovine sera. Vet. Parasitol. 67:133-42.

Oberle SM, Palmer GH, Barbet AF, McGuire TC. 1988. Molecular size variations in an immunoprotective protein complex among isolates of *Anaplasma marginale*. Infect. Immun. 56:1567-73.

Oberle SM, Palmer GH, Barbet AF. 1993. Expression and immune recognition of the conserved MSP4 outer membrane protein of *Anaplasma marginale*. Infect. Immun. 61:5245-51.

Obonyo M, Munderloh UG, Fingerle V, Wilske B, Kurtti T. 1999. *Borrelia burgdorferi* in tick cell culture modulates expression of outer surface proteins A and C in response to temperature. J. Clin. Microbiol. 37:2137-41.

Ohashi N, Rikihisa Y, Unver A. 2001. Analysis of transcriptionally active gene clusters of major outer membrane protein multigene family in *Ehrlichia canis* and *E. chaffeensis*. Infect. Immun. 69:2083-91.

Pal U, de Silva AM, Montgomery RR, Fish D, Anguita J, Anderson JF, Lobet Y, Fikrig E. 2000. Attachment of *Borrelia burgdorferi* within *Ixodes scapularis* mediated by outer surface protein A. J. Clin. Invest. 106:561-9.

Palmer GH. 1989. *Anaplasma* vaccines. In: IG Wright (ed.), Veterinary Protozoan and Hemoparasite Vaccines, CRC Press, Boca Raton, Florida, pp. 1-29.

Palmer GH. 2002. The highest priority: what microbial genomes are telling us about immunity. Vet. Immunol. Immunopathol. 85:1-8.

Palmer GH, McGuire TC. 1984. Immune serum against *Anaplasma marginale* initial bodies neutralizes infectivity for cattle. J. Immunol. 133:1010-5.

Palmer GH, McElwain TF. 1995. Molecular basis for vaccine development against anaplasmosis and babesiosis. Vet. Parasitol. 57:233-53.

Palmer GH, Kocan KM, Barron SJ, Hair JA, Barbet AF, Davis WC, McGuire TC. 1985. Presence of common antigens, including major surface protein epitopes, between the cattle (intraerythrocytic) and tick stages of *Anaplasma marginale*. Infect. Immun. 50:881-6.

Palmer GH, Barbet AF, Kuttler KL, McGuire TC. 1986a. Detection of an *Anaplasma marginale* common surface protein present in all stages of infection. J. Clin. Microbiol. 23:1078-83.

Palmer GH, Barbet AF, Davis WC, McGuire TC. 1986b. Immunization with an isolate-common surface protein protects cattle against anaplasmosis. Science 231:1299-302.

Palmer GH, Waghela SD, Barbet AF, Davis WC, McGuire TC. 1987. Characterization of a neutralization sensitive epitope on the AM 105 surface protein of *Anaplasma marginale*. Int. J. Parasitol. 17:1279-85.

Palmer GH, Oberle SM, Barbet AF, Goff WL, Davis WC, McGuire TC. 1988. Immunization of cattle with a 36-kilodalton surface protein induces protection against homologous and

heterologous *Anaplasma marginale* challenge. Infect. Immun. 56:1526-31.

Palmer GH, Barbet AF, Cantor GH, McGuire TC. 1989. Immunization of cattle with the MSP-1 surface protein complex induces protection against a structurally variant *Anaplasma marginale* isolate. Infect. Immun. 57:3666-9.

Palmer GH, Eid G, Barbet AF, McGuire TC, McElwain TF. 1994. The immunoprotective *Anaplasma marginale* major surface protein 2 is encoded by a polymorphic multigene family. Infect. Immun. 62:3808-16.

Palmer GH, Rurangirwa FR, Kocan KM, Brown WC. 1999. Molecular basis for vaccine development against the ehrlichial pathogen *Anaplasma marginale*. Parasitol. Today 15:281-6.

Palmer GH, Rurangirwa FR, McElwain TF. 2001. Strain composition of the ehrlichia *Anaplasma marginale* within persistently infected cattle, a mammalian reservoir for tick transmission. J. Clin. Microbiol. 39:631-5.

Peregrine AS. 1994. Chemotherapy and delivery systems: haemoparasites. Vet. Parasitol. 54:223-48.

Phillips ZE, Strauch MA. 2002. *Bacillus subtilis* sporulation and stationary phase gene expression. Cell. Mol. Life Sci. 59:392-402.

Piercy PL. 1938. Fifty-first Annual Report, Texas Agricultural Experiment Station, pp. 12.

Piercy PL, Schmidt H. 1941. Fifty-fourth Annual Report, Texas Agricultural Experiment Station, pp. 110-1.

Pipano E. 1995. Live vaccines against hemoparasitic diseases in livestock. Vet. Parasitol. 57:213-31.

Popov VL, Yu X, Walker DH. 2000. The 120 kDa outer membrane protein of *Ehrlichia chaffeensis*: preferential expression on dense-core cells and gene expression in *Escherichia coli* associated with attachment and entry. Microb. Pathog. 28:71-80.

Potgieter FT, van Rensburg L. 1987. The persistence of colostral *Anaplasma* antibodies and incidence of *in utero* transmission of *Anaplasma* infections in calves under laboratory conditions. Onderstepoort J. Vet. Res. 54:557-60.

Potgieter FT, Sutherland B, Biggs HC. 1981. Attempts to transmit *Anaplasma marginale* with *Hippobosca rufipes* and *Stomoxys calcitrans*. Onderstepoort J. Vet. Res. 48:119-22.

Potgieter FT, Kocan KM, McNew RW, Ewing SA. 1983. Demonstration of colonies of *Anaplasma marginale* in the midgut of *Rhipicephalus simus*. Am. J. Vet. Res. 44:2256-61.

Reddy GR, More T, Sharma SP, Singh LN. 1988. The oxidant defense system in water-buffaloes (*Bubalus bubalis*) experimentally infected with *Anaplasma marginale*. Vet. Parasitol. 27:245-9.

Rees CW. 1930. Experimental transmission of anaplasmosis by *Rhipicephalus sanguineus*. North Am. Veterinarian 11:17-20.

Rees CW. 1932. The experimental transmission of anaplasmosis by *Dermacentor variabilis*. Science 75:318-20.

Rees CW. 1933. The experimental transmission of anaplasmosis by *Dermacentor andersoni.* Parasitology 21:569-73.

Rees CW. 1934. Transmission of anaplasmosis by various species of ticks. United States Department of Agriculture, Technical Bulletin 418, Washington, DC.

Rees CW, Avery JL. 1939. Experiments on the hereditary transmission of anaplasmosis by ticks. North Am. Veterinarian 20:35-6.

Renshaw HW, Vaughn HW, Magonigle RA, Davis WC, Stauber EH, Frank FW. 1977. Evaluation of free-roaming mule deer as carriers of anaplasmosis in an area of Idaho where bovine anaplasmosis is enzootic. J. Am. Vet. Med. Assoc. 130:334-9.

Renshaw HW, Magonigle RA, Vaughn HW. 1979. Evaluation of the anaplasmosis rapid card agglutination test for detecting experimentally-infected elk. J. Wildl. Dis. 15:379-86.

Rikihisa Y, Ewing SA, Fox JC. 1994. Western immunoblot analysis of *Ehrlichia chaffeensis*, *E. canis*, or *E. ewingii* infections in dogs and humans. J. Clin. Microbiol. 32:2107-12.

Ristic M. 1977. Bovine anaplasmosis. In: JP Krier (ed.), Parasitic Protozoa IV. Academic Press, New York, pp. 235-349.

Ristic M, Watrach AM. 1963. Anaplasmosis. VI. Studies and a hypothesis concerning the cycle of development of the causative agent. Am. J. Vet. Res. 24:267-76.

Ristic M, Carson CA. 1977. Methods of immunoprophylaxis against bovine anaplasmosis with emphasis on use of the attenuated *Anaplasma marginale* vaccine. In: LH Miller, JA Pino and JJ McKelvey (eds.), Immunity to Blood Parasites of Animals and Man. Plenum Publishing Corp., New York, pp. 151-88.

Roberts RH, Love JN. 1977. Infectivity of *Anaplasma marginale* after ingestion by potential insect vectors. Am. J. Vet. Res. 38:1629-30.

Rosenbusch F, Gonzalez R. 1927. Die Tristeza Uebertragung durch Zecken und dessen Immunitatsprobleme. Arch. f. Protistenk. 58:300-20.

Rozeboom LE, Stiles GW, Moe LH. 1940. Anaplasmosis transmission by *Dermacentor andersoni* Stiles. J. Parasitol. 26:95-100.

Rurangirwa FR, Stiller D, French DM, Palmer GH. 1999. Restriction of major surface protein 2 (MSP2) variants during tick transmission of the ehrlichia *Anaplasma marginale.* Proc. Natl. Acad. Sci. U. S. A. 96:3171-6.

Rurangirwa FR, Brayton KA, McGuire TC, Knowles DP, Palmer GH. 2002. Conservation of the unique rickettsial rRNA gene arrangement in *Anaplasma.* Int. J. Syst. Evol. Microbiol. 52:1405-9.

Saliki JT, Blouin EF, Rodgers SJ, Kocan KM. 1998. Use of tick cell culture-derived *Anaplasma marginale* antigen in a competitive ELISA for serodiagnosis of anaplasmosis. Ann. N. Y. Acad. Sci. 849:273-81.

Sambri V, Stefanelli C, Cevenini R. 1992. Detection of glycoproteins in *Borrelia burgdorferi*. Arch. Microbiol. 157:205-8.

Sambri V, Massaria F, Ardizzoni M, Stefanelli C, Cevenini R. 1993. Glycoprotein patterns in *Borrelia* spp. Zentralbl Bakteriol. 279:330-5.

Sanborn CE, Moe LH. 1934. Anaplasmosis investigations. In: Report of the Oklahoma Agricultural and Mining College and Agricultural Experiment Station, 1932-1934, pp. 275-9.

Sanborn CE, Stiles GW, Moe LH. 1938. Anaplasmosis transmission by naturally infected *Dermacentor andersoni* male and female ticks. North Am. Veterinarian 19:31-2.

Sanders DA. 1933. Notes on the experimental transmission of bovine anaplasmosis in Florida. J. Am. Vet. Med. Assoc. 88:799-805.

Schmidt H, Piercy PL. 1937. Fiftieth Annual Report of the Texas Agricultural Experiment Station, pp. 12-4.

Schreuder BE, Uilenberg G, Tondeur W. 1977. Studies on Theileriidae (Sporozoa) in Tanzania. VIII. Experiments with African buffalo (*Syncerus caffer*). Tropenmed. Parasitol. 28:367-71.

Schwan TG, Piesman J. 2000. Temporal changes in outer surface proteins A and C of the Lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice. J. Clin. Microbiol. 38:382-8.

Senitzer D, Dimopoullos GT, Brinkley BR, Mandel M. 1972. Deoxyribonucleic acid of *Anaplasma marginale*. J. Bacteriol. 109:434-6.

Sergent E, Dontien A, Parrot L, Lestoquard F. (In Memoriam). 1945. Etudes sur les piroplasmoses bovines. Institut Pasteur d'Algerie, pp. 816.

Shkap V, Pipano E, McGuire TC, Palmer GH. 1991. Identification of immunodominant polypeptides common between *Anaplasma centrale* and *Anaplasma marginale*. Vet. Immunol. Immunopathol. 29:31-40.

Siegmund OH. 1979. Anaplasmosis. In: OH Siegmund (ed.) Merck Veterinary Manual, Merck & Co. Inc., New Jersey.

Skerman VBD, McGowan V, Sneath PHA. 1980. Approved lists of bacterial names. Int. J. Syst. Bacteriol. 30:225-420.

Sleator RD, Hill C. 2002. Bacterial osmoadaptation: the role of osmolytes in bacterial stress and virulence. FEMS Microbiol. Rev. 26:49-71.

Smith RD, Woolf A, Hungerford LL, Sundberg JP. 1982. Serologic evidence of *Anaplasma marginale* infection in Illinois white-tailed deer. J. Am. Vet. Med. Assoc. 181:1254-6.

Stauber EH, Autenrieth R, Markham OD, Whitbeck V. 1980. A seroepidemiologic survey of three pronghorn (*Antilocapra americana*) populations in southeastern Idaho, 1975-1977. J. Wildl. Dis. 16:109-15.

Stich RW, Kocan KM, Palmer GH, Ewing SA, Hair JA, Barron SJ. 1989. Transstadial and attempted transovarial transmission of *Anaplasma marginale* by

Dermacentor variabilis. Am. J. Vet. Res. 50:1377-80.

Stich RW, Bantle JA, Kocan KM, Fekete A. 1993. Detection of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in hemolymph of *Dermacentor andersoni* (Acari: Ixodidae) with the polymerase chain reaction. J. Med. Entomol. 30:781-8.

Stiller D, Coan ME. 1995. Recent developments in elucidating tick vector relationships for anaplasmosis and equine piroplasmosis. Vet. Parasitol. 57:97-108.

Stiller D, Leatch G, Kuttler K. 1981. Experimental transmission of bovine anaplasmosis by the winter tick, *Dermacentor albipictus* (Packard). In: Proceedings of the National Anaplasmosis Conference, Mississippi State, Mississippi, pp. 463-75.

Storey JR, Doros-Richert LA, Gingrich-Baker C, Munroe K, Mather TN, Coughlin RT, Beltz GA, Murphy CI. 1998. Molecular cloning and sequencing of three granulocytic *Ehrlichia* genes encoding high-molecular-weight immunoreactive proteins. Infect. Immun. 66:1356-63.

Swanson AF, Kuo CC. 1991. Evidence that the major outer membrane protein of *Chlamydia trachomatis* is glycosylated. Infect. Immun. 59:2120-5.

Szymanski CM, Burr DH, Guerry P. 2002. *Campylobacter* protein glycosylation affects host cell interactions. Infect. Immun. 70:2242-4.

Taylor SK, Lane VM, Hunter DL, Eyre KG, Kaufman S, Frye S, Johnson MR. 1997. Serologic survey for infectious pathogens in free-ranging American bison. J. Wildl. Dis. 33:308-11.

Tebele N, McGuire TC, Palmer GH. 1991. Induction of protective immunity by using *Anaplasma marginale* initial body membranes. Infect. Immun. 59:3199-204.

Theiler A. 1910. *Anaplasma marginale* (gen. and spec. nov.). The marginal points in the blood of cattle suffering from a specific disease. In: Report to the Government, Transvaal, South Africa. Veterinary Bacteriology, Department of Agriculture (1908-9,1910) pp. 7-64.

Theiler A. 1912a. Gallsickness of imported cattle and the protective inoculation against this disease. Agric. J. Union S. Africa 3:1-2.

Theiler A. 1912b. Uebertragung der anaplasmosis mittels Zecken. Ztschr f Infektionskr 12:105-16.

Thibault P, Logan SM, Kelly JF, Brisson JR, Ewing CP, Trust TJ, Guerry P. 2001. Identification of the carbohydrate moieties and glycosylation motifs in *Campylobacter jejuni* flagellin. J. Biol. Chem. 276:34862-70.

Torioni De Echaide S, Knowles DP, McGuire TC, Palmer GH, Suarez CE, McElwain TF. 1998. Detection of cattle naturally infected with *Anaplasma marginale* in a region of endemicity by nested PCR and a competitive enzyme-linked immunosorbent assay using recombinant major surface protein 5. J. Clin. Microbiol. 36:777-82.

Trueblood ES, McGuire TC, Palmer GH. 1991. Detection of *Anaplasma marginale*

rickettsemia prior to onset of clinical signs by using an antigen capture enzyme-linked immunosorbent assay. J. Clin. Microbiol. 29:1542-4.

Turton JA, Katsande TC, Matingo MB, Jorgensen WK, Ushewokunze-Obatolu U, Dalgliesh RJ. 1998. Observations on the use of *Anaplasma centrale* for immunization of cattle against anaplasmosis in Zimbabwe. Onderstepoort J. Vet. Res. 65:81-6.

Unver A, Ohashi N, Tajima T, Stich RW, Grover D, Rikihisa Y. 2001. Transcriptional analysis of *p30* major outer membrane multigene family of *Ehrlichia canis* in dogs, ticks, and cell culture at different temperatures. Infect. Immun. 69:6172-8.

Unver A, Rikihisa Y, Stich RW, Ohashi N, Felek S. 2002. The *omp-1* major outer membrane multigene family of *Ehrlichia chaffeensis* is differentially expressed in canine and tick hosts. Infect. Immun. 70:4701-4.

Vaz Junior Ida S, Martinez RH, Oliveira A, Heck A, Logullo C, Gonzales JC, Dewes H, Masuda A. 1996. Functional bovine immunoglobulins in *Boophilus microplus* hemolymph. Vet. Parasitol. 62:155-60.

Vidotto MC, McGuire TC, McElwain TF, Palmer GH, Knowles DP Jr. 1994. Intermolecular relationships of major surface proteins of *Anaplasma marginale*. Infect. Immun. 62:2940-6.

Viseshakul N, Kamper S, Bowie MV, Barbet AF. 2000. Sequence and expression analysis of a surface antigen gene family of the rickettsia *Anaplasma marginale*. Gene 253:45-53.

Visser ES, McGuire TC, Palmer GH, Davis WC, Shkap V, Pipano E, Knowles DP Jr. 1992. The *Anaplasma marginale msp5* gene encodes a 19-kilodalton protein conserved in all recognized *Anaplasma* species. Infect. Immun. 60:5139-44.

Vizcaino O, Carson CA, Lee AJ, Ristic M. 1978. Efficacy of attenuated *Anaplasma marginale* vaccine under laboratory and field conditions in Colombia. Am. J. Vet. Res. 39:229-33.

Wickwire KB, Kocan KM, Barron SJ, Ewing SA, Smith RD, Hair JA. 1987. Infectivity of three isolates of *Anaplasma marginale* for *Dermacentor andersoni*. Am. J. Vet. Res. 48:96-9.

Wilson AJ, Trueman KF, Spinks G, McSorley AF. 1978. A comparison of 4 serological tests in the detection of humoral antibodies to anaplasmosis in cattle. Aust. Vet. J. 54:383-6.

Wilson AJ, Parker R, Trueman KF. 1980. Susceptibility of *Bos indicus* crossbred and *Bos taurus* cattle to *Anaplasma marginale* infection. Trop. Anim. Health Prod. 12:90-4.

World Organization for Animal Health. 2000. Manual of standards for diagnostic tests and vaccines. World Organization for Animal Health, Paris, France.

Yago T, Leppanen A, Carlyon JA, Akkoyunlu M, Karmakar S, Fikrig E, Cummings RD, McEver RP. 2003. Structurally distinct requirements for binding of P-selectin glycoprotein ligand-1 and sialyl Lewis x to *Anaplasma phagocytophilum* and P-selectin. J. Biol. Chem. 278:37987-97.

Young NM, Brisson JR, Kelly J, Watson DC, Tessier L, Lanthier PH, Jarrell HC, Cadotte N, St Michael F, Aberg E, Szymanski CM. 2002. Structure of the *N*-linked glycan present on multiple glycoproteins in the gram-negative bacterium, *Campylobacter jejuni*. J. Biol. Chem. 277:42530-9.

Yu XJ, Crocquet-Valdes P, Walker DH. 1997. Cloning and sequencing of the gene for a 120-kDa immunodominant protein of *Ehrlichia chaffeensis*. Gene 184:149-54.

Zaugg JL, Kuttler KL. 1985. *Anaplasma marginale* infections in American bison: experimental infection and serologic study. Am. J. Vet. Res. 46:438-41.

Zaugg JL, Goff WL, Foreyt W, Hunter DL. 1996. Susceptibility of elk (*Cervus elaphus*) to experimental infection with *Anaplasma marginale* and *A. ovis*. Am. J. Wildl. Dis. 32:62-6.

Zeller H, Helm R. 1923. Versuche zur Frage der Uebertragbarkeit des Texasfiebers auf Deutrsche Rinder durch die bei uns vorkommenden Zecken *Ixodes ricinus* und *Haemaphysalis punctata* Cinabarina. Berl tieraztl Wchnschr 39:1-4.

Zhi N, Ohashi N, Tajima T, Mott J, Stich RW, Grover D, Telford SR 3rd, Lin Q, Rikihisa Y. 2002. Transcript heterogeneity of the *p44* multigene family in a human granulocytic ehrlichiosis agent transmitted by ticks. Infect. Immun. 70:1175-84.

RESEARCH PROBLEM

The research proposed herein focuses on the characterization of the antigenic determinants, expression and glycosylation of the *A. marginale* MSP1a. MSP1a has been shown to be an important surface protein because it is an adhesin for both bovine erythrocytes and tick cells. MSP1a also contains a neutralization-sensitive epitope and is involved in immune protection against *A. marginale* infection. Preliminary data that led to this research was the discovery that the antibody response of cattle immunized with *A. marginale* derived from bovine erythrocytes or tick cell culture differed. Cattle immunized with erythrocyte derived antigen had a preferential antibody response to MSP1a, while cattle immunized with tick cell culture-derived antigen developed a preferential antibody response to MSP1b. We also confirmed that the observed molecular weight of MSP1a was greater than the predicted molecular weight which led to the hypothesis that this difference may be due to the glycosylation of this protein. Both the regulation of the expression and the post-translational modifications of surface proteins may influence the ability of intracellular rickettsia to adhere to and infect both vertebrate and ticks cells during the parasite life cycle. In this research, we hypothesize that the regulation of the expression of MSP1a by *A. marginale* differs in bovine erythrocytes and tick cells and this differential expression influences the antibody response of cattle immunized with erythrocyte or tick cell-derived *A. marginale.* In addition, we hypothesize that immunized cattle develop an antibody response against B-cell epitopes of MSP1a and that this antibody response is involved in protection against *A. marginale* infection. We further

hypothesize that MSP1a is glycosylated and that the glycosylation may influence the adhesive properties of the protein.

The specific objectives of the research proposed herein are:

1. To characterize the expression of MSP1a on *A. marginale* derived from bovine erythrocytes and tick cells; and
2. To characterize the antibody response against MSP1a in cattle immunized with recombinant MSP1a protein or *A. marginale* derived from bovine erythrocytes and tick cells;
3. To determine whether the *A. marginale* MSP1a is glycosylated and, if so, whether glycosylation influences the adhesive properties of the protein.

Chapter 2

DIFFERENTIAL EXPRESSION OF THE *msp1a* GENE OF *ANAPLASMA MARGINALE* OCCURS IN BOVINE ERYTHROCYTES AND TICK CELLS

Garcia-Garcia JC, de la Fuente J, Blouin EF, Johnson TJ, Halbur T, Onet VC, Saliki JT, Kocan KM. *Veterinary Microbiology*, In press.

Abstract

Major surface proteins (MSP) 1a and 1b of the tick-borne pathogen *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) are conserved on *A. marginale* derived from bovine erythrocytes and tick cells. MSP1a and MSP1b form the MSP1 complex and are adhesins involved in infection of host cells. While both MSP1a and MSP1b are adhesins for bovine erythrocytes, only MSP1a is an adhesin for cultured and native tick cells. These studies were initiated because antibody responses to MSP1a and MSP1b differed in cattle immunized with killed *A. marginale* derived from bovine erythrocytes or cultured tick cells. A strong antibody response to MSP1a was observed in cattle immunized with erythrocyte-derived *A. marginale*, whereas cattle immunized with tick cell culture-derived *A. marginale* produced antibodies preferentially to MSP1b. The molecular basis of this differential antibody response was then studied using Western blot, confocal microscopy and reverse transcriptase (RT)-PCR. Whereas expression of MSP1b by *A. marginale* derived from both bovine and tick host cells was similar at the protein and RNA levels, expression of MSP1a by *A. marginale* in these cells differed. Low levels of MSP1a were observed in cultured tick cells and tick salivary glands, but high expression of MSP1a occurred on *A. marginale* derived from bovine erythrocytes. The analysis of the expression of the *msp1a* gene by RT-PCR suggests that the differential expression of

MSP1a is regulated at the transcriptional level and may influence the infectivity of *A. marginale* for host cells. Variation in the expression of MSP1a may also contribute to phenotypic and antigenic changes in the pathogen.

Introduction

The expression of surface proteins by rickettsial pathogens has been shown to vary with environmental conditions or the type of host cells. Selected surface proteins have been found to be involved in host cell invasion and in the generation of antigenic variants that contribute to the establishment of persistent infection (Unver *et al.*, 2001; 2002; Bekker *et al.*, 2002; IJdo *et al.*, 2002; Löhr *et al.*, 2002b).

Anaplasma marginale (Rickettsiales: Anaplasmataceae), the tick-borne pathogen that causes bovine anaplasmosis, replicates in bovine erythrocytes and tick cells. Major surface proteins (MSP) 1a and 1b of *A. marginale*, conserved upon growth on both bovine erythrocytes and tick cells (Palmer *et al.*, 1985; Barbet *et al.*, 1999; Blouin *et al.*, 2000), have been shown to be involved in host cell infection (McGarey and Allred, 1994; McGarey *et al.*, 1994; de la Fuente *et al.*, 2001a). Both MSP1a and MSP1b are adhesins for bovine erythrocytes, while only MSP1a is an adhesin for cultured and native tick cells (Palmer and McGuire, 1984; McGarey *et al.*, 1994; de la Fuente *et al.*, 2001a). Therefore, expression of these proteins may differ during the parasite life cycle as the pathogen adapts to bovine and tick environments. Recently, differential expression of *A. marginale* proteins associated with the *msp2* operon was reported. The regulation of expression appears to be post-transcriptional (Löhr *et al.*, 2002a), whereas the expression of specific *A. marginale* MSP2 variants was shown to be due to genetic recombination events (Brayton *et al*,. 2002). Differential regulation of the expression of *A. marginale* surface molecules encoded by a single copy gene such as *msp1a* has not been reported.

These studies were initiated because antibody responses to MSP1a and MSP1b differed in cattle immunized with killed *A. marginale* derived from bovine erythrocytes or cultured tick cells. Cattle immunized with erythrocyte-derived *A. marginale* elicited an antibody response primarily against MSP1a, but cattle immunized with tick cell culture-derived antigen produced antibodies preferentially to MSP1b (Kocan *et al.*, 2001; de la Fuente *et al.*, 2002a). The molecular basis of the differential immune response of cattle to *A. marginale* derived from bovine and tick cells was characterized by use of Western blot, confocal microscopy and reverse transcriptase (RT)-PCR. Definition of the expression pattern of major surface proteins involved in adhesion of host cells is important for understanding the mechanism of infection of *A. marginale* for bovine and tick cells and may influence development of more effective vaccine strategies for control of bovine anaplasmosis.

Experimental procedures

Anaplasma marginale isolates.

Oklahoma and Virginia isolates of *A. marginale* were used for these studies. These isolates have been shown to be transmissible by *Dermacentor andersoni* and *D. variabilis*, and have been propagated in tick cell culture by our laboratory (Munderloh *et al.*, 1996; Blouin *et al.*, 2000; de la Fuente *et al.*, 2001b; 2002b).

Infection of cattle with A. marginale and preparation of antigen from bovine erythrocytes.

Three splenectomized calves (3-4-month-old mixed breed) were experimentally infected with the Oklahoma or Virginia isolates of *A. marginale*. Calf PA479 was infected with blood stabilate (Oklahoma isolate) from PA407 (Blouin *et al.*, 2000) with a percent of parasitized erythrocytes (PPE) of 10% and calf PA408 was inoculated with Oklahoma isolate infected cultured tick cells (Blouin *et al.*, 2000). The Virginia isolate *A. marginale* was transmitted to calf PA433 by *D. variabilis* males that were acquisition-fed on PA432 (de la Fuente *et al.*, 2002b). The calves were maintained by the OSU Laboratory Animal Resources according to the Institutional Care and Use of Animal Committee guidelines. Infection was monitored by examination of stained blood smears and determination of the packed cell volume (PCV). Blood was collected from the calves at the peak parasitemia as follows: (i) calf PA479, PCV=18%, PPE=82%; (ii) calf PA408, PCV=12%, PPE=34%; and (iii) calf PA433, PCV=28.5%, PPE=12.2%. Blood samples were collected from calves PA479 and PA408 and total RNA was extracted for RT-PCR analysis of gene expression. Infected blood was collected for protein expression studies and preparation of antigen for cattle immunization studies, and the erythrocytes were washed three times in PBS, each time removing the buffy

coat, and stored at -70°C. Oklahoma isolate erythrocyte antigen from PA479 was thawed, quantified by use of an MSP5 antigen detection ELISA (Saliki *et al.*, 1998), inactivated with β-propiolactone (BPL), and doses of approximately 2×10^{10} *A. marginale* were prepared.

Propagation of A. marginale in tick cell culture and antigen preparation.

A. marginale was propagated in the tick cell line IDE8 (ATCC CRL 11973), derived from *Ixodes scapularis* embryos, as described previously (Munderloh *et al.*, 1996; Blouin *et al.*, 2000). Briefly, tick cells were maintained at 31°C in L-15 B medium, pH 7.2, supplemented with 5% heat inactivated fetal bovine serum (Sigma, USA), 10% tryptose phosphate broth (Difco, USA) and 0.1% lipoprotein concentrate (ICN, USA), and the culture medium was replaced weekly.

Monolayers of IDE8 cells were inoculated with the Oklahoma or Virginia isolate of *A. marginale* and monitored by stained smears and with phase contrast microscopy. Terminal cell cultures, in which approximately 100% of the cells were infected, were harvested by centrifugation. Samples of cultured tick cells were analyzed by RT-PCR, immunoblotting and confocal microscopy as described below. Cultured cells infected with Oklahoma isolate of *A. marginale* to be used for the cattle immunization studies were resuspended in PBS and stored at -70°C until used for antigen preparation. The antigen was quantified by use of an MSP5-specific antigen detection ELISA (Saliki *et al.*, 1998). Antigen doses were prepared that contained approximately 2×10^{10} *A. marginale* and were then inactivated with BPL.

Infection of ticks and collection of salivary glands.

Dermacentor variabilis and *D. andersoni* were obtained from the Oklahoma State University, Centralized Tick Rearing Facility. Larvae and nymphs were fed on rabbits and

sheep, respectively, and were then allowed to molt to the subsequent stage. Adult males were held in a humidity chamber (90-95% RH) at 25°C with a 14-hr photoperiod until used for these studies. Uninfected males were allowed to acquire infection (acquisition feeding) with the Oklahoma isolate by feeding for seven days on the infected calf PA479 during ascending parasitemia, after which the ticks were removed and held in humidity chamber at room temperature for seven days. The ticks were then allowed to transmission feed on a sheep for seven days to allow for infection of the salivary glands, after which they were removed, the salivary glands dissected and used for analysis of the expression of the *A. marginale* MSPs. Salivary glands from 10-20 ticks were pooled in 500 µl of RNALater (Ambion, USA) and processed as described below. Another group of infected salivary glands was collected and embedded in paraffin for confocal microscopy studies. Groups of uninfected ticks were allowed to feed in a manner similar to the infected ticks and the salivary glands were dissected and used as uninfected controls for the confocal microscopy studies.

Expression of recombinant MSP1a, MSP1b and MSP5, purification and antigen preparation.

The genes *msp1α*, *msp1β*$_1$ and *msp5* of the Oklahoma isolate of *A. marginale*, encoding for MSP1a, MSP1b$_1$ and MSP5, respectively, were cloned and expressed in *E. coli* as reported previously (de la Fuente *et al.*, 2001a). Expression of the recombinant proteins was confirmed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970).

The recombinant proteins expressed in *E. coli* were purified by FLAG-affinity chromatography (Sigma, USA) following the manufacturer's instructions. Doses of tick cell

culture-derived *A. marginale* were supplemented with 100 μg of purified recombinant MSP1a protein and used to vaccinate cattle.

Cattle immunization studies and serum collection.

Twenty, intact one-year-old Angus cattle, found to be seronegative for *A. marginale* by use of an *A. marginale* competitive ELISA (Saliki *et al.*, 1998), were randomly assigned into four groups of 5 animals each (Table 1). Animals were immunized by subcutaneous injection at weeks 1, 5 and 8 with a 5 ml dose containing test antigen in an oil based adjuvant (XTEND SP®, Novartis Animal Vaccines Inc., USA). Serum was collected from each animal at weeks 1, 5, 8, 10 and 12, and sera were stored at -70°C until assayed by ELISA and Western blotting.

Serologic evaluation of immunized cattle.

The levels of antibodies against MSP5, a surface protein that is conserved in the tick and erythrocytic stages of *A. marginale* and that was used for normalizing the amount of *A. marginale* antigen in the vaccine preparations, were measured using a competitive ELISA (Saliki *et al.*, 1998). Antibody levels to *A. marginale* MSP1a and MSP1b were detected by ELISA developed for these studies. Briefly, purified recombinant MSP1a and MSP1b were used to coat ELISA plates for 3 hours at 37°C, after which the plates were washed with TBST (0.05% Tween-20 in TBS) and blocked with 2% skim milk overnight at 4°C. Sera were serially diluted 1:2 from a 1:100 initial dilution. The plates were incubated with the diluted sera for 2 hours at 37°C, washed three times with TBST and then incubated with goat anti-bovine IgG-HRP conjugate (KPL, USA) diluted 1:2000 in TBS. Plates were washed again and were then developed with TMB (Sigma, USA) for 15 minutes and finally stopped with 25 μl of 2N H_2SO_4. The OD_{450nm} was determined in an ELISA reader. Antibody titers were expressed as

the maximum dilution of the serum that yielded an OD value at least twice as high as the negative control serum. Geometric mean titers were calculated for each experimental group. The antibody levels against MSP1a and MSP1b in each immunization group were compared using a paired Student's t-test.

Immunoblotting.

The antibody responses against MSP1a and MSP1b were analyzed by Western blot. Fifty μg of purified recombinant MSP1a and MSP1b proteins were solubilized in sample loading buffer (2% SDS, 1% β-mercaptoethanol, 10% glycerol, 0.5% bromophenol blue, 0.0625 M Tris, pH 6.8) and denatured for 3 min at 100°C. The protein samples were loaded in an 8% SDS-PAGE gel (Laemmli, 1970), using a preparative comb. The proteins on the gel were transferred to a nitrocellulose membrane for 60 min in a semi-dry transfer apparatus (Hoefer Scientific, USA). The membrane was blocked with 5% skim milk for 1 hr at room temperature. Sera from immunized animals were diluted 1:200 in TBS. Serum from a seronegative animal 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-bovine IgG alkaline phosphatase conjugate (KPL, USA) diluted 1:10,000. The membrane was washed again and color was developed using Sigma Fast BCIP/NBT alkaline phosphatase substrate (Sigma, USA). Finally, the membrane was examined for serum recognition of the MSP1a and MSP1b protein bands.

Expression of major surface proteins by *A. marginale* derived from infected cultured tick cells or bovine erythrocytes collected from calves PA479 and PA433 was also analyzed by

Western blot. The rickettsial protein concentration was adjusted for MSP5 that is encoded by a single copy gene highly conserved among *A. marginale* isolates (Visser *et al.*, 1992; Knowles *et al.*, 1996) in *A. marginale* derived from bovine erythrocytes, cultured tick cells and tick salivary glands (Barbet *et al.*, 1999). The protein samples were dissolved in sample buffer, separated by SDS-PAGE and transferred to a nitrocellulose membrane as described above. The membrane was then probed with a 10 μg/ml solution of anti-MSP1a monoclonal antibody (MAb ANA15D2, VMRD, USA), anti-MSP5 monoclonal antibody (MAb ANAF16C1, VMRD, USA) or MSP1b monospecific rabbit antiserum diluted 1:200. The membrane was washed and incubated with goat anti-mouse or anti-rabbit IgG alkaline phosphatase conjugate (KPL, USA) diluted 1:10,000. The membrane was washed again and color was developed using Sigma Fast BCIP/NBT alkaline phosphatase substrate (Sigma, USA). The relative amounts of MSP1a and MSP1b present in the *A. marginale* samples derived from both host cells were determined by densitometry and compared after normalizing for rickettsial protein content using MSP5.

Confocal microscopy.

Paraffin cross-sections of infected and uninfected salivary glands were used to study the expression of *A. marginale* MSPs in the different host cells. Salivary glands dissected from ticks fed on calf PA479 and from uninfected ticks were fixed with 4% paraformaldehyde in 0.2 M sodium cacodylate buffer, dehydrated in a graded series of ethanol and embedded in paraffin. Sections (4 μm) were cut and mounted on glass slides. The paraffin was removed from the sections with xylene and the sections were hydrated by successive 2 min washes with ethanol 100, 95, 80, 75 and 50%. Salivary gland sections were blocked for 1 hr with 1:100 mouse preimmune serum in TBS. The slides were then incubated for 6 hrs with anti-MSP1a and anti-MSP5 monoclonal antibodies labeled with Alexa Fluor 546 and Alexa Fluor 633

(Molecular Probes, USA), respectively. The slides were washed twice with TBS and mounted in ProLong Antifade reagent (Molecular Probes, USA). The sections were examined simultaneously for colonies of *A. marginale* labeled with both fluorochromes using a Leica SP2 laser scanning confocal microscope (Leica, USA). Sections of salivary glands from uninfected ticks were used as a control.

RNA extraction and RT-PCR.

Total RNA was extracted from *A. marginale*-infected bovine erythrocytes, cultured tick cells and salivary glands. One ml blood containing 1-3 $\times$ 10^9 infected bovine erythrocytes obtained from calves PA479 and PA408, approximately 10^{10} rickettsia from infected tick IDE8 cells and pools of tick salivary glands from 10-20 transmission-fed *D. andersoni* and *D. variabilis* were used for these studies. RNA was extracted from the infected cells using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. The final RNA pellet was resuspended in DNase I buffer (Gibco BRL; 20 mM Tris-HCl, 2 mM $MgCl_2$, 50 mM KCl, pH 8.4) and treated with 6 U of RNAse-free DNAse I (Gibco BRL, USA) per 6 μg RNA in a 60 μl reaction volume at room temperature for 15 min. After adding 6 μl of 25 mM EDTA the reaction was incubated at 65°C for 10 min. DNase I was removed from the RNA samples using the RNeasy mini kit for RNA stabilization and isolation (Qiagen, USA) according to the manufacturer's instructions. Half of the total RNA eluted from the column with diethyl pyrocarbonate-treated distilled deionized sterile water was heated at 70°C for 10 min and reverse transcribed in a 20 μl reaction mixture 1.5 mM $MgSO_4$, 1$\times$ avian myeloblastosis virus (AMV) RT/*Thermus flavus* (Tfl) reaction buffer (Promega, USA), 10 mM random hexamer, 0.5 mM each deoxynucleoside triphosphate (dNTP), 30 U RNAse inhibitor (Promega, USA), 5 U AMV RT (Promega, USA) at 48°C for 45 min. PCR was performed separately for each gene in

a 50 μl reaction mixture including 1 μl of the cDNA product, 10 pmol of each primer (Table 2), 1.5 mM $MgSO_4$, 0.2 mM dNTP, 1× AMV RT/Tfl reaction buffer, 5 u Tfl DNA polymerase, employing the Access RT-PCR system (Promega, USA). PCR conditions and primers used for the amplification of each cDNA are listed in Table 2. Control reactions were performed with the other half of the total RNA eluted from the column using the same procedures but without RT to rule out DNA contamination in the RNA preparations. Reactions without cDNA were also included to control contaminations of the PCR reaction. Positive control reactions for the PCR were performed with DNA from bovine erythrocytes infected with the Oklahoma isolate of *A. marginale*. To normalize rickettsial RNA in the samples, a PCR was performed with primers specific for *A. marginale* 16S rRNA and *msp4*, which have been used previously to quantify levels of *A. marginale* infection (de la Fuente *et al.*, 2001b).

Results

Immunization of cattle and immune response to A. marginale MSP1a, MSP1b and MSP5.

To study the anti-MSP antibody response of cattle immunized with erythrocyte- or tick cell culture-derived *A. marginale,* the levels of antibodies against MSP5, MSP1a and MSP1b were determined by ELISA. Antibody titers to MSP5, the protein used to normalize the amount of *A. marginale* antigen in the vaccine preparations, were similar in cattle immunized with erythrocyte- or tick cell culture-derived *A. marginale* (Fig. 1A). The level of MSP5-specific antibodies peaked approximately two weeks after the last immunization. Sera from control cattle that received adjuvant alone were negative for antibodies to MSP5 (Fig. 1A). The antibody response against MSP1a and MSP1b differed in cattle immunized with erythrocyte and tick cell culture-derived *A. marginale* antigen (Fig. 1B). Cattle immunized with *A. marginale* derived from bovine erythrocytes had a preferential response to MSP1a, whereas cattle immunized with tick cell culture *A. marginale* developed antibodies primarily to MSP1b. Cattle immunized with tick cell culture-derived *A. marginale* supplemented with recombinant MSP1a developed an antibody response against both MSP1a and MSP1b, with a response against MSP1a similar to the response obtained in the group immunized with erythrocyte-derived antigen (Fig. 1B).

Analysis of expression of MSPs in infected erythrocytes, cultured tick cells and salivary glands.

The Oklahoma isolate *A. marginale* derived from bovine erythrocytes and tick cells was analyzed by Western blot specific for MSP5, MSP1a and MSP1b (Fig. 2). The amounts of rickettsial proteins were normalized using MSP5 because this protein is encoded by a single copy gene and is conserved among different isolates and life stages of *A. marginale.* The

amount of MSP1b detected in the infected erythrocytes was similar to the amount of MSP1b detected in *A. marginale* derived from tick cells (Fig. 2, lanes 5 and 6), but detection of MSP1a was notably lower in *A. marginale* harvested from infected tick cells (Fig. 2, lanes 3 and 4). After quantification of the Western blot signals by densitometry scan of membranes, the erythrocyte-to-tick cell culture *A. marginale* protein ratio was equal to 69 and 2 for MSP1a and MSP1b, respectively. Multiple bands for MSP1a that result from proteolysis or internal translation start sites were detected in the Western blot for both erythrocyte (Fig. 2, lane 3) and cultured tick cell-derived antigens when higher protein amounts were loaded onto the gel (data not shown). A similar pattern of expression was observed using a second *A. marginale* isolate from Virginia (data not shown). The staining of cross-sections of *A. marginale*-infected salivary glands with MSP1a or MSP5 monoclonal antibodies labeled separately with different Alexafluor fluorescent dyes confirmed the low expression of MSP1a on *A. marginale* in tick salivary glands (Fig. 3). Although MSP5 expression could be readily detected on *A. marginale* within colonies in tick salivary gland cells (Fig. 3B), expression of MSP1a could not be detected in the same infected cells (Fig. 3A). MSP5 was not detected in uninfected salivary gland sections (Fig. 3C) processed at the same time to serve as negative controls.

Transcription of msp1α in infected erythrocytes and cultured tick cells.

The transcriptional levels of *msp1a*, *msp1β*, *msp4* and *msp5* in erythrocytes (calves PA408 and PA479) and cultured tick cells infected with *A. marginale* (Oklahoma isolate) were compared using RT-PCR. Similar amounts of 16S rRNA and *msp4* transcripts were detected in the RNA samples of infected erythrocytes and tick cells (Fig. 4, lanes 4, 6), indicating that the number of *A. marginale* organisms analyzed were similar in both samples. Transcripts for *msp5*, *msp1β*$_1$ and *msp1β* were also detected in both RNA samples (Fig. 4, lanes 2, 3, 5). However, the

amount of *msp1a* transcripts in infected tick cells was notably lower than that detected in infected erythrocytes (Fig. 4, lane 1A, B). Amplification products were not detected in the negative controls, in which RT was not added to the RT-PCR reactions (Fig. 4, lanes 7, 8), confirming the absence of DNA contamination. The transcriptional analysis of *msp1a* expression with the RNA extracted from the infected erythrocytes of calves PA408 and PA479 produced similar results.

Expression of A. marginale msp1α in infected tick salivary glands.

A. marginale infection levels detected in the RNA samples from *D. variabilis* and *D. andersoni* salivary glands were similar, as indicated by the amount of 16S rRNA and *msp4* transcripts (Fig. 5, lanes 5, 6). All the control reactions without RT were negative, demonstrating that contamination with *A. marginale* DNA did not occur in the RNA samples (data not shown). *A. marginale msp1a* gene expression in the salivary glands of *D. variabilis* or *D. andersoni* infected male adult ticks was not detectable (Fig. 5, lanes 1, 2). However, *msp1β* transcripts were detected in both tick species (Fig. 5, lanes 3, 4).

Discussion

Infection of *A. marginale* for host cells is mediated by adhesion of surface proteins to the host cell membrane (McGarey and Allred, 1994; McGarey *et al.*, 1994; de la Fuente *et al.*, 2001a), followed by endocytosis and internalization of the rickettsia within a parasitophorous vacuole into host cell cytoplasm (Blouin and Kocan, 1998). The MSP1 complex, formed by the MSP1a and MSP1b proteins, has been shown to be involved in the interactions between *A. marginale* and bovine erythrocytes and tick cells (McGarey and Allred, 1994; McGarey *et al.*, 1994; de la Fuente *et al.*, 2001a; 2003). These proteins were found to be conserved on the erythrocytic and tick stages of *A. marginale* (Palmer *et al.*, 1985; Barbet *et al.*, 1999; Blouin *et al.*, 2000).

In this study, we demonstrated that cattle immunized with *A. marginale* derived from bovine erythrocytes or tick cell culture develop a differential immune response to MSP1a and MSP1b, and these results were similar to two previous vaccination trials in which different breeds of cattle and immunization routes were used (Kocan *et al.*, 2001; de la Fuente *et al.*, 2002a). Cattle immunized with *A. marginale* harvested from bovine erythrocytes developed a preferential response to MSP1a, while cattle immunized with tick cell culture-derived *A. marginale* responded mainly against MSP1b. Moreover, in the present study cattle immunized with recombinant MSP1a in combination with tick cell culture-derived *A. marginale* developed high titers to MSP1a. Furthermore, the antibody response to MSP1a in cattle immunized with erythrocyte-derived *A. marginale* or infected tick cell culture-derived antigens plus recombinant MSP1a correlated with higher levels of packed cell volume after challenge with *A. marginale* infected blood (unpublished results). In previous studies, antibodies against MSP1a were shown to reduce infection of tick cells and erythrocytes by *A. marginale* (Palmer

and McGuire, 1984; Blouin *et al.*, 2003; de la Fuente *et al.*, 2003b). These studies suggest that the antibody response against MSP1a may be important in development of protective immunity and reduction of tick infections.

To study the expression of MSP1a and MSP1b in infected tick cells and bovine erythrocytes, we compared the relative amounts of MSP1a and MSP1b expressed by *A. marginale* derived from erythrocytes, cultured tick cells and salivary glands infected with the Oklahoma isolate of *A. marginale*. Although semi-quantitative assays, the amounts of MSP1b were similar in *A. marginale* grown in both host cells, but the amount of MSP1a was greater in *A. marginale* harvested from infected erythrocytes. These results were confirmed using a different geographic isolate of *A. marginale* from Virginia, which varies in molecular weight and MSP1a properties (de la Fuente *et al.*, 2001b; 2001c). The up-regulation of the expression of MSP1a in bovine erythrocytes could account for the preferential antibody response against MSP1a in cattle immunized with erythrocyte-derived antigen. The differences in the antibody response to MSP1b between erythrocyte and cultured tick cell-immunized cattle could be explained by the immunodominance of MSP1a, resulting in lower antibody titers against MSP1b when MSP1a was present in higher amounts in the erythrocyte-derived antigen. Nevertheless, other factors including antigen conformation could contribute to the differences in the antibody response.

The regulation of the expression of bacterial surface proteins often occurs at the transcriptional level (Huang *et al.*, 1999), although instances of post-transcriptional regulation of the expression of membrane proteins have also been reported (Löhr *et al.*, 2002a). We compared the levels of transcripts for *msp* genes in order to determine whether the amount of

msp1a transcripts is different in *A. marginale*-infected erythrocytes and tick cells. Although *msp5*, *msp4*, and *msp1β*$_1$ were detected at similar levels in both host cells, the amount of *msp1a* transcripts was much lower in infected cultured tick cells and tick salivary glands than in infected erythrocytes, most likely as a result of differences in the rate of transcription of *msp1a* (transcriptional regulation) or in the half-life of its transcripts. Increased levels of *msp1a* transcripts were detected in bovine erythrocytes from cattle infected with erythrocyte (calf PA479) or tick cell culture-derived (calf PA408) *A. marginale*, whereas low levels of *msp1a* transcript were detected in cultured *I. scapularis* cells and salivary glands from *D. variabilis* and *D. andersoni*, both vectors of *A. marginale* in the U.S. These results suggest that the regulation of the levels of *msp1a* transcript is influenced by the host cell environment. Temperature regulation of outer membrane protein expression has been described in other pathogenic bacteria (Konkel and Tilly, 2000), including the tick-borne pathogens *A. marginale* (Rurangirwa *et al.*, 1999; Löhr *et al.*, 2002a; 2002b), *A. phagocytophilum* (IJdo *et al.*, 2002), *E. canis*, *E. chaffeensis* and *E. ruminantium* (Unver *et al.*, 2001; 2002; Bekker *et al.*, 2002), and *Borrelia burgdorferi* (Schwan and Piesman, 2000; Indest *et al.*, 2000).

The mechanism by which infectivity of *A. marginale* increases during tick transmission feeding is unknown (Kocan, 1986, Löhr *et al.*, 2002b). Although the pattern of expression of *A. marginale* MSPs in different tick tissues has not been studied, differences in the level of expression of specific adhesion molecules may affect the infectivity of the rickettsia for bovine erythrocytes. Since MSP1a is an adhesin for tick cells and bovine erythrocytes (de la Fuente *et al.*, 2001a), its differential expression may influence the infectivity of *A. marginale* for these host cells. Our results suggest that the differential expression of MSP1a results in changes in the

stoichiometry of the MSP1 complex formed with MSP1b. These changes may affect the adhesive properties of the MSP1 complex, which are known to be different from the properties of the MSP1a and MSP1b proteins alone (McGarey *et al.*, 1994). The up-regulation of the expression of MSP1a in the intraerythrocytic stages may also result in the production of MSP1a molecules that do not complex with MSP1b. This increased expression of MSP1a, the adhesin for tick cells, may be a mechanism by which infectivity of *A. marginale* for ticks is increased in the bovine host, thereby enhancing the opportunity for biological transmission of the pathogen.

The differential regulation of the expression of *msp1a* may contribute to changes in the infectivity of *A. marginale* for bovine erythrocytes and tick cells by regulating the stoichiometry of the MSP1 complex. We do not know when changes in expression of *msp1a* occur in tick cells and bovine erythrocytes but its regulation may be coordinated with the life cycle of the rickettsia. Differential expression of surface proteins may also be involved in the generation of phenotypic and antigenic diversity, and in diverting the bovine immune response from the functionally important proteins in the different host cells. Understanding the factors involved in the regulation of the expression of these surface molecules will contribute to the development of more effective strategies for the control of anaplasmosis and its transmission.

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REFERENCES

Barbet, A.F., Blentlinger, R., Lundgren, A.M., Blouin, E.F., Kocan, K.M., 1999. Comparison of surface proteins of *Anaplasma marginale* grown in tick cell culture, tick salivary glands and cattle. Infect. Immun. 67, 102-107.

Bekker, C.P., Bell-Sakyi, L., Paxton, E.A., Martinez, D., Bensaid, A., Jongejan, F., 2002. Transcriptional analysis of the major antigenic protein 1 multigene family of *Cowdria ruminantium*. Gene 285, 193-201.

Blouin, E.F., Kocan, K.M., 1998. Morphology and development of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in cultured *Ixodes scapularis* (Acari: Ixodidae) cells. J. Med. Entomol. 35, 788-797.

Blouin, E.F., Barbet, A.F., Jooyoung, Yi, Kocan, K.M., Saliki, J.T., 2000. Establishment and characterization of an Oklahoma isolate of *Anaplasma marginale* in cultured *Ixodes scapularis* cells. Vet. Parasitol. 87, 301-313.

Blouin E.F., Saliki, J.T., de la Fuente, J., Garcia-Garcia, J.C., Kocan, K.M., 2003. Antibodies to *Anaplasma marginale* major surface proteins 1a and 1b inhibit infection for cultured tick cells. Vet. Parasitol. 111, 247-260.

Brayton, K.A., Palmer, G.H., Lundgren, A., Jooyoung, Yi, Barbet, A.F., 2002. Antigenic variation of *Anaplasma marginale msp2* occurs by combinatorial gene conversion. Mol. Microbiol. 43, 1151-1159.

de la Fuente, J., Garcia-Garcia, J.C., Blouin, E.F., Kocan, K.M., 2001a. Differential adhesion of major surface proteins 1a and 1b of the ehrlichial cattle pathogen *Anaplasma marginale* to bovine erythrocytes and tick cells. Int. J. Parasitol. 31, 145-153.

de la Fuente, J., Garcia-Garcia, J.C., Blouin, E.F., Kocan, K.M., 2001b. Major surface protein 1a effects tick infection and transmission of the ehrlichial pathogen *Anaplasma marginale*. Int. J. Parasitol. 31, 1705-1714.

de la Fuente, J., Garcia-Garcia, J.C., Blouin, E.F., Rodríguez, S.D., García, M.A., Kocan, K.M., 2001c. Evolution and function of tandem repeats in the major surface protein 1a of the ehrlichial pathogen *Anaplasma marginale*. Anim. Health Res. Rev. 2, 163-713.

de la Fuente, J., Kocan, K.M., Garcia-Garcia, J.C., Blouin, E.F., Claypool, P.L., Saliki, J.T., 2002a. Vaccination of cattle with *Anaplasma marginale* derived from tick cell culture and bovine erythrocytes followed by challenge-exposure with infected ticks. Vet. Microbiol. 89, 239-251.

de la Fuente, J., Garcia-Garcia, J.C., Blouin, E.F., Saliki, J.T., Kocan, K.M., 2002b. Infection of tick cells and bovine erythrocytes with one genotype of the intracellular ehrlichia *Anaplasma marginale* excludes infection with other genotypes. Clin. Diag. Lab. Immunol. 9, 658-668.

de la Fuente, J., Garcia-Garcia, J.C., Blouin, E.F., Kocan, K.M., 2003. Characterization of the functional domain of major surface protein 1a involved in adhesion of the rickettsia *Anaplasma marginale* to host cells. Vet. Microbiol. 91, 265-283.

Huang, L., Guan, R.J., Pardee, A.B., 1999. Evolution of transcriptional control from prokaryotic beginnings to eukaryotic complexities. Crit. Rev. Eukaryot. Gene Expr. 9, 175-182.

IJdo, J.W., Wu, C., Telford 3rd, S.R., Fikrig, E., 2002. Differential expression of the *p44* gene family in the agent of human granulocytic ehrlichiosis. Infect. Immun. 70, 5295-5298.

Indest, K.J., Ramamoorthy, R., Philipp, M.T., 2000. Transcriptional regulation in spirochetes. J. Mol. Microbiol. Biotechnol. 2, 473-481.

Knowles, D., Torioni de Echaide, S., Palmer, G., McGuire, T., Stiller, D., McElwain, T., 1996. Antibody against an *Anaplasma marginale* MSP5 epitope common to tick and erythrocyte stages identifies persistently infected cattle. J. Clin. Microbiol. 34, 2225-2230.

Kocan, K.M., 1986. Development of *Anaplasma marginale*: coordinated development of a rickettsial organisms and its tick host. In: . Sauer, J.R., Hair, J.A. (Eds.), Morphology, Physiology and Behavioral Ecology of Ticks, Ellis Horwood, New York, pp. 472-505.

Kocan, K.M., Halbur, T., Blouin, E.F., Onet, V., de la Fuente, J., Garcia-Garcia, J.C., Saliki, J.T., 2001. Immunization of cattle with *Anaplasma marginale* derived from tick cell culture. Vet. Parasitol. 102, 151-161.

Konkel, M.E., Tilly, K., 2000. Temperature-regulated expression of bacterial virulence genes. Microbes Infect. 2, 157-166.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

Löhr, C.V., Brayton, K.A., Shkap, V., Molad, T., Barbet, A.F., Brown, W.C., Palmer, G.H., 2002a. Expression of *Anaplasma marginale* major surface protein 2 operon-associated proteins during mammalian and arthropod infection. Infect. Immun. 70, 6005-6012

Löhr, C.V., Rurangirwa, F.R., McElwain, T.F., Stiller, D., Palmer, G.H., 2002b. Specific expression of *Anaplasma marginale* major surface protein 2 salivary gland variants occurs in the midgut and is an early event during tick transmission. Infect. Immun. 70, 114-120.

McGarey, D.J., Allred, D.R., 1994. Characterization of hemagglutinating components on the *Anaplasma marginale* initial body surface and identification of possible adhesins. Infect. Immun. 62, 4587-4593.

McGarey, D.J., Barbet, A.F., Palmer, G.H., McGuire, T.C., Allred, D.R., 1994. Putative adhesins of *Anaplasma marginale*: major surface polypeptides 1a and 1b. Infect. Immun. 62, 4594-4601.

Munderloh, U.G., Blouin, E.F., Kocan, K.M., Ge, N.L., Edwards, W.L., Kurtti, T.J., 1996. Establishment of the tick

(Acari: Ixodidae)-borne cattle pathogen *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in tick cell culture. J. Med. Entomol. 33, 656-664.

Palmer, G.H., McGuire, T.C., 1984. Immune serum against *Anaplasma marginale* initial bodies neutralizes infectivity for cattle. J. Immunol. 133, 1010-1015.

Palmer, G.H., Kocan, K.M., Barron, S.J., Hair, J.A., Barbet, A.F., Davis, W.C., McGuire, T.C., 1985. Presence of common antigens, including major surface protein epitopes, between the cattle (intraerythrocytic) and tick stages of *Anaplasma marginale*. Infect. Immun. 50, 881-886.

Rurangirwa, F.R., Stiller, D., French, D.M., Palmer, G.H., 1999. Restriction of major surface protein 2 (MSP2) variants during tick transmission of the ehrlichia *Anaplasma marginale*. Proc. Natl. Acad. Sci. USA 96, 3171-3176.

Saliki, J.T., Blouin, E.F., Rodgers, S.J., Kocan, K.M., 1998. Use of tick cell culture-derived *Anaplasma marginale* antigen in a competitive ELISA for serodiagnosis of anaplasmosis. Ann. NY Acad. Sci. 849, 273-281.

Schwan, T.G., Piesman, J., 2000. Temporal changes in outer surface proteins A and C of the Lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice. J. Clin. Microbiol. 38, 382-388.

Unver, A., Ohashi, N., Tajima, T., Stich, R.W., Grover, D., Rikihisa, Y., 2001. Transcriptional analysis of *p30* major outer membrane multigene family of *Ehrlichia canis* in dogs, ticks, and cell culture at different temperatures. Infect. Immun. 69, 6172-6178.

Unver, A., Rikihisa, Y., Stich, R.W., Ohashi, N., Felek, S., 2002. The *omp-1* major outer membrane multigene family of *Ehrlichia chaffeensis* is differentially expressed in canine and tick hosts. Infect. Immun. 70, 4701-4704.

Visser, E.S., McGuire, T.C., Palmer, G.H., Davis, W.C., Shkap, V., Pipano, E., Knowles Jr, D.P., 1992. The *Anaplasma marginale msp5* gene encodes a 19-kilodalton protein conserved in all recognized *Anaplasma* species. Infect. Immun. 60, 5139-5144.

TABLE 1. Immunogens used for cattle immunization.

Group	Immunogen[a]	No. Animals
1	Oklahoma isolate of *A. marginale* propagated in tick cell culture	5
2	Oklahoma isolate of *A. marginale* harvested from infected erythrocytes	5
3	Oklahoma isolate of *A. marginale* propagated in tick cell culture plus pure recombinant MSP1a	5
4	Adjuvant alone	5

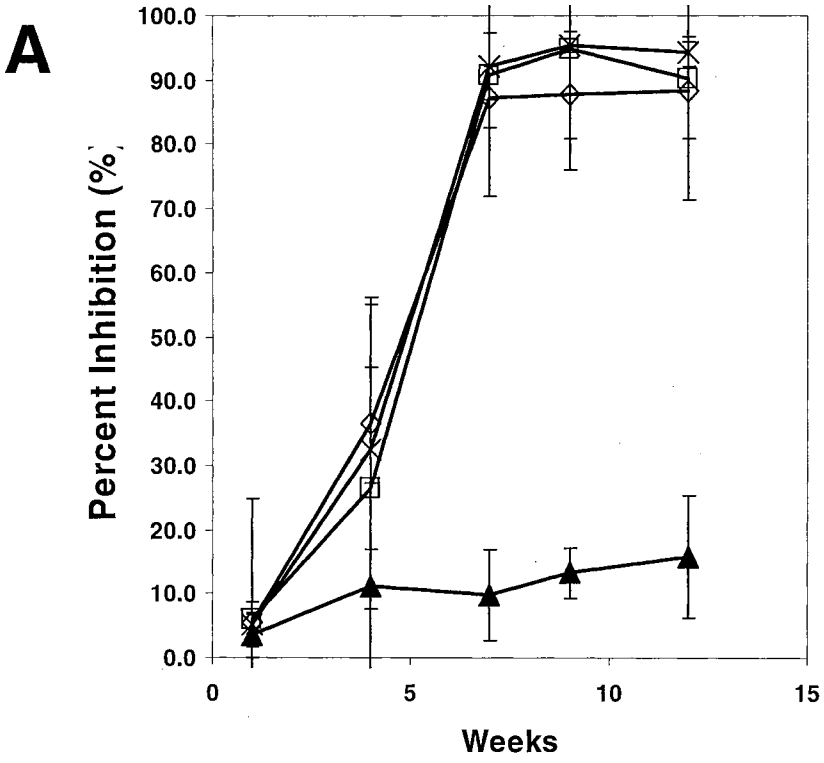
[a] Animals were immunized subcutaneously at weeks 1, 4 and 7. Doses contained approximately 2×10^{10} *A. marginale* organisms. One hundred μg recombinant MSP1a protein were added to the immunogen for group 3.

TABLE 2. Sequence of oligonucleotide primers and PCR conditions for the amplification of *A. marginale* cDNAs.

Amplified cDNA	Oligonucleotide Name: Sequence (5' – 3')	Amplification conditions[a]
msp1a	MSP1aATG: ATGTTAGCGGAGTATGTGTCCCCCCAG	94°C, 30 sec
	MSP1a3: GCTTTACGCCGCCGCCTGCGCC	68°C, 2.5 min
msp1ß	MSP1b125: GCCATCTCGGCCGTATTCCAGCGC	94°C, 30 sec
	MSP1b123: GATGGTCTTAATGGTTTCAGTCCC	68°C, 2.5 min
msp1ß$_1$	MSP1b125: GCCATCTCGGCCGTATTCCAGCGC	94°C, 30 sec
	MSP1b13: GGTGATGACGAGCTGAAGCTGTTCATG	68°C, 2.5 min
msp4	MSP45:	94°C, 30 sec
	GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	60°C, 30 sec
	MSP43: CCGGATCCTTAGCTGAACAGGAATCTTGC	68°C, 1 min
msp5	F55: CCGCTCGAGATGAGAATTTTCAAGATTGTGTC	94°C, 30 sec
	F53: AGATCTAGAATTAAGCATGTGACCGC	56°C, 30 sec
		68°C, 2 min
16S rRNA	AM16S5: AGAGTTTGATCCTGGCTCAG	94°C, 30 sec
	AM16S3: TACCTTGTTACGACTTCACC	56°C, 30 sec
		68°C, 2 min

[a] PCR reactions were incubated at 94°C for 30 sec before the 35 cycles of amplification and were terminated at 4°C. Control reactions without RT or cDNA were also performed.

Figure 1. Antibody response against *A. marginale* MSPs in immunized and control cattle. Four groups of five animals each were immunized with erythrocyte-derived *A. marginale* (×, eda), cell culture-derived *A. marginale* with (□, a+ccda) or without (◊, ccda) recombinant MSP1a, or adjuvant alone (▲, control).at 1, 4 and 7 weeks of the experiment. (A) Antibody levels against MSP5 were measured by competitive ELISA and are expressed as the geometric mean ± S.D. of the percent inhibition of each group. (B) The antibody response against MSP1a and MSP1b at week 9 of the experiment was measured by ELISA. Bars represent the geometric mean titer (mean ± S.D.) of the five animals of each group. Asterisks denote statistically significant differences between the mean antibody levels against MSP1a and MSP1b for each immunization group, using a paired Student's t-test with the Bonferroni correction for multiple pairwise comparisons and overall α=0.05.


A
100.0
90.0
80.0
70.0
60.0
50.0
40.0
30.0
20.0
10.0
0.0
Percent Inhibition (%)
0
5
10
15
Weeks

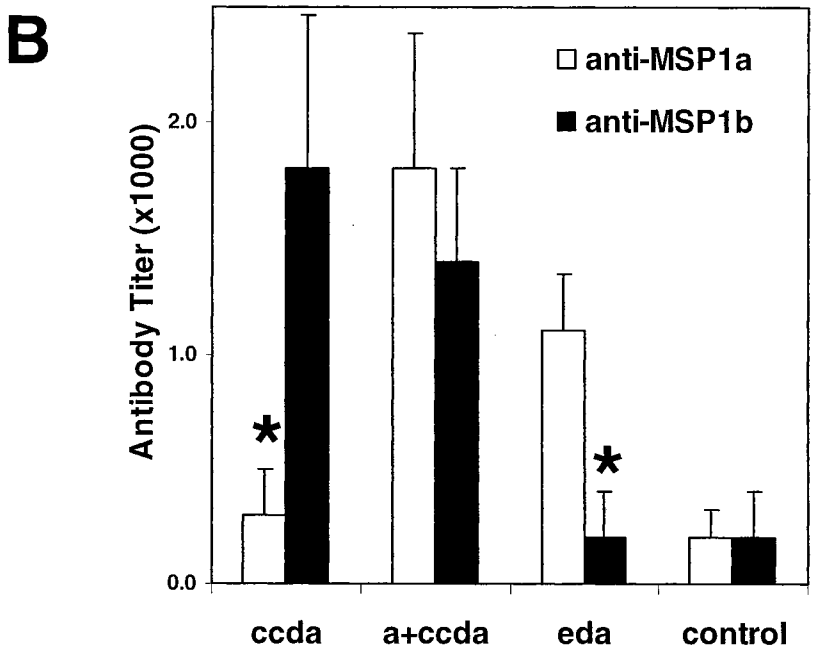

B
anti-MSP1a
anti-MSP1b
2.0
1.0
0.0
Antibody Titer (x1000)
*
*
ccda
a+ccda
eda
control

Figure 2. Western blot analysis of the expression of MSP5, MSP1a and MSP1b in *A. marginale* infected bovine erythrocytes (lanes 1, 3, 5) and culture tick cells (lanes 2, 4, 6). Proteins on the nitrocellulose membrane were probed with MAb ANAF16C1 (anti-MSP5, lanes 1, 2), MAb ANA15D2 (anti-MSP1a, lanes 3, 4), or anti-MSP1b monospecific rabbit antiserum (lanes 5, 6). The amount of MSP1a (arrowheads) was higher in infected bovine erythrocytes (lane 3) than in tick cells (lane 4).

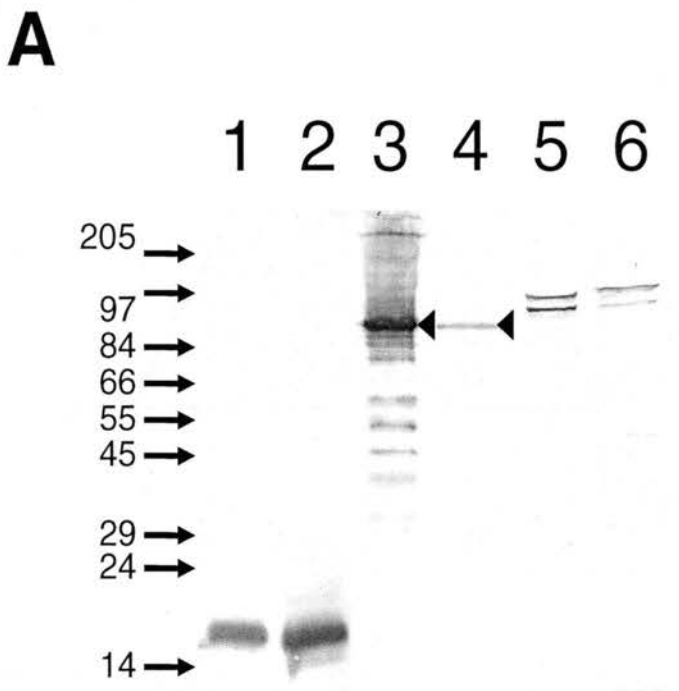

A
1 2 3 4 5 6
205
97
84
66
55
45
29
24
14

Figure 3. Confocal microscopy of (A, B) a cross-section of *A. marginale* infected *D. variabilis* salivary glands and (C) a cross-section of uninfected *D. variabilis* salivary glands.

Samples were probed with (A) MAb ANA15D2 (anti-MSP1a) or (B, C) MAb ANAF16C1 (anti-MSP5), labeled with Alexa Fluor 546 and Alexa Fluor 633, respectively. Arrows indicate expression of MSP5 in tick salivary gland colonies of *A. marginale*. Cross-sections in panels A and B correspond to the same cross-section of infected salivary glands that was simultaneously incubated with labeled anti-MSP1a and anti-MSP5 MAbs and examined for the presence of both labels.

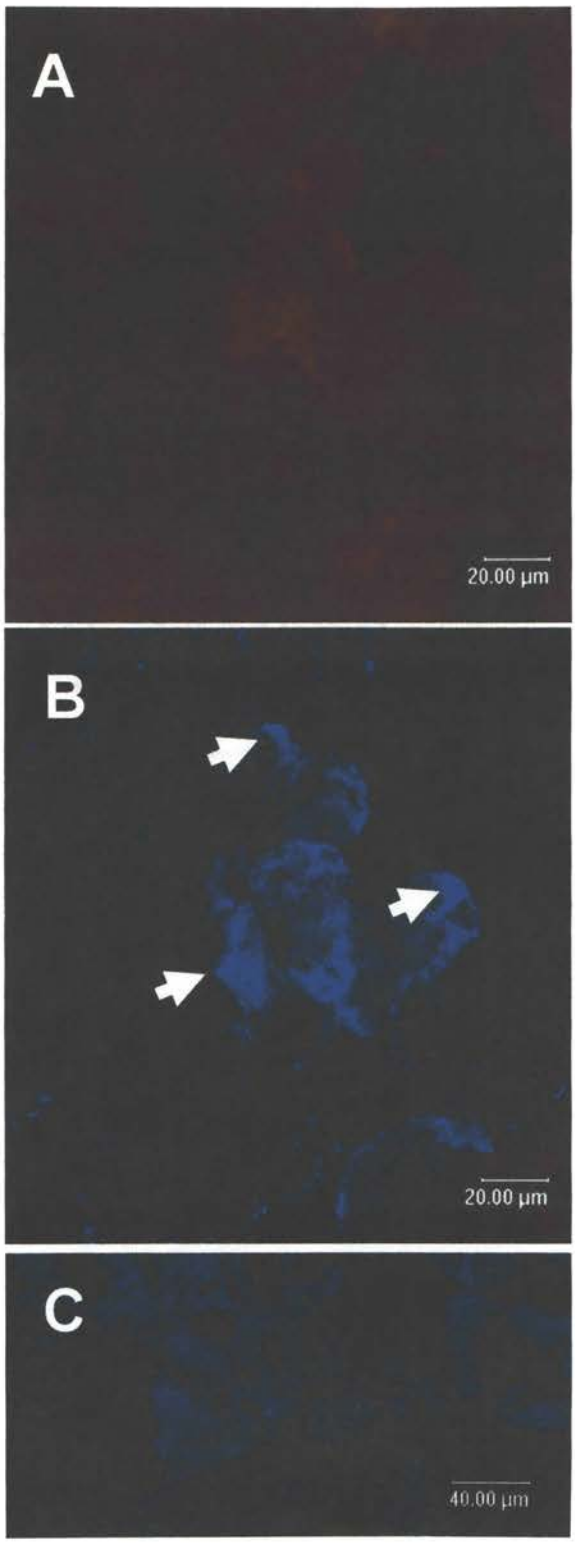

A
20.00 µm
B
20.00 µm
C
40.00 µm

Figure 4. RT-PCR analysis of the expression of *A. marginale msp* genes in culture tick cells and bovine erythrocytes.

Total RNA was extracted from samples of (A) erythrocytes from calf PA479 or (B) culture tick cells infected with the Oklahoma isolate of *A. marginale*. The RNA samples were normalized for rickettsial RNA content using 16S rRNA and *msp4*. The cDNAs *msp1a* (lane 1), *msp1β* (lane 2), *msp1β1* (lane 3), *msp4* (lane 4), *msp5* (lane 5) and 16S rRNA (lane 6) were amplified using the oligonucleotides and amplification conditions described in Table 2. Negative control reactions without RT (lane 7) and without DNA (lane 8) were also performed. Higher levels of *msp1a* transcripts were detected in bovine erythrocytes (lane 1A) than in infected culture tick cells (lane 1B).

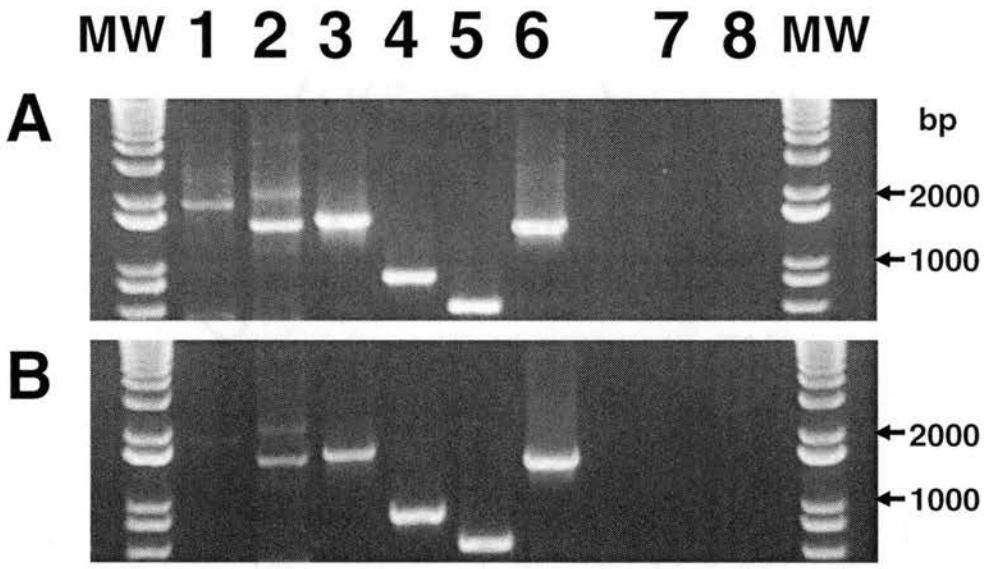
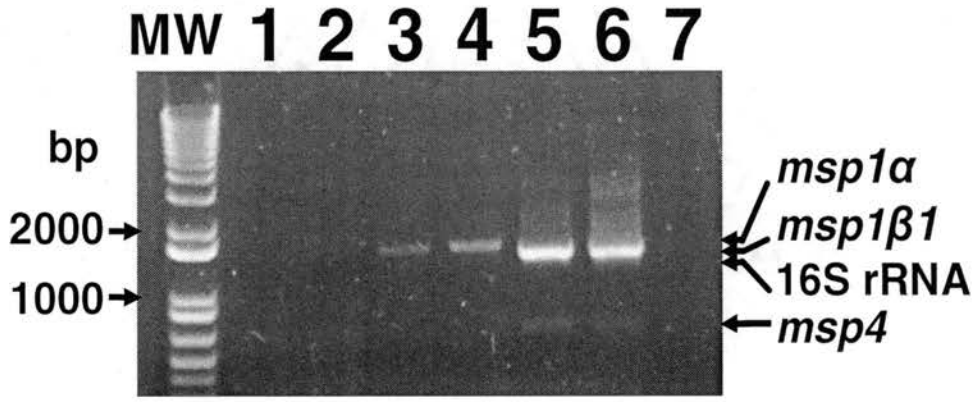

MW 1 2 3 4 5 6 7 8 MW
A
B
bp
2000
1000
2000
1000

Figure 5. RT-PCR analysis of the expression of *A. marginale msp* genes in tick salivary glands. Total RNA was extracted from pools of 10-20 salivary glands of *D. variabilis* (lanes 1, 3, 5) and *D. andersoni* (lanes 2, 4, 6) ticks fed on calf PA479 infected with the Oklahoma isolate of *A. marginale*. The cDNAs *msp1a* (lanes 1, 2), *msp1β1* (lanes 3, 4), and *msp4* and 16S rRNA (lanes 5, 6) were amplified using the oligonucleotides and amplification conditions described in Table 2. A control reaction without RT (lane 7) was also performed. No expression of *msp1a* was detected in the salivary glands of these ticks (lanes 1, 2). Arrows on the side of the gel indicate the molecular weight of the marker (MW) and amplification products.


MW 1 2 3 4 5 6 7
bp
2000
1000
msp1α
msp1β1
16S rRNA
msp4

Chapter 3

MAPPING OF B-CELL EPITOPES IN THE N-TERMINAL REPEATED PEPTIDES OF THE MAJOR SURFACE PROTEIN 1A OF *ANAPLASMA MARGINALE* AND CHARACTERIZATION OF THE HUMORAL IMMUNE RESPONSE OF CATTLE IMMUNIZED WITH RECOMBINANT AND WHOLE ORGANISM ANTIGENS.

Garcia-Garcia JC, de la Fuente J, Kocan KM, Blouin EF, Halbur T, Onet VC, Saliki JT. *Veterinary Immunology and Immunopathology*, In press.

Abstract

Major surface protein (MSP) 1a of the genus type species *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) together with MSP1b forms the MSP1 complex. MSP1a has been shown to be involved in adhesion, infection and tick transmission of *A. marginale*, as well as to contribute to protective immunity in cattle. A differential antibody response to MSP1a and MSP1b was observed in cattle immunized with *A. marginale* derived from bovine erythrocytes (anti-MSP1a response) or cultured tick cells (anti-MSP1b response). In this study, we further characterized the MSP1a antibody response of cattle using several immunogens, including recombinant MSP1a (rMSP1a) protein, erythrocyte- or tick cell culture-derived *A. marginale*, or a combination of tick cell culture-derived *A. marginale* and rMSP1a. The MSP1a antibody response to all these immunogens was directed primarily against the N-terminal region of MSP1a that contains tandemly repeated peptides, whereas low antibody levels were detected against the C-terminal portion. Linear B-cell epitopes of MSP1a were mapped using synthetic peptides representing the entire sequence of the protein that were prepared by SPOT synthesis technology. Only two peptides in the N-terminal repeats were recognized by sera from immunized cattle. These peptides shared the sequence SSAGGQQQESS, which is likely

to contain the linear B-cell epitope that was recognized by the pools of bovine sera. The average differential of antibody titers against MSP1a minus those against MSP1b correlated with lower percent reductions in PCV. A preferential antibody response to MSP1a was observed in cattle immunized with erythrocyte-derived, cell culture-derived plus rMSP1a or rMSP1a alone, and the percent reduction PCV was significantly lower in these cattle as compared with the other immunization groups. These results provide insight into the bovine antibody response against *A. marginale* and the role of MSP1a in protection of cattle against *A. marginale* infection.

Introduction

Bovine anaplasmosis is a tick-borne disease of cattle caused by the obligate intraerythrocytic rickettsia *Anaplasma marginale* (Rickettsiales: Anaplasmataceae). During the course of infection the number of infected erythrocytes increases geometrically and removal of these infected cells by phagocytosis results in severe anemia, weight loss, abortion, and often death (Kuttler, 1984). Cattle that recover from acute infection remain persistently infected with *A. marginale*, are protected from homologous challenge (Kuttler, 1984), and serve as reservoirs for mechanical and biological transmission of *A. marginale* (as reviewed by Dikmans, 1950 and Ewing, 1981).

Five major surface proteins (MSPs) have been identified on erythrocytic and tick stages of *A. marginale*. Four of these MSPs, designated MSP1, MSP2, MSP3 and MSP4, were identified initially using neutralizing polyclonal antibodies (Palmer and McGuire, 1984), and subsequently MSP5 was identified (Visser et al., 1992). All of these MSPs were shown to be structurally conserved in *A. marginale* derived from bovine erythrocytes and tick cells (Barbet et al., 1999).

The MSP1 complex is composed of two covalently linked unrelated polypeptides, MSP1a and MSP1b, which have been shown to be involved in adhesion of *A. marginale* to host cells. MSP1a is an adhesin for both bovine erythrocytes and tick cells, whereas MSP1b is an adhesin only for bovine erythrocytes (McGarey & Allred, 1994; McGarey et al., 1994, de la Fuente et al., 2001a). MSP1a has also been shown to be involved in infection and transmission of *A. marginale* by ticks (de la Fuente et al., 2001a; Blouin et al., 2003). The molecular size of MSP1a varies among isolates of *A. marginale* due to a different number of tandemly repeated peptides in the N-terminal region of MSP1a. These repeated peptides are surface-exposed,

contain a neutralization-sensitive epitope (Palmer et al., 1987; de la Fuente et al., 2001c), and were shown to be necessary and sufficient for adhesion of *A. marginale* to host cells (de la Fuente et al., 2003b).

Methods for the control of *A. marginale* have included vector control, vaccination and the use of antibiotics (reviewed by Kocan et al., 2003). Vaccination induces protective immunity in cattle, however anaplasmosis vaccines (live, attenuated or killed whole-organism) using erythrocyte-derived antigen may have the disadvantages of being contaminated with erythrocyte stroma, bear the risk of transmitting other pathogens, and these vaccines are expensive to produce because they require the use of cattle as a source of infected erythrocytes. *A. marginale* derived from cultured tick cell lines provides an alternate source of antigen that overcomes these drawbacks and the cell culture derived *A. marginale* has recently been shown to induce a protective immune response in cattle (reviewed by Kocan et al., 2003). Other approaches tested for the immunological control of anaplasmosis are based on vaccination with native or recombinant *A. marginale* surface proteins or naked DNA (Palmer et al., 1986; 1988; 1989; Tebele et al., 1991; McGuire et al., 1994; de la Fuente et al., 2003a; reviewed by Kocan et al., 2003). A differential antibody response to MSP1a and MSP1b was observed in cattle immunized with *A. marginale* derived from bovine erythrocytes or cultured tick cells (Kocan et al., 2001; de la Fuente et al., 2002a; Garcia-Garcia et al., 2003). Cattle immunized with erythrocyte-derived *A. marginale* developed a preferential antibody response to MSP1a, whereas cattle immunized with tick cell culture-derived *A. marginale* developed a stronger anti-MSP1b response. This difference was found to result from the up-regulated expression of MSP1a by *A. marginale* in bovine erythrocytes and low-level expression of MSP1a by organisms in tick cells (Garcia-Garcia et al., 2003).

Clearance of *A. marginale* infection by the bovine immune system is mediated by the development of both a humoral immune response against surface-exposed epitopes and a $CD4^+$ T-cell-mediated response (reviewed by Palmer et al., 1999). Antibodies against *A. marginale* major surface proteins are involved in three main mechanisms of protection against *A. marginale* infection, including neutralization due to the direct action of antibodies, antibody-dependent cellular cytotoxicity by MHC non-restricted lymphocytes and macrophage phagocytosis mediated by opsonizing antibodies (reviewed by Palmer et al., 1999). Protective immunity against *A. marginale* can be stimulated by vaccination with live or killed organisms, initial body membranes, purified native or recombinant outer membrane proteins, or DNA encoding for *A. marginale* MSPs (Palmer et al., 1989; Montenegro-James et al., 1991; Tebele et al., 1991; Arulkanthan et al., 1999; Kocan et al., 2001; de la Fuente et al., 2002a). Protection against *A. marginale* infection has been shown to correlate with the level of antibodies specific for *A. marginale* MSPs (Tebele et al., 1991).

Antibodies to MSP1a have been shown to inhibit *A. marginale* infection of bovine erythrocytes (Palmer et al., 1986) and cultured tick cells (Blouin et al., 2003) and to decrease infection of salivary glands of ticks fed on cattle with antibodies to MSP1a (de la Fuente et al., 2003a). Polyclonal antibodies to MSP1a have also been shown to inhibit adhesion to bovine erythrocytes mediated by MSP1a (McGarey et al., 1994). MSP1a contains $CD4^+$ T-lymphocyte epitopes in the conserved C-terminal region (Brown et al., 2001; 2002), but bovine B-cell epitopes of MSP1a have not been described.

Herein, we characterized the antibody response in cattle immunized with rMSP1a, killed *A. marginale* derived from bovine erythrocytes or cultured tick cells or a combination of cell culture-derived *A. marginale* and rMSP1a. We also analyzed the correlation between

antibody levels and reduction of anemia, and we identified linear B-cell epitopes on the N-terminal part of MSP1a by peptide mapping using sera from the immunized cattle. The implications of these findings for development of more effective vaccine strategies for control of anaplasmosis are discussed.

Materials and methods

Anaplasma marginale isolates

The Oklahoma isolate of *A. marginale* was used for the cattle immunization studies and the Virginia isolate was used for challenge exposure of immunized cattle. Both of these isolates have been shown to be tick transmissible and have been propagated in cultured tick cells in our laboratory (Munderloh et al., 1996; Blouin et al., 2000; de la Fuente et al., 2001b; 2002b).

Bovine erythrocyte-derived A. marginale

A susceptible splenectomized 3-month-old calf (PA479) was experimentally infected with blood stabilate of the Oklahoma isolate of *A. marginale* that was collected from calf PA407 with a percent parasitized erythrocytes (PPE) of 10% (Blouin et al., 2000). Calf PA481 was infected with the Virginia isolate of *A. marginale* by intravenous inoculation of blood stabilate from PA433 (de la Fuente et al., 2002b) with a PPE of 12.2% and a packed cell volume (PCV) of 28.5%. The calves were maintained by the OSU Laboratory Animal Resources according to the Institutional Care and Use of Animals Committee guidelines. Infection of the calves was monitored by examination of stained blood smears. Bovine erythrocytes were collected from calf PA479 at a PPE of 32.2%, washed three times in PBS, each time removing the buffy coat, and stored at –70°C. Infected erythrocytes were thawed and *A. marginale* antigen quantified by use of an MSP5 antigen detection ELISA (Saliki et al., 1998), inactivated with β-propiolactone (BPL), and doses of approximately 2×10^{10} *A. marginale* were prepared for immunization of cattle (Table 1). Blood from calf PA481 was collected during ascending parasitemia and used for challenge-exposure of vaccinated cattle.

Tick cell culture-derived A. marginale

A. marginale was propagated in the tick cell line, IDE8 (ATCC CRL 11973), derived originally from *Ixodes scapularis* embryos as described previously (Munderloh et al., 1996; Blouin et al., 2000). Monolayers of IDE8 cells were inoculated with a blood stabilate of the Oklahoma isolate of *A. marginale* that was retrieved from liquid nitrogen. Approximately 10 days post-inoculation terminal cultures with >90% infected cells were harvested by centrifugation, resuspended in PBS and stored at -70°C until used for antigen preparation (Table 1). *A. marginale* antigen was quantified by use of an MSP5-specific antigen detection ELISA (Saliki et al., 1998) and then inactivated with BPL. Antigen doses were prepared that contained approximately 2×10^{10} *A. marginale.*

Recombinant MSP1 proteins, expression and purification

The *msp1*α and *msp1*β_1 genes of the Oklahoma isolate of *A. marginale*, encoding for MSP1a and MSP1b, respectively, were cloned by PCR, fused to the FLAG peptide and expressed in *E. coli* as reported previously (de la Fuente et al., 2001a). Recombinant *E. coli* cells expressing MSP1a and MSP1b proteins were collected and disrupted by sonication. The membrane fractions containing the recombinant proteins were used for preparation of immunogens (100 μg/dose) for cattle vaccination (Table 1) and for the purification of the recombinant proteins. The recombinant MSP1a and MSP1b proteins were extracted with 0.1% Triton X-100 in TBS and purified by FLAG-affinity chromatography (Sigma, USA) following the manufacturer's instructions. Affinity-purified recombinant proteins were used as ELISA coating antigen for the serological evaluation of vaccinated cattle. Expression and purification of the recombinant proteins was confirmed by SDS-PAGE (Laemmli, 1970) and immunoblotting. A protein complex mimicking the native MSP1 complex was obtained in

vitro by diluting equal amounts of rMSP1a and rMSP1b proteins in 6 M guanidine hydrochloride, 5 mM DTT and dialyzing against a 6 M urea solution that was slowly diluted with PBS. After 48 hours the sample was dialyzed against PBS for an extra 12 hours. This in vitro obtained MSP1 complex was used in the cattle vaccination experiment (Table 1).

Construction, expression and purification of msp1α mutants

Two *msp1α* mutants were obtained as described by de la Fuente et al. (2003b). One mutant (pF1ARO5) contained only the sequence encoding for the hydrophilic N-terminal region of the MSP1a protein that contains the tandem repeats, while the second mutant (pAFOR1) contained only the sequence encoding for the conserved C-terminal region of MSP1a. These mutants were obtained by PCR using the Oklahoma isolate *msp1a* gene. The mutant proteins were then expressed in *E. coli,* purified as described above for rMSP1a and rMSP1b and used to coat ELISA plates for evaluation of the antibody response by immunized cattle.

SDS-polyacrylamide gel electrophoresis and immunoblotting

Protein samples were loaded on 10% polyacrylamide gels that were stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk for 60 min at room temperature. Western blot analysis was performed using monoclonal antibodies ANA15D_2 (VMRD, USA) specific for the N-terminal repeats of MSP1a, anti-FLAG M2 (Sigma, USA) for detection of recombinant fusion proteins or MSP1b-monospecific rabbit serum for detection of MSP1b. After washing with TBS, the membranes were incubated with 1:10,000 goat anti-mouse IgG or goat anti-rabbit IgG alkaline phosphatase conjugate (KPL, USA).The membranes were washed again and the color was developed using BCIP/NBT alkaline phosphatase substrate (Sigma, USA).

Cattle immunization and challenge-exposure

Forty Holstein cattle, 12 to 24-month-old, were randomly distributed into eight groups of five animals each (Table 1). Cattle were immunized at weeks 0, 4 and 7 with a 5 ml dose of immunogen containing the antigen(s) in an oil-based adjuvant (Adjuvant XtendIII®, Novartis Animal Vaccines Inc., USA). Cattle in an additional group were immunized with saline and adjuvant to serve as controls. Cattle were challenge-exposed two weeks after the last immunization (week 9) by intravenous administration of 1.7 ml blood from calf PA481 with approximately 10^9 infected erythrocytes of the Virginia isolate of *A. marginale*. Blood samples in EDTA-treated vacutainers were collected from the immunized and control cattle twice a week and then daily after detection of *A. marginale* infected erythrocytes for determination of the parasitemia and PCV. Protection against *A. marginale* infection was expressed as the percent reduction PCV calculated from the lowest PCV after challenge with respect to the initial PCV.

Production of antibodies in mice and rabbits

Four groups of five Balb/c mice were immunized subcutaneously at weeks 0 and 2 with 5 μg rMSP1a antigen, tick cell culture derived *A. marginale*, erythrocyte-derived *A. marginale* or cell culture-derived *A. marginale* supplemented with rMSP1a (Table 1). Serum samples were collected two weeks after the second immunization (week 4) and used for the analysis of B-cell epitopes. Monoclonal antibody ANA15D_2 (VMRD, USA), known to react with the linear neutralization-sensitive epitope (Q/E)ASTSS of the MSP1a repeated peptides (Palmer et al., 1987; Allred et al., 1990), was used as a control in the B-cell epitope mapping experiment. A New Zealand White rabbit was immunized subcutaneously two times (weeks 0, 4) with approximately 50 μg denatured rMSP1a antigen extracted from an SDS-PAGE gel. A blood sample was collected at week 6 and the sera were stored at -70°C until used for the epitope mapping studies. Antiserum prepared previously in a rabbit immunized with a

synthetic peptide (R2FL) that mimics the MSP1a repeats (de la Fuente et al., 2003b) was also used in this study.

Serologic evaluation of immunized cattle

The antibody response against *A. marginale* MSPs in immunized and control cattle was analyzed by ELISA. Antibody levels to *A. marginale* MSP5 were determined by use of an *A. marginale* specific competitive ELISA (Saliki et al., 1998). Antibody levels to *A. marginale* MSP1a, MSP1b, and MSP1a mutants were detected by indirect ELISA (Garcia-Garcia et al., 2003). Briefly, purified recombinant MSP1a, MSP1b, MSP1a N-terminal repeats mutant and MSP1a C-terminal region mutant were used to coat ELISA plates for 3 hours at 37°C. The coated plates were blocked with 2% skim milk overnight at 4°C. Sera were serially diluted 1:2 from a 1:125 initial dilution. The plates were incubated with the diluted sera for 2 hours at 37°C and then incubated with 1:2000 goat anti-bovine IgG-HRP conjugate (KPL, USA) for 1 hour at 37°C. The color reaction was developed with TMB (Sigma, USA) and the OD_{450nm} was determined. Antibody titers were expressed as the maximum dilution of the serum that yielded an OD value at least twice as high as the negative control serum. Antisera with antibody levels not detectable at the lowest dilution (1:125) were assigned a titer of 10 for the statistical analysis. Geometric mean titers were calculated for each experimental group.

Linear B-cell epitope mapping

Equal volumes of serum samples collected at the peak antibody response from cattle or mice in each immunization group were pooled for mapping of linear B-cell epitopes. Seventy six overlapping 16-mer peptides covering the entire sequence of the Oklahoma isolate MSP1a were simultaneously synthesized on a cellulose membrane using SPOT synthesis technology (Sigma Genosys, USA). Before each use, the membrane was blocked with 5% skim milk in TBST for 1 h at room temperature. The membrane was then incubated for 1 h with a

1:200 dilution of pooled serum samples from groups of cattle or mice immunized with erythrocyte-derived *A. marginale* antigen, tick cell culture-derived *A. marginale*, rMSP1a protein or tick cell culture *A. marginale* antigen supplemented with rMSP1a. Sera from rabbits immunized with synthetic peptide R2FL that models the MSP1a N-terminal peptides (de la Fuente et al., 2003b) or with rMSP1a protein were also assayed. Monoclonal antibody ANA15D_2 (VMRD, USA) was used as a positive control. After washing with TBST, the membrane was incubated for 1 h with a 1:250,000 dilution of goat anti-bovine IgG, anti-rabbit IgG or anti-mouse IgG horseradish peroxidase conjugate (KPL, USA). The membrane was washed five times with TBST, incubated with SuperSignal® West Pico peroxidase substrate (Pierce, USA) for 5 min and exposed to X-ray film for 1 min. The membrane was regenerated to remove bound antibodies by incubating with Restore™ Western Blot Stripping buffer (Pierce, USA) for 15 minutes at room temperature. The membrane was tested for complete removal of antibodies by being reincubated between assays with horseradish peroxidase conjugate and substrate and then exposed to film.

Protein sequence and prediction of protein topology

The amino acid sequence of the MSP1a protein from the Oklahoma isolate of *A. marginale* were obtained from GenBank, accession number AY010247, and used for the design of the peptides synthesized for the mapping of B-cell epitopes. Protein topology was predicted using the TMHMM2 algorithm for the prediction of transmembrane helices (Krogh et al., 2001).

Statistical analysis

The antibody titers among immunization groups were compared using analysis of variance and a Student's t-test with the Bonferroni correction for multiple comparisons. The

percent reduction PCV in cattle with a preferential antibody response to MSP1a was compared to that of cattle with a preferential antibody response to MSP1b using a Student's t-test with the Bonferroni correction. A correlation analysis was performed using Microsoft Excel to study the correlation of antibody titers to MSP1a or MSP1b and the percent reduction PCV, an indicator of anemia and thus clinical disease. The group mean differential of the antibody titers against MSP1a minus the MSP1b antibody titers was also included in the correlation analysis.

Results

Immunization of cattle and antibody response to the A. marginale MSPs.

To study the MSP antibody response of immunized and control cattle, the antibody titers against MSP5, MSP1a, MSP1b, and MSP1a mutants were determined by ELISA. All cattle immunized with immunogens that contained erythrocyte- or tick cell culture-derived *A. marginale* seroconverted to MSP5, the surface protein used to normalize the amount of *A. marginale* antigen contained in the vaccine preparations. The level of MSP5 specific antibodies peaked at weeks 9-10, approximately two weeks after the last immunization (Fig. 1). Although the peak antibody levels were close to saturation of the assay, the peak MSP5 antibody levels (weeks 9-10) in cattle immunized with tick cell culture-derived *A. marginale* were significantly lower ($p<0.05$) than that of cattle immunized with erythrocyte-derived *A. marginale* (Fig. 1). Sera from cattle that received recombinant proteins or adjuvant alone were negative for antibodies against MSP5 from weeks 0 to 10, but developed an antibody response to MSP5 after challenge-exposure with *A. marginale* infected erythrocytes on week 9 (Fig. 1). This boost in the antibody response was not observed in cattle vaccinated with erythrocyte- or tick cell culture-derived *A. marginale*, which may indicate that either these animals were protected against challenge or that the antibody levels were already high enough to detect small variations in antibody levels using a competitive ELISA. Cattle that were immunized with MSP1, rMSP1a, rMSP1b, or rMSP1a and rMSP1b developed a strong antibody response against these respective proteins (Fig. 2). The antibody response of cattle immunized with erythrocyte-derived *A. marginale* was predominantly against MSP1a (Fig. 2), consistent with previous studies. The antibody levels against MSP1a and MSP1b developed by vaccine preparations containing tick cell culture-derived *A. marginale* were very low.

Mapping of bovine B-cell epitopes of MSP1a

The antibody titers against MSP1a was higher in cattle vaccinated with immunogens containing rMSP1a or erythrocyte-derived *A. marginale* antigen (Fig. 2). This antibody response was primarily directed against the N-terminal repeated peptides of MSP1a (Fig. 3), indicating that this region contains immunodominant B-cell epitopes. In contrast, antibodies directed to the C-terminal region of MSP1a were detected at very low levels in all the immunization groups (Fig. 3), suggesting that bovine B-cell epitopes may not be present in the conserved region of MSP1a. In order to identify the epitopes recognized by sera from immunized cattle and to determine if recombinant and whole-organism vaccines elicit a response against the same or different B-cell epitopes of *A. marginale* MSP1a, sera from immunized cattle were reacted with synthetic peptides that spanned the entire sequence of MSP1a. Sera from groups of cattle immunized with rMSP1a, erythrocyte-derived *A. marginale*, tick cell culture-derived *A. marginale*, or cell culture-derived *A. marginale* plus rMSP1a, were pooled and used in this experiment. The four pools of sera recognized the same two peptides in the N-terminal repeats of MSP1a (Fig. 4). These two peptides share the sequence SSAGGQQQESS that is likely to contain the linear epitope recognized by the pools of bovine antisera. The epitopes recognized by these sera were the same regardless of the immunogen used. Interestingly, the pattern of response was very homogenous despite the fact that the experiments were carried out using mixed breed cattle.

Linear B-cell epitopes recognized by mouse and rabbit antibodies

Pooled sera from groups of Balb/c mice immunized with erythrocyte- or culture-derived *A. marginale* supplemented or not with rMSP1a recognized a set of epitopes different from those recognized by bovine sera (Fig. 4), but the epitopes were the same among immunization groups. None of the peptides recognized by mouse sera were located in the

repeated peptides of MSP1a. Therefore, the mouse neutralization-sensitive epitope reported previously (Palmer et al., 1987), was not recognized by sera from immunized mice. Serum from a rabbit immunized with denatured rMSP1a antigen reacted with three peptides, only one of which was in the N-terminal repeats of MSP1a (Fig. 4). Another rabbit immunized with a synthetic peptide that models the N-terminal repeats recognized four consecutive peptides spanning a single repeat. Monoclonal antibody ANA15D_2, used as a positive control in the epitope mapping experiment, recognized two peptides in the N-terminal repeats that contained the reported sequence (QASTSS) of the neutralization-sensitive epitope. All the epitopes recognized by bovine, rabbit and mouse antisera were predicted to be exposed on the surface of the outer membrane of *A. marginale* using the TMHMM2 algorithm (Fig. 4).

Protection against A. marginale infection

Immunized and control cattle were challenge-exposed with Virginia isolate *A. marginale* two weeks after the last immunization. All control animals, immunized with saline and adjuvant alone, developed signs of infection, with PPE ranging from 2.7% to 7.0 % and an average 33% reduction PCV. The reduction in PCV was monitored as a measure of protection against heterologous *A. marginale* challenge and was significantly lower ($p<0.05$) in cattle with preferential anti-MSP1a response as compared to control animals or cattle with a preferential response against MSP1b (Table 2). However, the average percent reduction PCV did not correlate with the mean antibody titers against MSP1a or MSP1b (data not shown). The percent reduction in PCV of animals that developed a preferential response to MSP1b was not significantly different from that of control cattle. In addition, the mean differential of the antibody response against MSP1a and MSP1b (anti-MSP1a titer minus anti-MSP1b titer) correlated with the average percent reduction of PCV (Fig. 5). This correlation was accurately

modeled by the fitted curve of a non-linear (third degree polynomial) equation. The highest degree of protection (lowest PCV reduction) was obtained in cattle immunized with erythrocyte-derived *A. marginale* antigen, followed by the group of cattle immunized with cell culture-derived *A. marginale* plus rMSP1a antigen (Fig. 5). Percent reduction PCV after heterologous challenge in animals vaccinated with immunogens that contained rMSP1b protein was not significantly different from percent reduction PCV in control cattle.

Discussion

The *A. marginale*/tick cell culture system provided an alternative source of *A. marginale* antigen for serologic tests and vaccine development. The efficacy of a vaccine preparation based on *A. marginale* derived from this tick cell culture system has been reported recently (Kocan et al., 2001; de la Fuente et al., 2002a). The MSPs of *A. marginale* derived from cultured tick cells and infected bovine erythrocytes have been shown to be structurally conserved (Barbet et al., 1999), but MSP1a was recently shown to be differentially expressed in *A. marginale* derived from bovine erythrocytes and tick cells (Garcia-Garcia et al., 2003). As a result, cattle immunized with erythrocyte-derived *A. marginale* develop a preferential response to MSP1a, whereas cattle immunized with tick cell culture-derived *A. marginale* respond preferentially to MSP1b (Kocan et al., 2001; de la Fuente et al., 2002a; Garcia-Garcia et al., 2003). Antibodies against *A. marginale* MSPs have been shown to be involved in different mechanisms of immune protection (reviewed by Palmer et al., 1999 and Kocan et al., 2003). MSP1a-specific antibodies appear to be particularly important in the inhibition of *A. marginale* adhesion to and invasion of cultured tick cells, tick salivary glands and bovine erythrocytes (McGarey et al., 1994; Blouin et al., 2003; de la Fuente et al., 2003a; 2003b). Therefore, we characterized the antibody response to *A. marginale* MSP1a elicited by recombinant and whole-organism vaccine preparations. Consistent with previous observations, the antibody response to MSP1a was higher in cattle immunized with erythrocyte-derived *A. marginale* or with vaccine preparations that contained rMSP1a protein. Moreover, the antibody response in these groups of cattle was directed primarily against the MSP1a repeats, and very low level of antibodies were detected against the conserved C-terminal region. Both the repeats and C-terminal regions of MSP1a were previously reported to contain B-cell epitopes and similar levels of

antibodies were reported to be elicited against each region in response to immunization of cattle with purified native MSP1 complex (Brown et al., 2001). However, the present study suggests that most of the bovine B-cell epitopes of MSP1a are located in the N-terminal hydrophilic region that contains the repeated peptides. Although the discrepancy might be due to the use of different antigen preparations, it is likely that differences are also due to variation in the immunoassays used to measure antibody levels. In this study we used quantitative immunoassays and all the antigens used for coating the ELISA plates were recombinant proteins expressed in *E. coli*. In the previous study, semiquantitative immunoblotting and dot blot assays, using synthetic versus recombinant antigens, respectively, were used for antibody quantification (Brown et al., 2001). The data reported here suggested that immunodominant bovine B-cell epitopes are located in the N-terminal repeats of MSP1a, which we have previously shown to be necessary and sufficient for adhesion to bovine erythrocytes and tick cells (de la Fuente et al., 2003b). Antibodies against these repeats were also shown to inhibit binding to and infection of erythrocytes (Palmer et al., 1986; McGarey et al., 1994; de la Fuente et al., 2003b) and tick cells (Blouin et al., 2003; de la Fuente et al., 2003b). Therefore, the development of a strong MSP1a repeat specific antibody response may contribute to a protective response against *A. marginale* infection.

A neutralization-sensitive epitope recognized by mouse monoclonal antibody ANA15D$_2$ has been mapped to the repeated peptides of MSP1a (Allred et al., 1990). Although the presence of B-cell epitopes in the MSP1a repeats and C-terminal had been suggested (Brown et al., 2001), these epitopes have not been characterized previously. Using overlapping synthetic peptides covering the entire MSP1a sequence, we mapped linear B-cell epitopes recognized by pooled sera from cattle immunized with rMSP1a, or *A. marginale* derived from

infected bovine erythrocytes or cultured tick cells. Only two peptides, located in the N-terminal repeats of MSP1a and containing the sequence SSAGGQQQESS, were recognized by the four different pools of sera. These sera likely recognized the same linear B-cell epitope represented twice in the tandemly repeated peptides of the Oklahoma isolate MSP1a. This result is consistent with the observation that the main antibody response in immunized cattle was directed against the repeated peptides and not the C-terminal region of MSP1a. Moreover, $CD4^+$ T-lymphocyte epitopes have been identified in the hydrophilic N-terminal region of MSP1a that contains the repeated peptides (Brown et al., 2002). Collectively, these results indicate that this region contains the T- and B-cell epitopes necessary for developing a protective immune response, and suggests the utility of the hydrophilic N-terminal portion of MSP1a for immunization and assessment of its protective capacity.

When sera from immunized mice and rabbits were used for epitope mapping, the linear epitopes recognized were different from the bovine B-cell epitopes described above, suggesting MHC-restriction or at least species-specificity of the B-cell epitopes of MSP1a. However, the same peptides were recognized by sera from all the immunization groups, suggesting that the B-cell epitopes of MSP1a are the same in the recombinant and whole organism vaccine preparations included in this study. Interestingly, immunized Balb/c mice did not develop antibodies against the linear mouse neutralization-sensitive epitope recognized by mAb $ANA15D_2$. This monoclonal antibody was likely obtained in a different mouse strain and, as discussed above, there may be MHC-restriction of the B-cell epitopes of MSP1a. The peptides containing the neutralizing epitope recognized by mAb $ANA15D_2$ did not react with bovine sera from any immunization group. A rabbit serum, known to inhibit adhesion of MSP1a to tick cells in an in vitro assay (de la Fuente et al., 2003b), reacted with four

consecutive peptides that covered the whole sequence of a single repeat. Since the 16-mer peptides used for epitope mapping were synthesized with 8 amino acid overlaps, at least two different epitopes were recognized by this rabbit serum. The peptides recognized by this rabbit serum overlap with one of the peptides recognized by antisera from vaccinated cattle, but whether the antibodies react with the same or different epitope sequences is not known. In other experiments, sera from immunized cattle inhibited infection of tick cells by *A. marginale* (Blouin et al., 2003). All the linear epitopes identified in this study were predicted to be surface exposed by the TMHMM2 algorithm. These data validates the topology predicted for MSP1a in which only four transmembrane helices are present, in contrast to other models that predict five transmembrane domains in MSP1a (de la Fuente et al., 2001c).

Although all the immunogens tested produced an antibody response against the same two linear B-cell epitopes in immunized cattle, a significant difference in the average percent reduction PCV was observed among immunization groups. These results suggest that differences in protective efficacy might not be due to the development of an antibody response against different linear B-cell epitopes but rather due to differences in the amount of antibodies generated against these linear epitopes or other conformational epitopes. However, the antibody titers against MSP1a or MSP1b did not correlate with the level of protection against *A. marginale* infection. Since MSP1a is an integral membrane protein covalently linked to MSP1b (Vidotto et al., 1994), some of the B-cell epitopes of MSP1a may be involved in interactions with other molecules in the *A. marginale* membrane and therefore not be accessible for the development of an effective antibody response when cattle are immunized with whole organism preparations. Alternatively, overexpression of MSP1a in erythrocytic stages of *A. marginale* (Garcia-Garcia et al., 2003) may prevent antibodies from complete neutralization.

In an attempt to study the effect of the presence of MSP1b on the protective response mediated by MSP1a antibodies, we performed a correlation analysis between the differential of the anti-MSP1a minus anti-MSP1b antibody titers and the protection against *A. marginale* infection as determined by the percent reduction PCV. The fact that protection against heterologous challenge in cattle with a preferential antibody response against MSP1a was significantly higher than in cattle that developed a preferential response against MSP1b may indicate that MSP1a-specific antibodies are involved in the protective response against *A. marginale* infection, but the presence of other immunodominant proteins interacting with MSP1a, including MSP1b, may make inaccessible or alter the conformation of specific B-cell epitopes of MSP1a that are necessary for protection, which might be more relevant when MSP1a is expressed at low levels by *A. marginale* growing in tick cells (Garcia-Garcia et al., 2003). Even if the same epitopes were recognized and similar antibody levels were produced, the quality of the immune response and therefore the protective immunity could be affected by components of the whole organism vaccine preparations. A high titer of opsonizing IgG2 antibodies directed to surface exposed epitopes of *A. marginale* has been associated with a protective immune response (Brown et al., 1998). Whether the antibody response to MSP1a is affected by these mechanisms of immunomodulation remains unknown. In addition, there might be differences in the $CD4^+$ T-lymphocyte epitopes in the recombinant and whole organism vaccines evaluated in this study. A $CD4^+$ T-lymphocyte response has been shown to be involved in the development of protection against *A. marginale* infection (Brown et al., 1998; 2001; 2002).

Although the main goal of this research was to characterize the linear B-cell epitopes of MSP1a, conformational or non-peptidic epitopes of MSP1a might also be involved in

protection against *A. marginale* infection. MSP1a was recently shown to be glycosylated (Garcia-Garcia, J. C., de la Fuente, J., Bell, G., Blouin, E. F., Kocan, K. M., submitted for publication), and these carbohydrate modifications were suggested to play a role in adhesion of *A. marginale* to tick cells. Therefore, an antibody response against the glycans of MSP1a may also contribute to the neutralization of the function of MSP1a as an adhesin for host cells.

Collectively the results of this study suggest that immunization of cattle with *A. marginale* antigens that elicit a strong and preferential antibody response against MSP1a induce protection in vaccinated cattle. Since other *A. marginale* antigens may have a synergistic effect on protection, a vaccine preparation that contains whole *A. marginale* organisms supplemented with rMSP1a might induce a protective immune response mediated by antibodies against MSP1a as well as other protective antigens. Because antibodies to MSP1a have been shown to inhibit the infection of tick cells and bovine erythrocytes by *A. marginale*, the development of a vaccine based on a combination of rMSP1a with whole *A. marginale* organisms derived from tick cell culture to include the contribution of other antigens, might provide protection against bovine anaplasmosis and its transmission by the tick vector.

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REFERENCES

Allred, D. R., McGuire, T. C., Palmer, G. H., Leib, S. R., Harkins, T. M., McElwain, T. F., Barbet, A. F., 1990. Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*. Proc. Natl. Acad. Sci. 87, 3220-3224.

Arulkanthan, A., Brown, W. C., McGuire, T. C., Knowles, D. P., 1999. Biased immunoglobulin G1 isotype responses induced in cattle with DNA expressing *msp1a* of *Anaplasma marginale*. Infect. Immun. 67, 3481-3487.

Barbet, A. F., Blentlinger, R., Lundgren, A. M., Blouin, E. F., Kocan, K. M., 1999. Comparison of surface proteins of *Anaplasma marginale* grown in tick cell culture, tick salivary glands and cattle. Infect. Immun. 67, 102-107.

Blouin, E. F., Barbet, A. F., Jooyoung Yi, Kocan, K. M., Saliki, J. T., 2000. Establishment and characterization of an Oklahoma isolate of *Anaplasma marginale* in cultured *Ixodes scapularis* cells. Vet. Parasitol. 87, 301-313.

Blouin, E. F., Saliki, J. T., de la Fuente, J., Garcia-Garcia, J. C., Kocan, K. M., 2003. Antibodies to *Anaplasma marginale* major surface proteins 1a and 1b inhibit infectivity for cultured tick cells. Vet. Parasitol. 111, 247-260.

Brown, W. C., Shkap, V., Zhu, D., McGuire, T. C., Tuo, W., McElwain, T. F., Palmer, G. H., 1998. $CD4^+$ T-lymphocyte and immunoglobulin G2 responses in calves immunized with *Anaplasma marginale* outer membranes and protected against homologous challenge. Infect. Immun. 66, 5406-5413.

Brown, W. C., Palmer, G. H., Lewin, H. A., McGuire, T. C., 2001. CD4(+) T lymphocytes from calves immunized with *Anaplasma marginale* major surface protein 1 (MSP1), a heteromeric complex of MSP1a and MSP1b, preferentially recognize the MSP1a carboxyl terminus that is conserved among strains. Infect. Immun. 69, 6853-6862.

Brown, W. C., McGuire, T. C., Mwangi, W., Kegerreis, K. A., Macmillan, H., Lewin, H. A., Palmer, G. H., 2002. Major histocompatibility complex class II DR-restricted memory CD4(+) T lymphocytes recognize conserved immunodominant epitopes of *Anaplasma marginale* major surface protein 1a. Infect. Immun. 70, 5521-5532.

de la Fuente, J., Garcia-Garcia, J. C., Blouin, E. F., Kocan, K. M., 2001a. Differential adhesion of major surface proteins 1a and 1b of the ehrlichial cattle pathogen *Anaplasma marginale* to bovine erythrocytes and tick cells. Int. J. Parasitol. 31, 145-153.

de la Fuente, J., Garcia-Garcia, J. C., Blouin, E. F., Kocan, K. M., 2001b. Major surface protein 1a effects tick infection and transmission of the ehrlichial pathogen *Anaplasma marginale*. Int. J. Parasitol. 31, 1705-1714.

de la Fuente, J., Garcia-Garcia, J. C., Blouin, E. F., Rodríguez, S. D., García, M. A., Kocan, K. M., 2001c. Evolution and function of tandem repeats in the major surface protein 1a of the ehrlichial pathogen *Anaplasma marginale*. Anim. Health Res. Rev. 2, 163-173.

de la Fuente, J., Kocan, K. M., Garcia-Garcia, J. C., Blouin, E. F., Claypool, P. L., Saliki, J. T., 2002a. Vaccination of cattle with *Anaplasma marginale* derived from tick cell culture and bovine erythrocytes followed by challenge-exposure with infected ticks. Vet. Microbiol. 89, 239-251.

de la Fuente, J., Garcia-Garcia, J. C., Blouin, E. F., Saliki, J. T., Kocan, K. M., 2002b. Infection of tick cells and bovine erythrocytes with one genotype of the intracellular ehrlichia *Anaplasma marginale* excludes infection with other genotypes. Clin. Diagn. Lab. Immunol. 9, 658-668.

de la Fuente, J., Kocan, K. M., Garcia-Garcia, J. C., Blouin, E. F., Halbur, T., Onet, V., 2003a. Antibodies to *Anaplasma marginale* major surface protein 1a reduce infectivity for ticks. Acta Tropica, in press.

de la Fuente, J., Garcia-Garcia, J. C., Blouin, E. F., Kocan, K. M., 2003b. Characterization of the functional domain of major surface protein 1a involved in adhesion of the rickettsia *Anaplasma marginale* to host cells. Vet. Microbiol. 91, 265-283.

Dikmans, G., 1950. The transmission of anaplasmosis. Am. J. Vet. Res. 11, 5-16.

Ewing, S. A., 1981. Transmission of *Anaplasma marginale* by arthropods. In: Proceedings of the 7th National Anaplasmosis Conference, Mississippi State University, MS, USA, pp. 395-423.

Garcia-Garcia, J. C., de la Fuente, J., Blouin, E. F., Johnson, T. D., Halbur, T., Onet, V., Saliki, J. T., Kocan, K. M., 2003. Differential expression of the *msp1a* gene of *Anaplasma marginale* occurs in bovine erythrocytes and tick cells. Vet. Microbiol, in press.

Kocan, K. M, Halbur, T., Blouin, E. F., Onet, V., de la Fuente, J., Garcia-Garcia, J. C., Saliki, J. T., 2001. Immunization of cattle with *Anaplasma marginale* derived from tick cell culture. Vet. Parasitol. 102, 151-161.

Kocan, K. M, de la Fuente, J., Guglielmone, A. A., Meléndez, R. D., 2003. Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. Clin. Microbiol. Rev. 16, 698-712.

Kuttler, K. L., 1984. *Anaplasma* infections in wild and domestic ruminants: a review. J. Wildl. Dis. 20, 12-20.

Krogh, A., Larsson, B., von Heijne, G., Sonnhammer, E. L., 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J. Mol. Biol. 305, 567-580.

Laemmli, U. K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680-685.

McGarey, D. J., Allred, D. R., 1994. Characterization of hemagglutinating components on the *Anaplasma marginale* initial body surface and identification of possible adhesins. Infect. Immun. 62, 4587-4593.

McGarey, D. J., Barbet, A. F., Palmer, G. H., McGuire, T. C., Allred, D. R., 1994. Putative adhesins of *Anaplasma marginale*: major surface polypeptides 1a and 1b. Infect. Immun. 62, 4594-4601.

McGuire, T. C., Stephens, E. B., Palmer, G. H., McElwain, T. F., Leichtensteiger, C. A., Leib, S. R., Barbet, A. F., 1994. Recombinant vaccinia virus expression of *Anaplasma marginale* surface protein MSP-1a: effect of promoters, leader

sequences and GPI anchor sequence on antibody response. Vaccine 12, 465-471.

Montenegro-James, S., James, M. A., Toro Benitez, M., Leon, E., Baek, B. K., Guillen, A.T., 1991. Efficacy of purified *Anaplasma marginale* initial bodies as a vaccine against anaplasmosis. Parasitol. Res. 77, 93-101.

Munderloh, U. G., Blouin, E. F., Kocan, K. M., Ge, N. L., Edwards, W. L., Kurtti, T. J., 1996. Establishment of the tick (Acari: Ixodidae)-borne cattle pathogen *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in tick cell culture. J. Med. Entomol. 33, 656-664.

Palmer, G. H., McGuire, T. C., 1984. Immune serum against *Anaplasma marginale* initial bodies neutralizes infectivity for cattle. J. Immunol. 133, 1010-1015.

Palmer, G. H., Barbet, A. F., Davis, W. C., McGuire, T. C., 1986. Immunization with an isolate-common surface protein protects cattle against anaplasmosis. Science 231, 1299-1302.

Palmer, G. H., Waghela, S. D., Barbet, A. F., Davis, W. C., McGuire, T. C., 1987. Characterization of a neutralization sensitive epitope on the AM 105 surface protein of *Anaplasma marginale*. Int. J. Parasitol. 17, 1279-1285.

Palmer, G. H., Oberle, S. M., Barbet, A. F., Goff, W. L., Davis, W. C., McGuire, T. C., 1988. Immunization of cattle with a 36-kilodalton surface protein induces protection against homologous and heterologous *Anaplasma marginale* challenge. Infect. Immun. 56, 1526-1531.

Palmer, G. H., Barbet, A. F., Cantor, G. H., McGuire, T. C., 1989. Immunization of cattle with the MSP-1 surface protein complex induces protection against a structurally variant *Anaplasma marginale* isolate. Infect. Immun. 57, 3666-3669.

Palmer, G. H., Rurangirwa, F. R., Kocan, K. M., Brown, W. C., 1999. Molecular basis for vaccine development against the ehrlichial pathogen *Anaplasma marginale*. Parasitol. Today 15, 281-286.

Saliki, J. T., Blouin, E. F., Rodgers, S. J., Kocan, K. M., 1998. Use of tick cell culture-derived *Anaplasma marginale* antigen in a competitive ELISA for serodiagnosis of anaplasmosis. Ann. N. Y. Acad. Sci. 849, 273-281.

Tebele, N., McGuire, T. C., Palmer, G. H., 1991. Induction of protective immunity by using *Anaplasma marginale* initial body membranes. Infect. Immun. 59, 3199-3204.

Vidotto, M. C., McGuire, T. C., McElwain, T. F., Palmer, G. H., Knowles, D. P. Jr., 1994. Intermolecular relationships of major surface proteins of *Anaplasma marginale*. Infect. Immun. 62, 2940-2946.

Visser, E. S., McGuire, T. C., Palmer, G. H., Davis, W. C., Shkap, V., Pipano, E., Knowles, D. P. Jr., 1992. The *Anaplasma marginale msp5* gene encodes a 19-kilodalton protein conserved in all recognized *Anaplasma* species. Infect. Immun. 60, 5139-5144.

TABLE 1. Immunization groups and immunogen composition.

Immunization Group[a]	Immunogen[b]
rMSP1a+1b	Recombinant MSP1a and MSP1b antigens
MSP1	Recombinant MSP1 complex obtained *in vitro*
rMSP1a	Recombinant MSP1a antigen
rMSP1b	Recombinant MSP1b antigen
CCDA	Tick cell culture-derived *A. marginale*
rMSP1a+CCDA	Tick cell culture-derived *A. marginale* plus recombinant MSP1a
EDA	Erythrocyte-derived *A. marginale*
Saline	Adjuvant alone

[a] Only immunogens rMSP1a, rMSP1a+CCDA and EDA were used in the mouse immunization experiment. All eight groups were included in the cattle immunization experiment.

[b] Doses of immunogen contained 10^{10} *A. marginale* organisms and/or 100 μg recombinant antigen in 5 ml oil adjuvant for cattle and 10^{8} *A. marginale* and/or 5 μg recombinant antigen in 100 μl for mice. Cattle were immunized at weeks 0, 4 and 7 and challenged at week 9 while mice were immunized at weeks 0 and 2 with blood collection at week 4.

TABLE 2. Percent reduction PCV and antibody response against MSP1a and MSP1b in vaccinated cattle.

Differential Titer[a] MSP1a – MSP1b		Immunization Group	Reduction PCV[b] (%)
Negative	-1000	MSP1	30.4
	-1000	MSP1	29.8
	-500	MSP1	34.7
	-1500	MSP1	30.8
	-1000	MSP1	34.5
	-6000	rMSP1a+1b	32.0
	-1750	rMSP1b	32.7
	-990	rMSP1b	36
	-1750	rMSP1b	26.2
	-3500	rMSP1b	36.0
	-490	rMSP1b	28.7
Mean±SD	**-1332±704**		**32.0±3.2**
Positive	500	rMSP1a+1b	43.5
	1500	rMSP1a+1b	34.8
	750	rMSP1a	23.5
	490	rMSP1a	30.7
	990	rMSP1a	38.1
	990	rMSP1a	18.0
	990	rMSP1a	27.0
	490	CCDA	22.4
	990	rMSP1a +CCDA	22.6
	240	rMSP1a +CCDA	34.7
	990	rMSP1a +CCDA	14.0
	240	rMSP1a +CCDA	29.2
	490	rMSP1a +CCDA	27.6
	490	EDA	6.9
	990	EDA	22.2
	750	EDA	27.5
	490	EDA	28.6
	1990	EDA	18.5
Mean±SD	**691±299**		**26.1±8.8**

Neutral[c]	Saline	33.0
	Saline	26.2
	Saline	42.8
	Saline	24.6
	Saline	36.4
	rMSP1a+1b	22.3
	rMSP1a+1b	34.6
	CCDA	29.2
	CCDA	43.5
	CCDA	37.7
	CCDA	27.4
Mean±SD		**32.5±7.2**

[a] The differential titer was calculated subtracting the MSP1b antibody titer from the MSP1a antibody titer for each individual animal. Geometric mean antibody titers were calculated for each group of cattle.

[b] The percent reduction PCV was calculated from the lowest PCV after heterologous *A. marginale* challenge-exposure and the PCV prior to challenge, %Reduction PCV = 100 × (1-Initial PCV / Lowest PCV).

[c] Control animals, immunized with saline and adjuvant only, and animals in which the antibody response against MSP1a and MSP1b was not biased (differential = 0), were grouped for this analysis.

Figure 1. Antibody response against *A. marginale* MSP5 in immunized and control cattle. Eight groups of five animals each were immunized with MSP1 complex (MSP1), rMSP1a, rMSP1b, rMSP1a plus rMSP1b, cell culture-derived *A. marginale* with (rMSP1a+CCDA) or without (CCDA) rMSP1a, erythrocyte-derived *A. marginale* (EDA), or adjuvant alone (Saline) at 1, 4 and 7 weeks of the experiment (arrows). Antibody levels against MSP5 were measured by competitive ELISA and expressed as the mean ± SEM. All cattle were challenge exposed (indicated by star) at week 9 with 10^9 *A. marginale.*

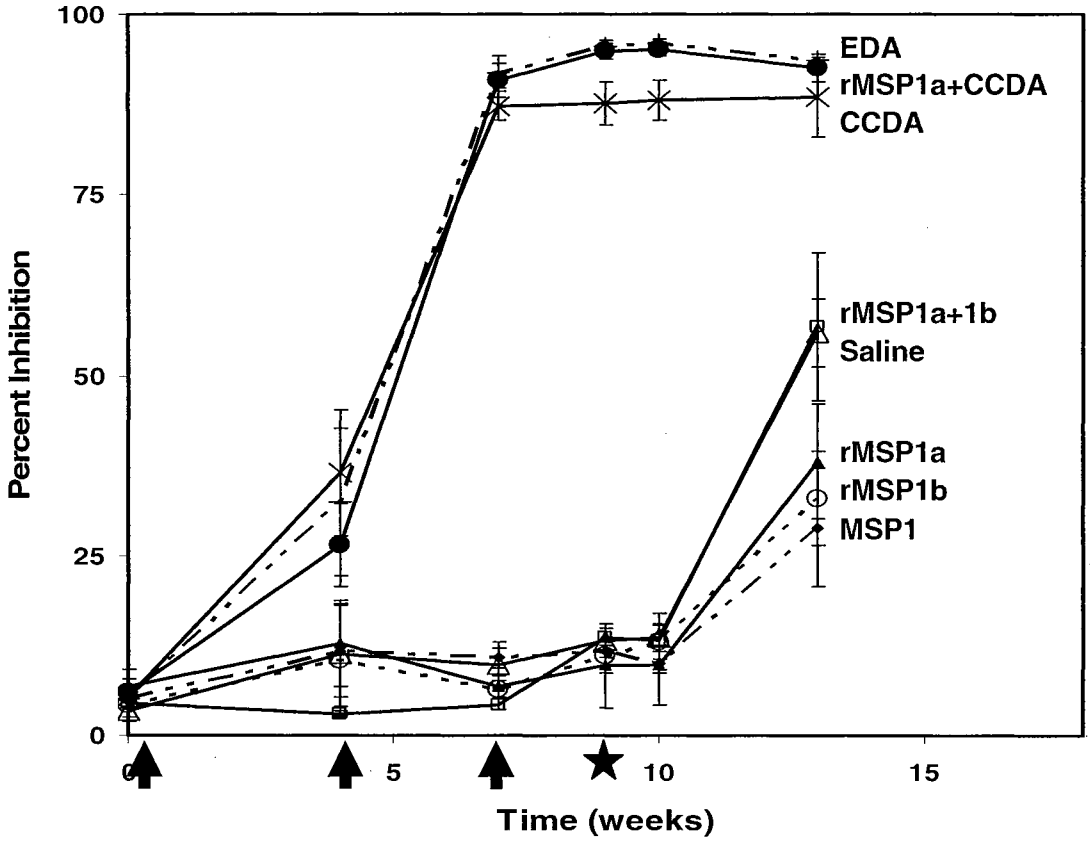
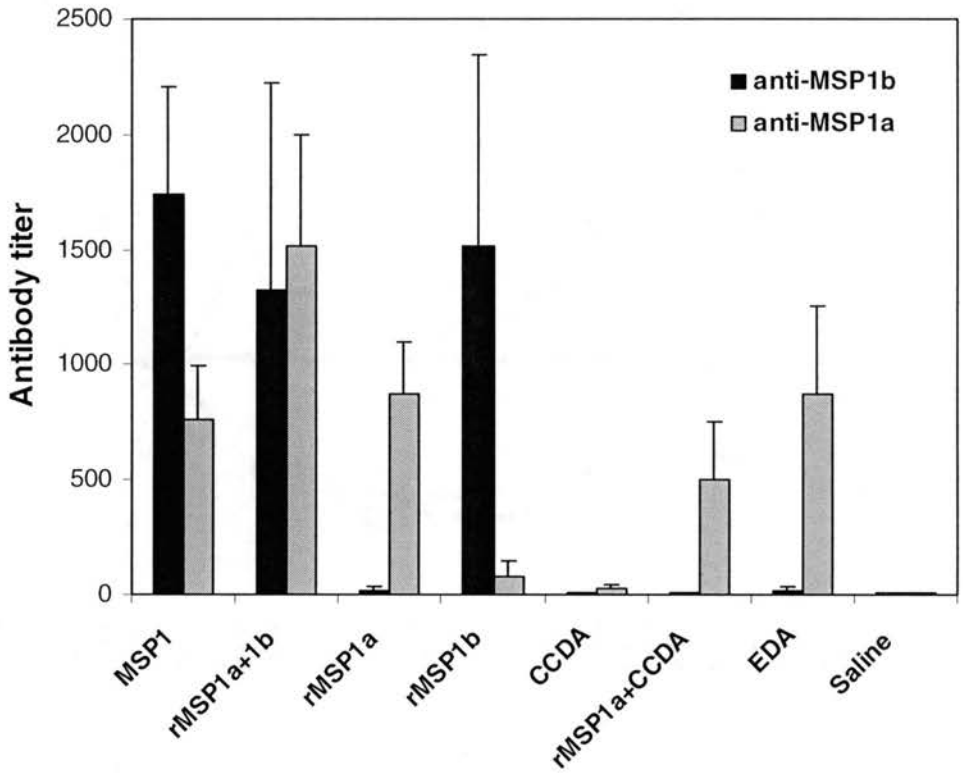

100
75
50
25
0
Percent Inhibition
0
5
10
15
Time (weeks)
EDA
rMSP1a+CCDA
CCDA
rMSP1a+1b
Saline
rMSP1a
rMSP1b
MSP1

Figure 2. Antibody response against *A. marginale* MSP1a and MSP1b in immunized and control cattle. Serum samples were collected at the peak antibody response (week 9), approximately two weeks after the last immunization and prior to challenge. The antibody levels against MSP1a and MSP1b were measured by ELISA. Bars represent the geometric mean titer (mean ± S.D.) of the five cattle of each group.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2000
1500
1000
500
0
Antibody titer
anti-MSP1b
anti-MSP1a
MSP1
rMSP1a+1b
rMSP1a
rMSP1b
CCDA
rMSP1a+CCDA
EDA
Saline

Figure 3. Antibody response against *A. marginale* MSP1a and its N-terminal repeated peptides and C-terminal regions at the peak antibody response (week 9). Recombinant MSP1a, MSP1a repeats and C-terminal region were expressed in *E. coli* and used for coating ELISA plates. Bars represent the geometric mean titer (mean ± S.D.) of the five cattle per group.

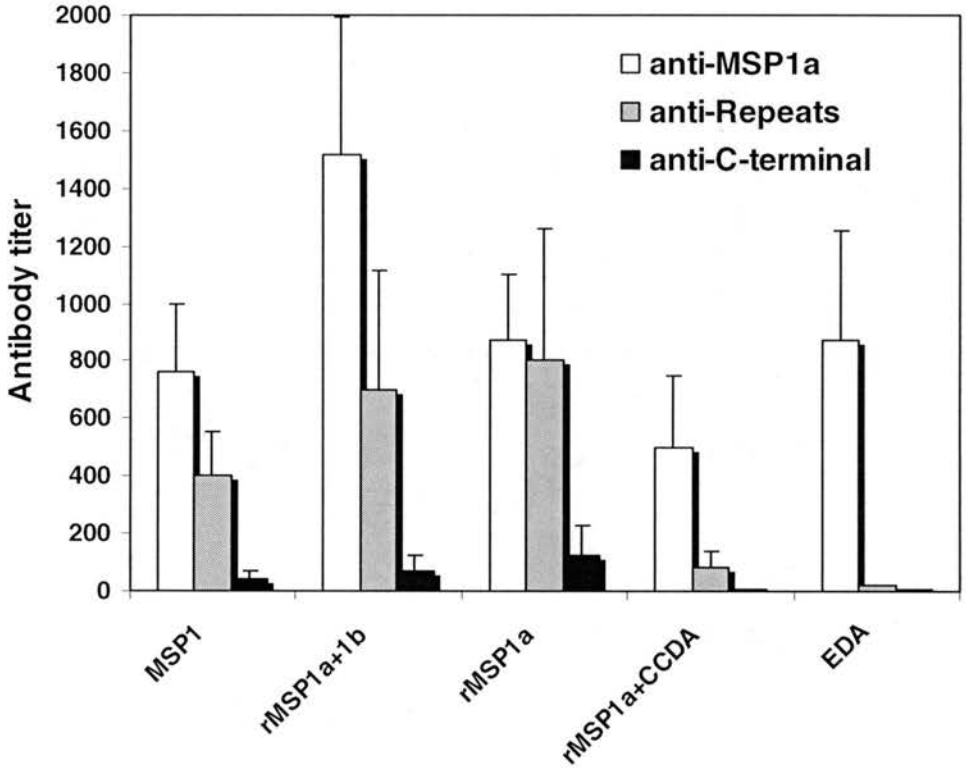

2000
1800
1600
1400
1200
1000
800
600
400
200
0
Antibody titer
anti-MSP1a
anti-Repeats
anti-C-terminal
MSP1
rMSP1a+1b
rMSP1a
rMSP1a+CCDA
EDA

Figure 4. Linear B-cell epitope mapping of MSP1a. Pools of sera from cattle (Bov) and mice immunized with rMSP1a, cell culture-derived *A. marginale* with (rMSP1a+CCDA) or without (CCDA) rMSP1a or erythrocyte-derived *A. marginale* (EDA) were allowed to react with peptides synthesized using SPOTs technology. The amino acid sequences of the overlapping synthetic peptides that span the whole MSP1a protein from the Oklahoma isolate of *A. marginale* are indicated. Sera from rabbits (Rab) immunized with rMSP1a or a synthetic peptide, R2FL, that models the MSP1a repeats were also analyzed. Mouse monoclonal antibody $15D_2$ was used as a positive control. Black boxes represent recognition of the peptide by the corresponding antibodies. The location of each peptide in the predicted topology of the MSP1a protein is indicated in the left column. Residues in the inner (In) or outer (Out) side of the membrane (M) are indicated. Transmembrane helices (in boldface) were predicted using the TMHMM2 algorithm.

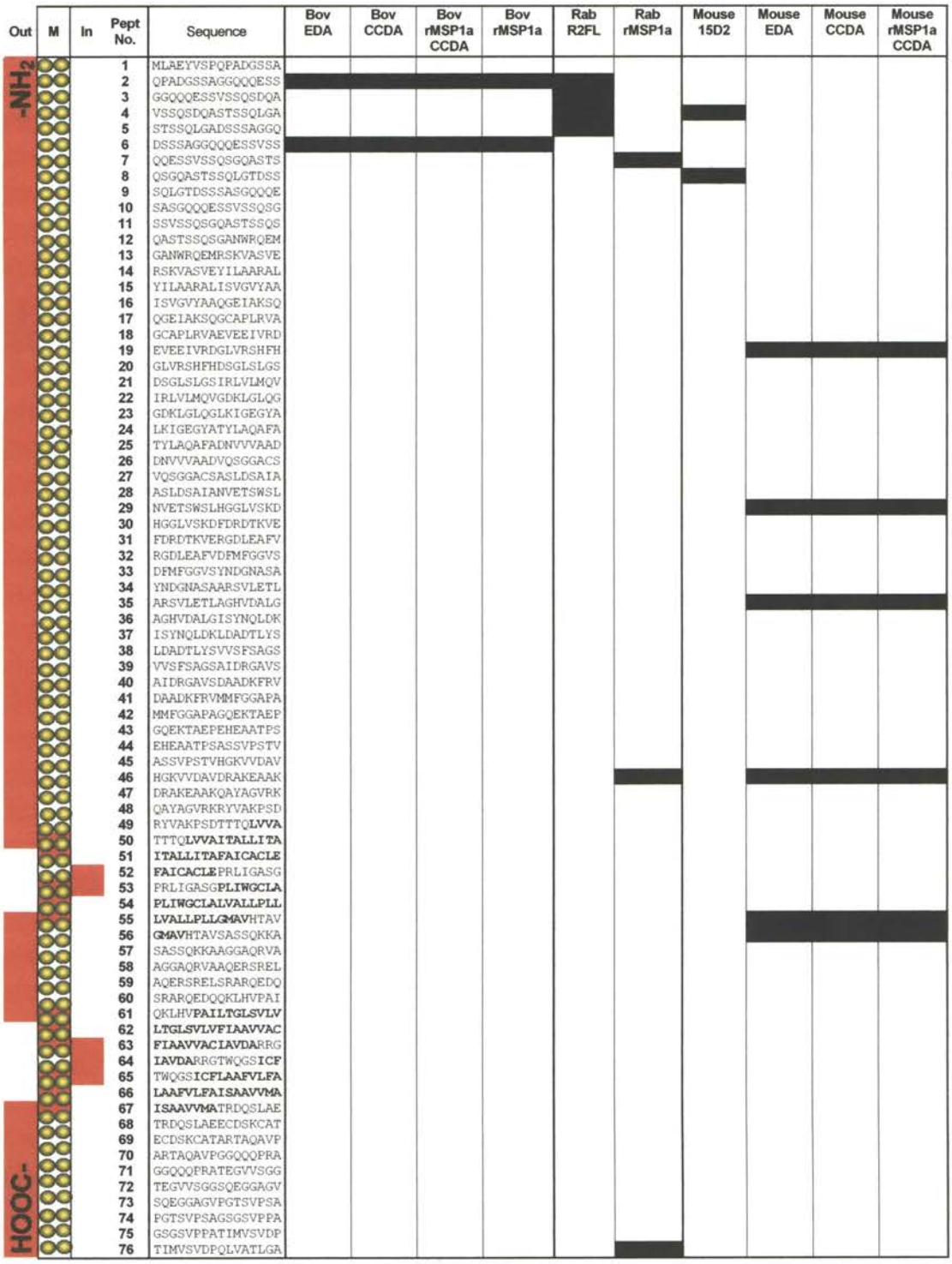
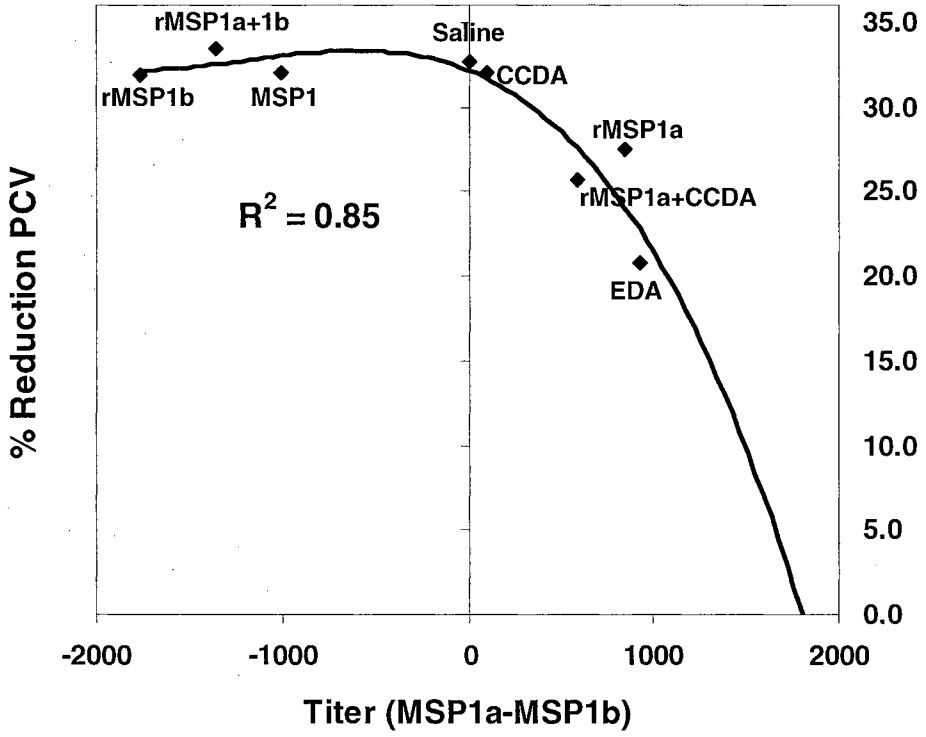

Out
M
In
Pept No.
Sequence
Bov EDA
Bov CCDA
Bov rMSP1a CCDA
Bov rMSP1a
Rab R2FL
Rab rMSP1a
Mouse 15D2
Mouse EDA
Mouse CCDA
Mouse rMSP1a CCDA
-NH2
HOOC-
1 MLAEYVSPQPADGSSA
2 QPADGSSAGGQQQESS
3 GGQQQESSVSSQSDQA
4 VSSQSDQASTSSQLGA
5 STSSQLGADSSSAGGQ
6 DSSSAGGQQQESSVSS
7 QQESSVSSQSGQASTS
8 QSGQASTSSQLGTDSS
9 SQLGTDSSSASGQQQE
10 SASGQQQESSVSSQSG
11 SSVSSQSGQASTSSQS
12 QASTSSQSGANWRQEM
13 GANWRQEMRSKVASVE
14 RSKVASVEYILAARAL
15 YILAARALISVGVYAA
16 ISVGVYAAQGEIAKSQ
17 QGEIAKSQGCAPLRVA
18 GCAPLRVAEVEEIVRD
19 EVEEIVRDGLVRSHFH
20 GLVRSHFHDSGLSLGS
21 DSGLSLGSIRLVLMQV
22 IRLVLMQVGDKLGLQG
23 GDKLGLQGLKIGEGYA
24 LKIGEGYATYLAQAFA
25 TYLAQAFADNVVVAAD
26 DNVVVAADVQSGGACS
27 VQSGGACSASLDSAIA
28 ASLDSAIANVETSWSL
29 NVETSWSLHGGLVSKD
30 HGGLVSKDFDRDTKVE
31 FDRDTKVERGDLEAFV
32 RGDLEAFVDFMFGGVS
33 DFMFGGVSYNDGNASA
34 YNDGNASAARSVLETL
35 ARSVLETLAGHVDALG
36 AGHVDALGISYNQLDK
37 ISYNQLDKLDADTLYS
38 LDADTLYSVVSFSAGS
39 VVSFSAGSAIDRGAVS
40 AIDRGAVSDAADKFRV
41 DAADKFRVMMFGGAPA
42 MMFGGAPAGQEKTAEP
43 GQEKTAEPEHEAATPS
44 EHEAATPSASSVPSTV
45 ASSVPSTVHGKVVDAV
46 HGKVVDAVDRAKEAAK
47 DRAKEAAKQAYAGVRK
48 QAYAGVRKRYVAKPSD
49 RYVAKPSDTTTQLVVA
50 TTTQLVVAITALLITA
51 ITALLITAFAICACLE
52 FAICACLEPRLIGASG
53 PRLIGASGPLIWGCLA
54 PLIWGCLALVALLPLL
55 LVALLPLLGMAVHTAV
56 GMAVHTAVSASSQKKA
57 SASSQKKAAGGAQRVA
58 AGGAQRVAAQERSREL
59 AQERSRELSRARQEDQ
60 SRARQEDQQKLHVPAI
61 QKLHVPAILTGLSVLV
62 LTGLSVLVFIAAVVAC
63 FIAAVVACIAVDARRG
64 IAVDARRGTWQGSICF
65 TWQGSICFLAAFVLFA
66 LAAFVLFAISAAVVMA
67 ISAAVVMATRDQSLAE
68 TRDQSLAEECDSKCAT
69 ECDSKCATARTAQAVP
70 ARTAQAVPGGQQQPRA
71 GGQQQPRATEGVVSGG
72 TEGVVSGGSQEGGAGV
73 SQEGGAGVPGTSVPSA
74 PGTSVPSAGSGSVPPA
75 GSGSVPPATIMVSVDP
76 TIMVSVDPQLVATLGA

Figure 5. Effect of antibodies specific for MSP1a and MSP1b on protection against *A. marginale* infection. The group mean percent reduction PCV was correlated with the group mean differential antibody titers (MSP1a minus MSP1b). Percent reduction PCV was calculated from the lowest PCV after challenge and the average PCV prior to challenge. The trendline was fitted to a cubic (third degree) polynomial equation using Microsoft Excel. Immunization groups are indicated.


rMSP1a+1b
Saline
rMSP1b
MSP1
CCDA
rMSP1a
rMSP1a+CCDA
EDA
R2 = 0.85
% Reduction PCV
35.0
30.0
25.0
20.0
15.0
10.0
5.0
0.0
-2000
-1000
0
1000
2000
Titer (MSP1a-MSP1b)

Chapter 4

GLYCOSYLATION OF *ANAPLASMA MARGINALE* MAJOR SURFACE PROTEIN 1A AND ITS PUTATIVE ROLE IN ADHESION TO TICK CELLS

Garcia-Garcia JC, de la Fuente J, Bell G, Blouin EF, Kocan KM.
Infection and Immunity, Submitted.

Abstract

Anaplasma marginale, the causative agent of bovine anaplasmosis, is a tick-borne rickettsial pathogen of cattle that multiplies in erythrocytes and tick cells. Major surface protein (MSP) 1a and MSP1b form the MSP1 complex of *A. marginale* which is involved in adhesion of the pathogen to host cells. In this study we tested the hypothesis that MSP1a and MSP1b were glycosylated because the observed molecular weights of both proteins were greater than the deduced molecular masses. We further hypothesized that the glycosylation of MSP1a plays a role in adhesion of *A. marginale* to tick cells. Native and *Escherichia coli*-derived recombinant MSP1a and MSP1b proteins were shown by gas chromatography to be glycosylated and to contain neutral sugars. Glycosylation of MSP1a appeared to be mainly O-linked to Ser/Thr residues in the N-terminal repeated peptides. Glycosylation may play a role in adhesion of *A. marginale* to tick cells because chemical deglycosylation of MSP1a significantly reduced its adhesive properties. Although the MSP1a polypeptide backbone alone was adherent to tick cell extract, the glycans in the N-terminal repeats appeared to enhance binding and may cooperatively interact with one or more surface molecules on host cells. These results demonstrated that MSP1a and MSP1b are glycosylated and suggest that the glycosylation of MSP1a plays a role in the adhesion of *A. marginale* to tick cells.

Introduction

Anaplasmosis is a tick-borne disease of cattle caused by the obligate intraerythrocytic rickettsia *Anaplasma marginale* (Rickettsiales: Anaplasmataceae). The acute phase of the disease is characterized by severe anemia, weight loss, fever, abortion, lower milk production and often death [1]. The only known site of infection of *A. marginale* in cattle is within erythrocytes [2]. The number of infected erythrocytes increases geometrically and removal of these infected cells by phagocytosis results in development of anemia and icterus without hemoglobinemia and hemoglobinuria. Cattle that recover from acute infection remain persistently infected, are protected from clinical disease, and serve as reservoirs of *A. marginale* for mechanical transmission and for biological transmission by ticks [3, 4].

The process of infection of host cells by *A. marginale* is initiated by adhesion of the rickettsia to the host cell membrane [5], a process that appears to be mediated by surface-exposed proteins on the pathogen and host cell receptors. Of the five major surface proteins (MSPs) identified on erythrocytic and tick stages of *A. marginale*, the MSP1 complex, composed of two polypeptides, MSP1a and MSP1b, has been shown to be involved in adhesion of *A. marginale* to host cells [6-8]. Immunization of cattle with the MSP1 complex has also been shown to induce partial protective immunity [9].

The *A. marginale* MSP1a is encoded by a single gene, *msp1*α [10], while MSP1b is encoded by at least two genes, *msp1*β*1* and *msp1*β*2* [11-13]. MSP1a has been shown to contain a neutralization-sensitive epitope [14] and to be an adhesin for both bovine erythrocytes and tick cells, whereas MSP1b is an adhesin only for bovine erythrocytes [6-8]. The extracellular N-

terminal region of MSP1a contains tandemly repeated peptides [15, 16] which have been shown to be necessary and sufficient for adhesion of *A. marginale* to tick cells and bovine erythrocytes [16]. MSP1a has also been shown to be involved in infection and transmission of *A. marginale* by *Dermacentor* spp. [17, 18].

The molecular mass of both MSP1a and MSP1b was found to be greater than the molecular weight predicted from their respective amino acid sequences [10, 19, 20]. Surface proteins of other rickettsial organisms, specifically *Ehrlichia chaffeensis* P120 and *E. canis* P140, were shown to be glycosylated, which accounted for the difference between their expected and observed molecular masses [21]. In addition, surface proteins from other Gram-negative bacteria have been shown to be glycosylated and the glycosylation appears to be involved in their ability to adhere to and invade host cells [22]. In this study, we determined that MSP1a and MSP1b from *A. marginale* are glycosylated. We then characterized the glycosylation of the native and recombinant MSP1a and MSP1b proteins and studied the role of these carbohydrate moieties in the adhesive properties of MSP1a for tick cells.

Materials and methods

Anaplasma marginale isolates

Isolates of *A. marginale* derived originally from California, Saint Maries (Idaho), Texas, Virginia, Okeechobee (Florida) and Oklahoma were used in these studies (Table 1) [16, 23].

Isolation of A. marginale from bovine erythrocytes and tick cells

Two splenectomized calves (3 month old, mixed breed beef cattle) were experimentally infected with the Oklahoma or Virginia isolates of *A. marginale.* Calf PA479 was infected with blood stabilate (Oklahoma isolate) retrieved from liquid nitrogen that was collected from calf PA407 at 10% percent parasitized erythrocytes (PPE) [23]. Calf PA433 was infected with the Virginia isolate of *A. marginale* by allowing *Dermacentor variabilis* males that acquired infection on calf PA432 [24] to feed on the calf and thus transmit *A. marginale.* The calves were maintained by the OSU Laboratory Animal Resources according to the Institutional Care and Use of Animals Committee guidelines. Infection of the calves was monitored by examination of stained blood smears. Bovine erythrocytes were collected from the calves at peak parasitemia (PA479, PPE 32.2%; PA433, PPE 18.9%), washed three times in phosphate-buffered saline (PBS), each time removing the buffy coat, and stored at –70°C.

A. marginale was propagated in the tick cell line, IDE8 (ATCC CRL 11973), derived originally from *Ixodes scapularis* embryos, as described previously [23, 25]. Briefly, tick cells were maintained at 31°C in L-15 B medium, pH 7.2, supplemented with 5% heat inactivated fetal bovine serum (Sigma, USA), 10% tryptose phosphate broth (Difco, USA) and 0.1% lipoprotein concentrate (ICN, USA), and the culture medium was replaced weekly. Monolayers of IDE8 cells were inoculated with the Oklahoma or Virginia isolate of *A. marginale* and monitored for infection by phase contrast microscopy and examination of stained smears.

Terminal cell cultures were harvested by centrifugation at approximately 10 days post-inoculation for analysis of MSP1a and MSP1b.

Infection of ticks and collection of salivary glands

Dermacentor variabilis were obtained from the Oklahoma State University, Centralized Tick Rearing Facility. Larvae and nymphs were fed on rabbits and sheep, respectively, and were then allowed to molt to the subsequent stage. Male ticks were held in a humidity chamber (90-95% RH) at 25°C with a 14-hr photoperiod until used for these studies. Uninfected males were allowed to acquire infection with the Oklahoma isolate by feeding for seven days on the infected calf PA479 during ascending parasitemia, after which the ticks were removed and held at room temperature in a humidity chamber for seven days. The ticks were then allowed to transmission feed on a sheep for seven days to allow for development of colonies of *A. marginale* in salivary glands, after which they were removed, the salivary glands dissected, pooled and used for analysis of *A. marginale* MSPs.

Cloning, expression and purification of recombinant MSP1a and MSP1b

The *msp1*α and *msp1*β_1 genes of the Oklahoma isolate of *A. marginale*, encoding for MSP1a and MSP1b, respectively, were cloned by PCR, fused to the FLAG peptide and expressed in *E. coli* as reported previously [8]. *E. coli* cells expressing recombinant MSP1a and MSP1b proteins were collected and disrupted by sonication in 0.1% Triton X-100 in Tris-buffered saline (TBS). The recombinant proteins were purified by FLAG-affinity chromatography (Sigma, USA) following the manufacturer's instructions. Expression and purification of the recombinant proteins was confirmed by SDS-polyacrylamide gel electrophoresis [26] and immunoblotting.

The *msp1α* genes from *A. marginale* isolates from California, Saint Maries (Idaho), Texas, Virginia and Okeechobee (Florida) were also cloned and expressed in *E. coli* as described previously for the Oklahoma isolate [16].

Construction of msp1α mutants

Two *msp1α* mutants were constructed for expression in *E. coli*. The first mutant contained only the sequence encoding for the N-terminal region of the MSP1a protein that includes the tandem repeats. The second mutant contained the sequence encoding for the conserved C-terminal region of MSP1a which lacks the tandemly repeated peptides. These mutants were obtained by PCR using the Oklahoma isolate *msp1a* gene as described previously [16].

SDS-polyacrylamide gel electrophoresis and immunoblotting

Protein samples were loaded on 10% polyacrylamide gels that were stained with Coomassie Brilliant Blue or transferred to nitrocellulose membranes. Membranes were blocked with 5% skim milk for 60 min at room temperature. Western blot analysis was performed using monoclonal antibodies ANA15D2 (VMRD, USA) and AFOR2.2F1 (produced in our laboratory), specific for the repeats and the conserved C-terminal region of MSP1a, respectively, anti-FLAG M2 monoclonal antibody for detection of recombinant fusion proteins or MSP1b-monospecific rabbit serum for detection of MSP1b. After washing with TBS, membranes were incubated with 1:10,000 goat anti-mouse IgG or goat anti-rabbit IgG alkaline phosphatase conjugate (KPL, USA). Membranes were washed again and the color developed using BCIP/NBT alkaline phosphatase substrate (Sigma, USA).

Identification of glycoproteins

Protein glycosylation was detected on blots of pure proteins or crude extracts by a modification of the method of Haselbeck and Hösel [27, 28]. Briefly, 10 μg total protein of crude extracts or 2 μg of purified protein was loaded, separated in a 10% SDS-PAGE gel, and transferred to a nitrocellulose membrane. The membrane was equilibrated for 10 min in 0.1 M acetic acid and carbohydrates were oxidized for 20 min at room temperature in the dark with 10 mM sodium metaperiodate in 0.1 M acetic acid. The membrane was washed twice with 0.1 M acetic acid and once with 0.05% Tween-20, 0.1 M acetic acid. Biotin-hydrazide (Bio-Rad, USA) in 0.05% Tween-20 and 0.1 M acetic acid was then added and allowed to react for 60 min at RT in order to label the aldehydes that resulted from oxidation of the carbohydrates. After three washes with 0.05% Tween-20 in TBS, the membrane was blocked for 30 min and incubated with a 1:2,000 solution of streptavidin-alkaline phosphatase conjugate (Bio-Rad, USA). The membrane was washed again with TBST and stained with BCIP/NBT (Sigma, USA) as substrate.

Estimation of glycoprotein carbohydrate content

The carbohydrate content of purified recombinant MSP1a was estimated using a glycoprotein carbohydrate estimation kit (Pierce, USA) according to the manufacturer's instructions.

Analysis of monosaccharide composition by gas chromatography

Analysis of the carbohydrate composition of pure MSP1a and MSP1b glycoproteins was performed using gas liquid chromatography of the trimethylsilyl glycoside derivatives [29]. Affinity-purified MSP1a and MSP1b glycoproteins were dialyzed extensively against water and then freeze-dried. Inositol was added prior to drying the samples to serve as an internal

standard. The protein samples were hydrolyzed with 1.5 M methanolic HCl and methyl acetate for 3 hours at 80°C. The samples were dried under a stream of N_2 and the sugars derivatized with a 3:1 trimethylsilyl: pyridine mixture for 15 minutes at room temperature. The trimethylsilyl sugar derivatives were dried, dissolved in isooctane and separated on a DB-1 fused silica capillary column (J & W Scientific Inc., USA) using a Varian 3300 gas chromatographer (Sunnyvale, USA). Monosaccharide amounts were calculated by relative comparison of the peak areas.

Analysis of monosaccharide composition by capillary electrophoresis

The monosaccharide composition of MSP1a and MSP1b was studied by capillary zone electrophoresis. Affinity-purified glycoproteins (2 μg) and 3-O-methyl glucose as internal standard were dried in a centrifugal vacuum evaporator. The glycans were hydrolyzed to monosaccharides with trifluoroacetic acid at 121°C for 60 min. The monosaccharides were then derivatized with a fluorescent label by adding 3 mg/ml anthranilic acid, 4% sodium acetate, 2% borate in methanol, and the labeling reaction was allowed to proceed at 80°C for 2 h. The methanol was evaporated and the samples were dissolved in water. Analytical separation of derivatized monosaccharides was performed in a Biofocus 2000 CZE instrument (Bio-Rad, USA) and detection was achieved by laser induced fluorescence. The amount of individual monosaccharides was estimated by comparison to the internal standard.

Enzymatic deglycosylation

Affinity-purified recombinant MSP1a and MSP1b proteins were denatured with SDS and β-mercaptoethanol prior to the enzymatic deglycosylation reaction to increase the efficiency of deglycosylation. Enzymes used in this study included endoglycosidases PNGase F

(*N*-glycosidase F) and O-glycosidase DS, specific for N-linked oligosaccharides and Gal(β-1,3) GalNAc(α1), respectively, and exoglycosidases GALase III (β1-4 galactosidase), HEXase I (β1-2,3,4,6 *N*-acetylhexosaminidase), NANase II (β2–3,6 neuraminidase), specific for β1-4 galactose, β-linked *N*-acetylglucosamine, and α2-3 and α2-6 *N*-acetylneuraminic acid residues, respectively. These enzymes were provided in the Enzymatic Deglycosylation Enhancement Kit (Bio-Rad, USA) and were used following the manufacturer's instructions.

Chemical deglycosylation with TFMS

Purified recombinant MSP1a protein (500 μg) was dialyzed extensively against 0.1% trifluoroacetic acid and then freeze-dried. The MSP1a protein was deglycosylated by anhydrous trifluoromethanesulfonic (TFMS) acid treatment according to the instructions of the GlycoFree Deglycosylation Kit (Glyko Inc., USA). The TFMS acid cleaves protein-linked glycans non-selectively from the glycoprotein while leaving the primary structure of the protein intact [30].

MALDI-TOF mass spectrometry analysis

To confirm that the MSP1a amino acids were not modified by the chemical deglycosylation with TFMS acid, native and deglycosylated MSP1a proteins were digested with trypsin and the proteolytic fragments analyzed by Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) mass spectrometry, which was performed using a Voyager DE PRO mass spectrometer (Applied Biosystems, USA) in the positive mode with reflectron, 20 kV accelerating voltage, 70% grid voltage with delayed extraction. Affinity-purified protein preparations were digested with Trypsin Gold (Promega, USA) and extracted following the manufacturer's instructions. The protein digest samples and

α-cyano-4-hydroxycinnamic acid (α-CHCA) matrix (in 50% acetonitrile, 0.3% trifluoroacetic acid) were spotted on the MALDI plate and allowed to dry at room temperature. External mass calibration was achieved using a mixture of peptide standards containing des-Arg^1-Bradykinin, Angiotensin I, Glu^1-Fibrinopeptide B and ACTH 1-17 (Sigma, USA) that was spotted next to the sample. Spectra from 250 laser shots were summed to obtain the accumulated spectrum. The peak lists generated from the analysis of native and deglycosylated MSP1a proteins were compared.

Tick cell binding assay

The capacity of glycosylated and deglycosylated recombinant MSP1a to bind to tick cell extract was determined using a modification of an in vitro binding assay that has been used in several studies to define MSP1a as an adhesin for tick gut and cultured tick cells [8, 16, 17, 33]. Cultured IDE8 tick cells were sonicated in 0.1% Triton X-100 and centrifuged at 12,000 x g. Tick cell proteins (1 μg/well) were used for coating a 96-well plate for 3 h at 37°C. The plate was washed three times with TBST and blocked for 2 h at 37°C with 2% skim milk. Serial 1:2 dilutions of native and deglycosylated pure recombinant MSP1a protein were added to the tick cell extract starting at 10 μg/well. Recombinant MSP1b was used as a negative control of binding. After incubating for 1 h at 37°C, the plate was washed with TBST and incubated with 1:1,000 anti-FLAG M2 monoclonal antibody (Sigma, USA) for 1 h at 37°C. The plate was washed and incubated with 1:2,000 goat anti-mouse IgG horseradish peroxidase conjugate (KPL, USA) for 1 h at 37°C. TMB in 0.05 M phosphate-citrate buffer, pH 5, containing 0.03% sodium perborate (Sigma, USA) was used for color development. The reaction was stopped with 2 N H_2SO_4 and the OD was read at 450 nm.

Protein sequence analysis and prediction of glycosylation sites

The amino acid sequences of MSP1a and MSP1b proteins from several isolates of *A. marginale* were obtained from GenBank (Table 1). The amino acid composition and the predicted molecular weight for each isolate were determined by use of the statistical analysis of protein sequences algorithm [31], and the observed molecular masses were estimated from the electrophoretic mobility in SDS-PAGE.

Prediction of potential *O*-glycosylation sites in the MSP1a and MSP1b protein sequences was performed using the NetOGlyc 2.0 algorithm [32]. Potential *N*-glycosylation sites were predicted by identifying Asn-X-Ser/Thr sequences present in the MSP1a and MSP1b amino acid sequences.

Results

Sequence analysis and prediction of potential glycosylation sites

The amino acid sequences of MSP1a and MSP1b, deduced from the Oklahoma isolate *msp1α* and *msp1β1* gene coding sequences, respectively, were analyzed for predicted *N*- and *O*-glycosylation sites, as well as for the amino acid composition. Oklahoma isolate MSP1a was found to be a Ser/Thr rich protein and contained 18% Ser/Thr (109 Ser +Thr / 623 a.a.). The Ser/Thr content was particularly high in the region containing the tandemly repeated peptides (43%), suggesting an O linkage for possible carbohydrate modifications. Most of the Ser/Thr residues were conserved among the MSP1a repeats of different *A. marginale* isolates (Fig. 1). Although every Ser or Thr residue may be a potential *O*-glycosylation site, we used NetOGlyc 2.0 algorithm to predict which Ser/Thr residues were more likely to be glycosylated (Fig. 2). Of the 25 residues predicted to be *O*-glycosylated, 14 sites were identified in the N-terminal tandem repeats (Fig. 2A). Only one Asn-Xaa-Ser/Thr, as indicated by analysis of the potential *N*-glycosylation sites in the MSP1a sequence, was found to be present in the Oklahoma isolate MSP1a (Fig. 2A), and this Asn residue is not located in the repeated peptides.

MSP1b contained only 12% Ser/Thr (90 S+T / 744 a.a.) and only one of these sites was predicted using NetOGly 2.0 to be *O*-glycosylated (Fig. 2B). However, seven Asn-Xaa-Ser/Thr sites were present in MSP1b (Fig. 2B), all of which may be potential *N*-glycosylation sites.

Molecular weights of native and recombinant MSP1a and MSP1b proteins

Although the molecular masses predicted from the deduced sequences of *A. marginale* MSP1a and MSP1b (Oklahoma isolate) were of 63 kDa and 79 kDa, respectively, the observed molecular weights of the recombinant *E. coli*-derived proteins were 90 kDa for MSP1a and 100

kDa for MSP1b (Fig. 3A, B). Native MSP1a and MSP1b proteins derived from *A. marginale*-infected cultured tick cells, erythrocytes and tick salivary glands had molecular weights similar to recombinant proteins (Fig. 3). The molecular weight of the MSP1a protein from a second isolate of *A. marginale* from Virginia, which contains a different number of tandemly repeated peptides, were also higher than predicted from their amino acid sequences (Fig. 3A, lanes 5-7). The recombinant MSP1a protein from the Virginia isolate (Fig. 3A, lane 5) had molecular weights similar to those of the native proteins (Fig. 3A, lanes 6, 7).

The *msp1a* gene that encodes for MSP1a was cloned from various geographic isolates of *A. marginale* and expressed in *E. coli*. The MSP1a proteins from these isolates contained 2-8 tandemly repeated peptides (Table 1). The deduced molecular masses of the proteins were calculated from their deduced primary sequence, and correlated with the number of repeated peptides in the same protein (Fig. 4). The correlation fit with the equation [MW(MSP1a) = 2.8 × N + 55.5] in which N is the number of repeats and the intercept, 55.5 kDa, is an estimate of the molecular mass of the C-terminal region of MSP1a that is conserved among isolates. The slope, 2.8 kDa, represents the average deduced molecular mass of a single repeat. The observed molecular weights of the recombinant MSP1a proteins from all of the isolates studied, estimated from their electrophoretic mobility (Fig. 5A), were greater than the predicted molecular weights (Fig. 4), and the dependency with the number of repeats was described by the equation [MW(MSP1a) = 10.5×N + 62.5] which demonstrated that the molecular weights of both the conserved region and the repeated N-terminal peptides are greater than their deduced molecular masses. This equation also indicated that the average weight of a single repeat was 10.5 kDa, approximately 8 kDa greater than the molecular mass

predicted from the amino acid sequence. The observed molecular weight of the MSP1a mutant that contained only the N-terminal repeats was approximately 30 kDa (Fig. 6, lane 1), similar to the molecular mass of 31.5 kDa predicted by the second equation, and was 3.6 times larger than the molecular mass predicted from the primary sequence and the first equation.

Detection of glycosylation and estimation of carbohydrate content

Glycosylation assays were performed in order to determine whether the difference between the deduced and observed molecular weights of MSP1a and MSP1b was due to glycosylation of the proteins. Crude extracts of recombinant *E. coli* cells expressing the recombinant proteins were labeled with biotin-hydrazide after oxidation with sodium periodate. Glycosylation was detected in the recombinant MSP1a proteins from all the *A. marginale* isolates analyzed (Fig. 5B), as well as in the recombinant MSP1b protein (Fig. 6B, lane 4). The carbohydrate content was estimated to be 17% for MSP1a and >40% for MSP1b. Furthermore, glycosylation was detected on the two mutant MSP1a proteins expressed in *E. coli* that contained either the conserved C-terminal region alone or the N-terminal repeats (Fig. 6B, lanes 1, 2).

Monosaccharide compositional analysis

The monosaccharide compositions of the recombinant MSP1a and MSP1b glycoproteins were determined by gas liquid chromatography. Four neutral sugars, glucose, galactose, mannose and xylose, were detected in the recombinant MSP1a (Table 2), while the recombinant MSP1b protein contained glucose, galactose and mannose (Table 2). Glucose was the most abundant monosaccharide in both recombinant proteins. These results were confirmed by capillary electrophoresis (data not shown).

Enzymatic deglycosylation analysis

The nature and structure of the glycans attached to MSP1a and MSP1b were characterized by treating affinity-purified recombinant MSP1a and MSP1b proteins with the endoglycosidases PNGase F, O-Glycosidase DS and the exoglycosidases GALase III, HEXase I and NANase II. Enzymatic treatment did not increase the electrophoretic mobility of MSP1a and MSP1b (data not shown). Therefore, these enzymes, which are specific for carbohydrate moieties commonly present in *N*- and *O*-glycoproteins, were not able to hydrolyze the glycans present in MSP1a and MSP1b glycoproteins.

Deglycosylation of MSP1a and binding to tick cells

Recombinant MSP1a protein was chemically deglycosylated with TFMS acid in order to determine the role of carbohydrate modifications in the adhesive properties of the MSP1a for tick cells. Deglycosylation was determined by the increased electrophoretic mobility of the deglycosylated protein (Fig. 7). The peptide backbone of the MSP1a protein did not appear to be altered after acid deglycosylation treatment because the deglycosylated protein was recognized by three monoclonal antibodies specific for epitopes in the N-terminal repeats, the conserved C-terminal region and the C-terminally fused FLAG peptide (Fig. 7, lanes 4-6). Moreover, the peptide masses generated by the tryptic digestion of deglycosylated MSP1a, analyzed by MALDI-TOF mass spectrometry, matched peptide masses of the reported Oklahoma isolate MSP1a protein, indicating that no covalent modifications were introduced in the MSP1a amino acid backbone by the chemical deglycosylation with TFMS acid.

Tick cell binding assays were conducted to compare the adhesive properties of the native and deglycosylated MSP1a protein. The native MSP1a glycoprotein bound to tick cells

(Fig. 8). Although the deglycosylated MSP1a protein also adhered to tick cells, its adhesive capacity was significantly reduced ($P<0.01$) with respect to native MSP1a.

Discussion

Several bacterial glycoproteins were reported recently and were shown to play a role in bacterial adhesion, invasion and pathogenesis. Glycosylation of outer membrane proteins was also described in several Gram-negative bacteria [22], including *E. coli* and the rickettsial tick-borne pathogens, *E. canis and E. chaffeensis.* [21]. In addition, recombinant proteins from *A. phagocytophilum*, *E. chaffeensis* and *E. ruminantium* expressed in *E. coli* were also found to be glycosylated [33].

Adhesion of *A. marginale* to host cells initiates the process of infection. Previous studies have demonstrated that polypeptides that compose the MSP1 complex, MSP1a and MSP1b, serve as *A. marginale* adhesins for tick cells and bovine erythrocytes [6-8]. We recently characterized the functional domain of MSP1a and have shown that the tandemly repeated peptides are necessary and sufficient to mediate adhesion of MSP1a to tick cells and bovine erythrocytes [16]. A critical role of specific amino acids in the adhesive capacity of MSP1a was determined by use of a synthetic peptide model system [16].

The molecular weights of MSP1a and MSP1b have been determined by SDS-PAGE to be greater than the predicted molecular masses [10, 19], and the difference between the expected and observed molecular weights was posited to be due to the presence of carbohydrate modifications on these proteins [14, 34]. In this study we demonstrated that both MSP1a and MSP1b from several *A. marginale* isolates are glycosylated. Glycosylation was particularly abundant in the N-terminal region of MSP1a that contains the repeated peptides. The repeated peptides of the Oklahoma isolate, which contain 43% Ser/Thr, were shown to be glycosylated and were predicted to be *O*-glycosylated using the NetOGlyc *O*-glycosylation

prediction algorithm. Most of these Ser/Thr residues were found to be conserved among the different MSP1a repeats, particularly the residues at or next to the neutralization sensitive epitope and the amino acid in position 20 that appears to be important for adhesion to tick cells [10, 14, 16]. Potential *N*-glycosylation sites were not present in this region, supporting the hypothesis that these glycans are O-linked. However, unusual modifications, known to occur in other bacterial glycoproteins [35], may also be present.

The number and type of potential glycosylation sites on MSP1a and MSP1b were different. While MSP1a contained a greater number of predicted *O*-glycosylation sites, MSP1b contained more potential *N*-glycosylation sites. Although only neutral sugars were detected in glycoproteins of both MSP1a and MSP1b, the difference in the number and type of glycosylation sites suggests that carbohydrate differences occur between the two proteins. The sugar composition of MSP1a and MSP1b indicates an unusual type of glycosylation in MSP1a and MSP1b. A similar carbohydrate composition has been described previously for the rickettsial recombinant proteins *E. chaffeensis* P120 and *E. canis* P140 expressed in *E. coli* [21]. The absence of amino sugars was also consistent with previous studies in which MSP1a did not label with ^{3}H-glucosamine [14]. While *N*-acetylglucosamine and *N*-acetylgalactosamine are commonly present in the core carbohydrate structure of eukaryotic glycoproteins, the types of glycosylation identified in prokaryotes have been variable [22]. Several lectins that recognize carbohydrate structures with *N*-acetylglucosamine and *N*-acetylgalactosamine did not bind to MSP1a [14], which provides further evidence of an unusual pattern of glycosylation. These results were also supported by the inability of exo- and endoglycosidases, specific for glycans that contain amino sugars, to deglycosylate recombinant MSP1a.

Although protein glycosylation in *E. coli* had been reported previously [22], glycosylation of heterologous recombinant proteins was thought not to occur until recently when a number of recombinant rickettsial proteins expressed in *E. coli* were shown to be glycosylated [21, 33]. The ability of *E. coli* to glycosylate heterologous proteins appears to be specific for prokaryotic proteins that are glycosylated in their native form and therefore contain the required glycosylation sites. These recombinant proteins are also transported to the appropriate cellular location, most likely the plasma membrane, to become glycosylated.

Although we demonstrated previously that several recombinant rickettsial proteins expressed in *E. coli* were glycosylated [33], only two of these proteins, the *A. marginale* MSP1a and the *E. ruminantium* mucin-like protein, proved to be adherent for tick cells using an in vitro adhesion assay [16, 33]. These two proteins had the highest content of Ser/Thr residues in the tandem repeats among those studied. These proteins appeared to be *O*-glycosylated and these O-linked glycans may be involved in adhesion to tick cells. In the present study, binding of recombinant MSP1a to tick cells was noticeably reduced when MSP1a was deglycosylated with TFMS acid, thus providing evidence that glycosylation plays a role in adhesion. Further studies are needed because the chemical deglycosylation may have introduced chemical modifications in amino acid residues of the protein that may have reduced the adhesive properties of the protein. However, chemical deglycosylation of other proteins did not affect their biological activity [30].

We also demonstrated that the deglycosylated peptide backbone of MSP1a was able to bind to tick cell extracts, although at reduced levels. The combined results of these and previous studies in which we used synthetic peptides that model the MSP1a repeats [16]

suggest that both the MSP1a peptidic backbone and its carbohydrate modifications are involved in the cooperative interaction with putative host cell receptors. Recent studies on a closely-related organism, *A. phagocytophilum,* demonstrated that *A. phagocytophilum* binds cooperatively to sites on the N-terminal peptide of human PSGL-1 and to carbohydrate moieties on the same or different molecules [36, 37]. In addition to MSP1a, MSP1b and MSP2 have been shown to be adhesins for bovine erythrocytes [6-8] and, therefore, these MSPs may cooperate in adhesion of *A. marginale* to erythrocytes.

Glycosylation of *A. marginale* surface proteins may also influence the capacity of the pathogen to generate antigenic diversity and to escape the host's immune response, as has been demonstrated for other bacterial and viral pathogens [38, 39]. While major amino acid changes may affect the conformation and thus function of the protein, minor amino acid changes may only alter the pattern of glycosylation, thus generating new antigenic variants that may allow pathogens to evade the host immune response [39]. In addition, glycosylation of proteins can occur in multiple forms, a phenomenon known as microheterogeneity, which may further contribute to antigenic diversity. Completion of the sequence of the *A. marginale* genome may provide the opportunity to identify genes encoding for the glycosylation machinery, as well as other glycosylated proteins. This approach has shown to be productive for the study of other pathogenic bacteria [40].

This research provides the first evidence of the role of glycosylation of *A. marginale* surface proteins in adhesion to host cells and may contribute to development of more effective vaccine strategies for control of this economically important pathogen of cattle.

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REFERENCES

1. Kuttler KL. *Anaplasma* infections in wild and domestic ruminants: a review. J Wild Dis 1984;20:12-20.

2. Ristic M, Watrach AM. Anaplasmosis. VI. Studies and a hypothesis concerning the cycle of development of the causative agent. Am J Vet Res 1963;24:267-276.

3. Dikmans G. The transmission of anaplasmosis. Am J Vet Res 1950;11:5-16.

4. Ewing SA. Transmission of *Anaplasma marginale* by arthropods. In: Proc. 7th National Anaplasmosis Conference, Mississippi State University, MS, 1981:395-423.

5. Blouin EF, Kocan KM. Morphology and development of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in cultured *Ixodes scapularis* (Acari: Ixodidae) cells. J Med Entomol 1998;35:788-797.

6. McGarey DJ, Barbet AF, Palmer GH, McGuire TC, Allred DR. Putative adhesins of *Anaplasma marginale*: major surface polypeptides 1a and 1b. Infect Immun 1994;62:4594-4601.

7. McGarey DJ, Allred DR. Characterization of hemagglutinating components on the *Anaplasma marginale* initial body surface and identification of possible adhesins. Infect Immun 1994;62:4587-4593.

8. de la Fuente J, Garcia-Garcia JC, Blouin EF, Kocan KM. Differential adhesion of major surface proteins 1a and 1b of the ehrlichial cattle pathogen *Anaplasma marginale* to bovine erythrocytes and tick cells. Int J Parasitol 2001;31:145-153.

9. Palmer GH, Barbet AF, Cantor GH, McGuire TC. Immunization of cattle with the MSP-1 surface protein complex induces protection against a structurally variant *Anaplasma marginale* isolate. Infect Immun 1989;57:3666-3669.

10. Allred DR, McGuire TC, Palmer GH, Leib SR, Harkins TM, McElwain TF, Barbet AF. Molecular basis for surface antigen size polymorphisms and conservation of a neutralization-sensitive epitope in *Anaplasma marginale*. Proc Natl Acad Sci 1990;87:3220-3224.

11. Barbet AF, Palmer GH, Myler PJ, McGuire TC. Characterization of an immunoprotective protein complex of *Anaplasma marginale* by cloning and expression of the gene coding for polypeptide Am105L. Infect Immun 1987;55:2428-2435.

12. Camacho-Nuez M, de Lourdes Munoz M, Suarez CE, McGuire TC, Brown WC, Palmer GH. Expression of polymorphic *msp1β* genes during acute *Anaplasma marginale* rickettsemia. Infect Immun 2000;68:1946-1952.

13. Bowie MV, de la Fuente J, Kocan KM, Blouin EF, Barbet AF. Conservation of major surface protein 1 genes of the ehrlichial pathogen *Anaplasma marginale* during cyclic transmission between ticks and cattle. Gene 2000;282:95-102.

14. Palmer GH, Waghela SD, Barbet AF, Davis WC, McGuire TC. Characterization of a neutralization sensitive epitope on the AM 105 surface protein of *Anaplasma marginale*. Int J Parasitol 1987;17:1279-1285.

15. de la Fuente J, Garcia-Garcia JC, Blouin EF, Rodriguez SD, Garcia MA, Kocan KM. Evolution and function of tandem repeats in the major surface protein 1a of the ehrlichial pathogen *Anaplasma marginale*. Anim Health Res Rev 2002;2:163-173.

16. de la Fuente J, Garcia-Garcia JC, Blouin EF, Kocan KM. Characterization of the functional domain of major surface protein 1a involved in adhesion of the rickettsia *Anaplasma marginale* to host cells. Vet Microbiol 2003;91:265-283.

17. de la Fuente J, Garcia-Garcia JC, Blouin EF, Kocan KM. Major surface protein 1a effects tick infection and transmission of the ehrlichial pathogen *Anaplasma marginale*. Int J Parasitol 2001;31:1705-1714.

18. Blouin EF, Saliki JT, de la Fuente J, Garcia-Garcia JC, Kocan KM. Antibodies to *Anaplasma marginale* major surface proteins 1a and 1b inhibit infection for cultured tick cells. Vet Parasitol 2003;111:247-260.

19. Oberle SM, Palmer GH, Barbet AF, McGuire TC. Molecular size variations in an immunoprotective protein complex among isolates of *Anaplasma marginale*. Infect Immun 1988;56:1567-1573.

20. Barbet AF, Allred DR. The msp1 beta multigene family of *Anaplasma marginale*: nucleotide sequence analysis of an expressed copy. Infect Immun 1991;59:971-976.

21. McBride JW, Yu XJ, Walker DH. Glycosylation of homologous immunodominant proteins of *Ehrlichia chaffeensis* and *Ehrlichia canis*. Infect Immun 2000;68:13-18.

22. Benz I, Schmidt MA. Never say never again: protein glycosylation in pathogenic bacteria. Mol Microbiol 2002;45: 267-276.

23. Blouin EF, Barbet AF, Jooyoung Yi, Kocan KM, Saliki JT. Establishment and characterization of an Oklahoma isolate of *Anaplasma marginale* in cultured *Ixodes scapularis* cells. Vet Parasitol 2000;87:301-313.

24. de la Fuente J, Garcia-Garcia JC, Blouin EF, Saliki JT, Kocan KM. Infection of tick cells and bovine erythrocytes with one genotype of the intracellular ehrlichia *Anaplasma marginale* excludes infection with other genotypes. Clin Diagn Lab Immunol 2001;9:658-668.

25. Munderloh UG, Blouin EF, Kocan KM, Ge NL, Edwards WL, Kurtti TJ. Establishment of the tick (Acari:

Ixodidae)-borne cattle pathogen *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in tick cell culture. J Med Ent 1996;33:656-664.

26. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-685.

27. Haselbeck A, Hösel W. Immunological detection of glycoproteins on blots based on labeling with digoxigenin. Methods Mol Biol 1993;14:161-73.

28. Bouchez-Mahiout I, Doyen C, Lauriere M. Accurate detection of both glycoproteins and total proteins on blots: control of side reactions occurring after periodate oxidation of proteins. Electrophoresis 1999;20:1412-1417.

29. Komalavilas P, Mort AJ. The acetylation of O-3 of galacturonic acid in the rhamnose-rich portion of pectins. Carbohyd Res 1989;189:261-272.

30. Tams JW, Welinder KG. Mild chemical deglycosylation of horseradish peroxidase yields a fully active, homogeneous enzyme. Anal Biochem 1995;228:48-55.

31. Brendel V, Bucher P, Nourbakhsh I, Blaisdell BE, Karlin S. Methods and algorithms for statistical analysis of protein sequences. Proc Natl Acad Sci USA 1992;89:2002-2006.

32. Hansen JE, Lund O, Tolstrup N, Gooley AA, Williams KL, Brunak S. NetOglyc: prediction of mucin type O-glycosylation sites based on sequence context and surface accessibility. Glycoconjugate J 1998;15:115-130.

33. de la Fuente J, Garcia-Garcia JC, Barbet AF, Blouin EF, Kocan KM. Adhesion of outer membrane proteins containing tandem repeats of *Anaplasma* and *Ehrlichia* species (Rickettsiales: Anaplasmataceae) to tick cells. Vet Microbiol 2003; In Press.

34. Brown, WC. Palmer GH, Lewin HA, Mcguire, TC. CD4(+) T lymphocytes from calves immunized with *Anaplasma marginale* major surface protein 1 (MSP1), a heteromeric complex of MSP1a and MSP1b, preferentially recognize the MSP1a carboxyl terminus that is conserved among strains. Infect Immun 2001;69:6853-6862.

35. Messner P, Christian R, Neuninger C, Schulz G. Similarity of "core" structures in two different glycans of tyrosine-linked eubacterial S-layer glycoproteins. J Bacteriol 1995;177:2188-2193.

36. Herron MJ, Nelson CM, Larson J, Snapp KR, Kansas GS, Goodman JL. Intracellular parasitism by the human granulocytic ehrlichiosis bacterium through the P-selectin ligand, PSGL-1. Science 2000;288:1653-1656.

37. Yago T, Leppanen A, Carlyon JA, Akkoyunlu M, Karmakar S, Fikrig E, Cummings R. D, McEver RP. Structurally distinct requirements for binding of P-selectin glycoprotein ligand-1 and sialyl Lewis x to *Anaplasma phagocytophilum* and P-

selectin. J Biol Chem 2003;278:37987-37997.

38. Alm RA, Guerry P, Power ME, Trust TJ. Variation in antigenicity and molecular weight of *Campylobacter coli* VC167 flagellin in different genetic backgrounds. J Bacteriol 1992;174:4230-4238.

39. Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, Salazar-Gonzalez JF, Salazar MG, Kilby JM, Saag MS, Komarova NL, Nowak MA, Hahn BH, Kwong PD, Shaw GM. Antibody neutralization and escape by HIV-1. Nature 2003;422:307-312.

40. Power PM, Jennings MP. The genetics of glycosylation in Gram-negative bacteria. FEMS Microbiol Lett 2003;218:211-222.

41. McGuire TC, Palmer GH, Goff WL, Johnson MI, Davis WC. Common and isolate-restricted antigens of *Anaplasma marginale* detected with monoclonal antibodies. Infect Immun 1984;45:697-700.

42. de la Fuente J, Van Den Bussche RA, Kocan KM. Molecular phylogeny and biogeography of North American isolates of *Anaplasma marginale* (Rickettsiaceae: Ehrlichieae). Vet Parasitol 2001;97:65-76.

43. Eriks IS, Stiller D, Goff WL, Panton M, Parish SM, McElwain TF, Palmer GH. Molecular and biological characterization of a newly isolated *Anaplasma marginale* strain. J Vet Diagn Invest 1994;6:435-441.

44. Palmer GH, Rurangirwa FR, McElwain TF. Strain composition of the ehrlichia *Anaplasma marginale* within persistently infected cattle, a mammalian reservoir for tick transmission. J Clin Microbiol 2001;39:631-635.

TABLE 1. *Anaplasma marginale* isolates and MSP1a proteins included in the study.

Isolate Name/Origin	MSP1a No. Repeats	Predicted Mol. Wt.[a]	GenBank Accession No.	Reference
Virginia	2	60.8	M32870	McGuire et al. [41]
Oklahoma	3	63.5	AY010247	Blouin et al. [23]
California	3	NR	AY010242	de la Fuente et al. [42]
St. Maries, ID	3	63.5	AF293062	Eriks et al. [43]
Rasmussen	3	63.5	AF293064	Palmer et al. [44]
South Dakota	3	63.7	AF293063	Palmer et al. [44]
Texas	4	NR	AF428091	McGuire et al. [41]
Washington	4	66.4	M32869	Allred et al. [10]
Okeechobee, FL	5	NR	AY010244	de la Fuente et al. [17]
Idaho	6	71.7	M32868	Allred et al. [10]
Florida	8	77.5	M32871	Allred et al. [10]

[a] The molecular mass of the MSP1a proteins was predicted from their amino acid sequences using the statistical analysis of protein sequences algorithm. NR indicates MSP1a proteins for which the complete coding sequence have not been reported.

TABLE 2. Monosaccharide composition of recombinant *Anaplasma marginale* MSP1a and MSP1b.

Monosaccharide	**MSP1a**	**MSP1b**
Glucose	66.5	67.3
Galactose	16.0	12.1
Mannose	6.0	20.6
Xylose	11.5	0.0

[a] Amounts of monosaccharides are expressed as the percent of total monosaccharides in the glycoprotein, as determined by gas chromatography of the trimethylsilyl glycoside derivatives.

Figure 1. Conservation of Ser/Thr residues (highlighted) in the tandem repeats encoded by *Anaplasma marginale msp1a* from different isolates. The amino acid positions are indicated above the sequences. The arrowhead points to the 20th amino acid, which is involved in interaction with tick cells. The neutralization-sensitive epitope recognized by monoclonal antibody ANA15D2 is indicated by the bracket. Sequences were obtained from de la Fuente J, Passos LMF, Van Den Bussche RA, Ribeiro MFB, Facury-Filho EJ, Kocan KM. Submitted for publication.

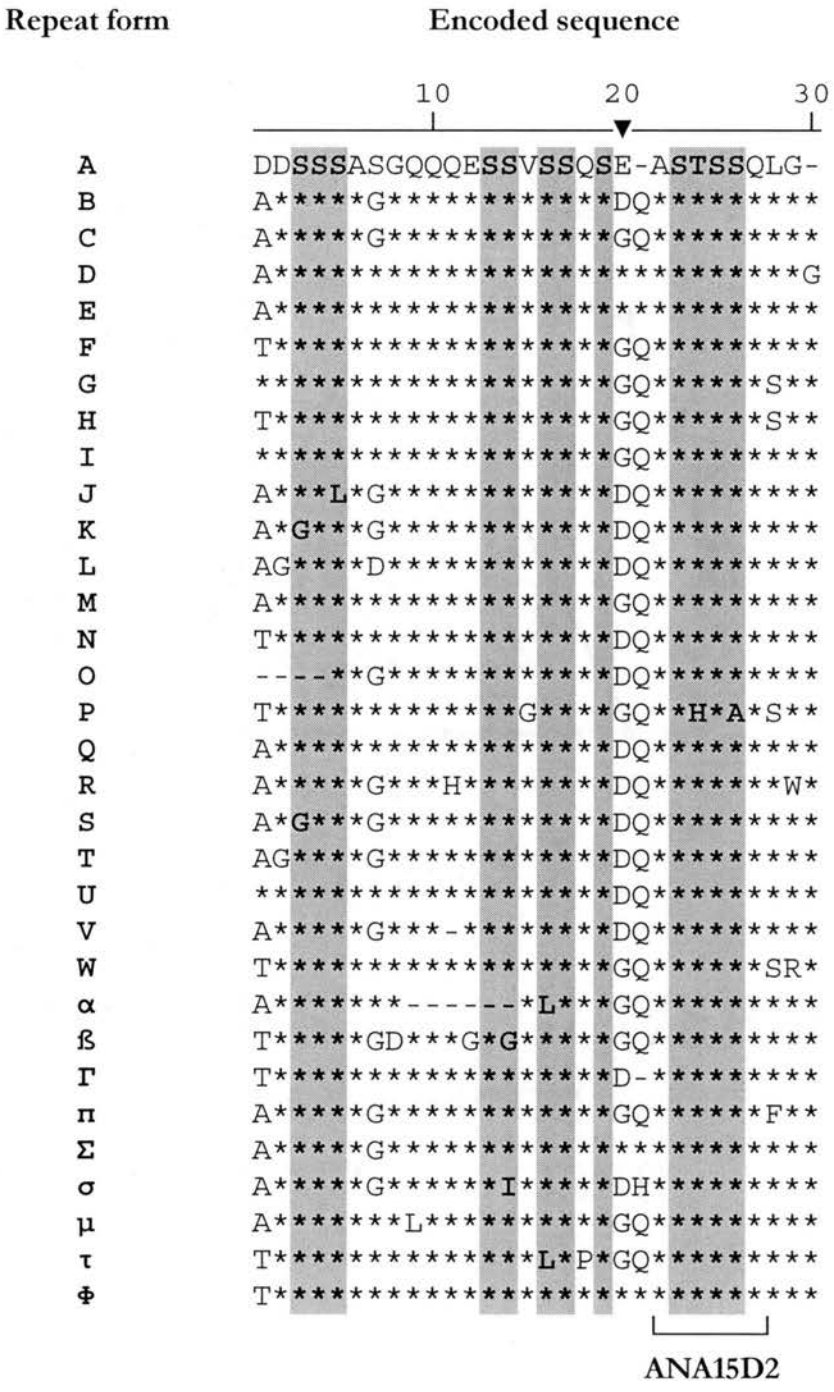
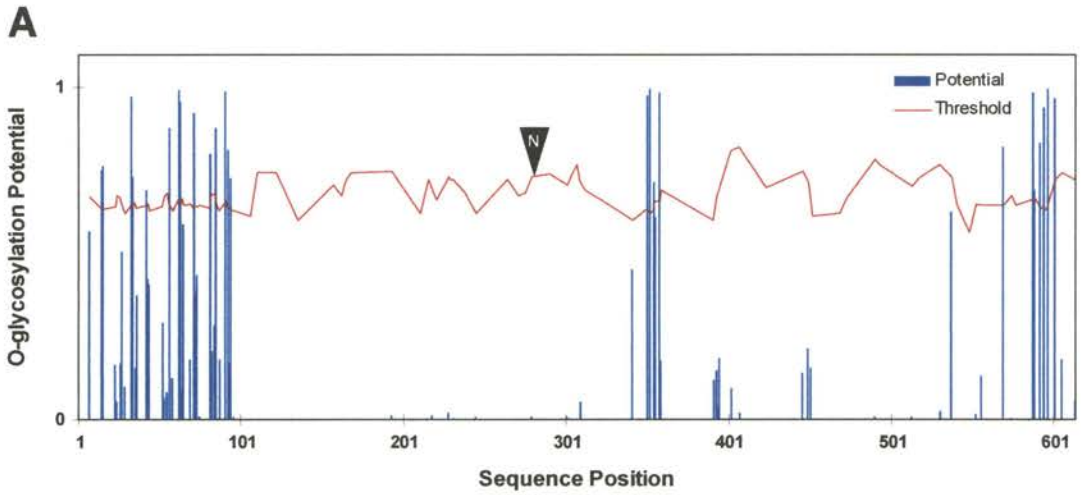

Repeat form
Encoded sequence
10
20
30
A DDSSSASGQQQESSVSSQSE-ASTSSQLG-
B A****G**********DQ*********
C A****G**********GQ*********
D A**********************G
E A**************************
F T****************GQ*********
G ****************GQ*****S**
H T****************GQ*****S**
I ****************GQ*********
J A***L*G**********DQ*********
K A*G***G**********DQ*********
L AG****D**********DQ*********
M A****************GQ*********
N T****************DQ*********
O ----**G**********DQ*********
P T**********G****GQ**H*A*S**
Q A****************DQ*********
R A****G***H*******DQ*******W*
S A*G***G**********DQ*********
T AG****G**********DQ*********
U ****************DQ*********
V A****G***-*******DQ*********
W T****************GQ*****SR*
α A******------**L***GQ*********
ß T****GD***G*G****GQ*********
Γ T****************D-*********
π A****G**********GQ*****F**
Σ A****G**********************
σ A****G******I*****DH*********
μ A******L**********GQ*********
τ T**********L*P*GQ*********
Φ T**********************
ANA15D2

Figure 2. Predicted glycosylation sites in (A) MSP1a and (B) MSP1b from the Oklahoma isolate of *A. marginale*. *O*-glycosylation was predicted using NetOGlyc 2.0 prediction algorithm to occur in the amino acid positions in which the *O*-glycosylation potential (blue bars) is greater than the threshold (red curve). Arrowheads indicate potential *N*-glycosylation sites (Asn-Xaa-Ser/Thr sequences).


A
Potential
Threshold
O-glycosylation Potential
1
0
N
1
101
201
301
401
501
601
Sequence Position

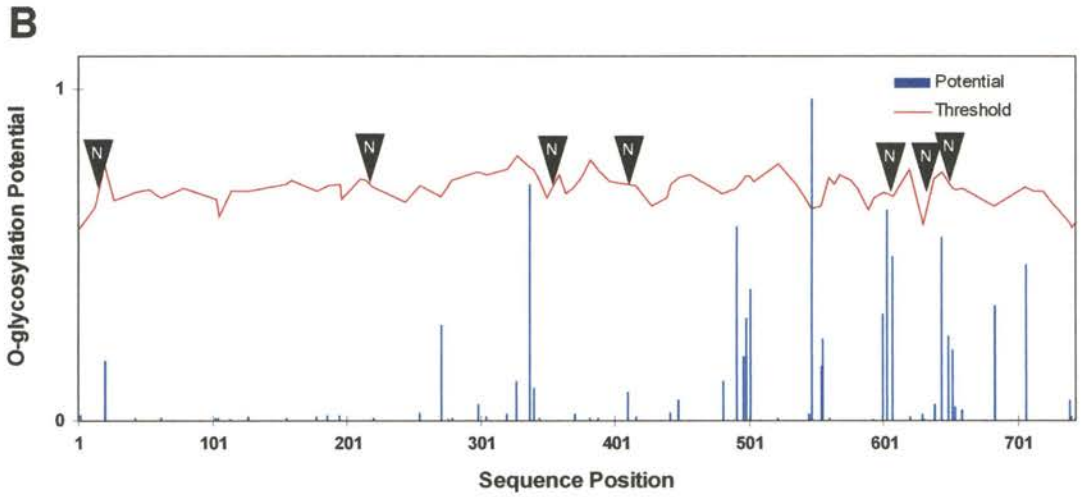
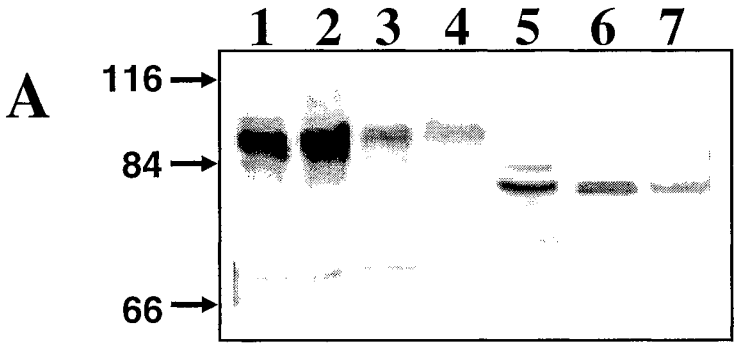

B
Potential
Threshold
O-glycosylation Potential
1
0
N
N
N
N
N
N
N
1
101
201
301
401
501
601
701
Sequence Position

Figure 3. Western blot analysis of native and recombinant (A) MSP1a and (B) MSP1b proteins from the Oklahoma isolate (lanes 1-4) and Virginia isolate (lanes 5-7) of *A. marginale.* Samples of recombinant proteins expressed in *E. coli* (lanes 1, 5), erythrocyte-derived *A. marginale* (lanes 2, 6), tick cell culture-derived *A. marginale* (lanes 3, 7) and infected tick salivary glands (lane 4) were separated by SDS-PAGE and reacted with (A) anti-MSP1a MAb ANA15D2 or (B) rabbit polyclonal anti-MSP1b serum. Arrows on the left indicate mol. wt. markers in kDa.


A
1 2 3 4 5 6 7
116
84
66

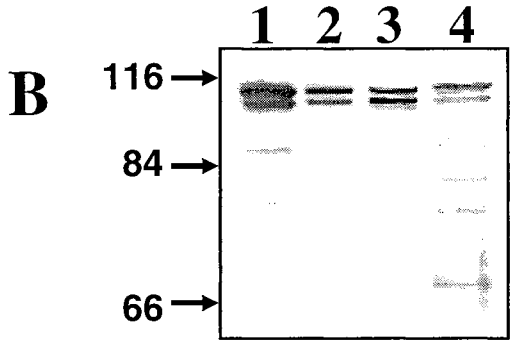

B
1 2 3 4
116
84
66

Figure 4. Dependence of the MSP1a molecular mass upon the number of tandem repeats. The predicted (squares) and observed (triangles) molecular masses of recombinant MSP1a from different *A. marginale* isolates expressed in *E. coli* were calculated from the reported amino acid sequence or estimated from the electrophoretic mobility, respectively. The intercept indicates the molecular mass of the conserved C-terminal region and the slope the average molecular mass of a single repeat.

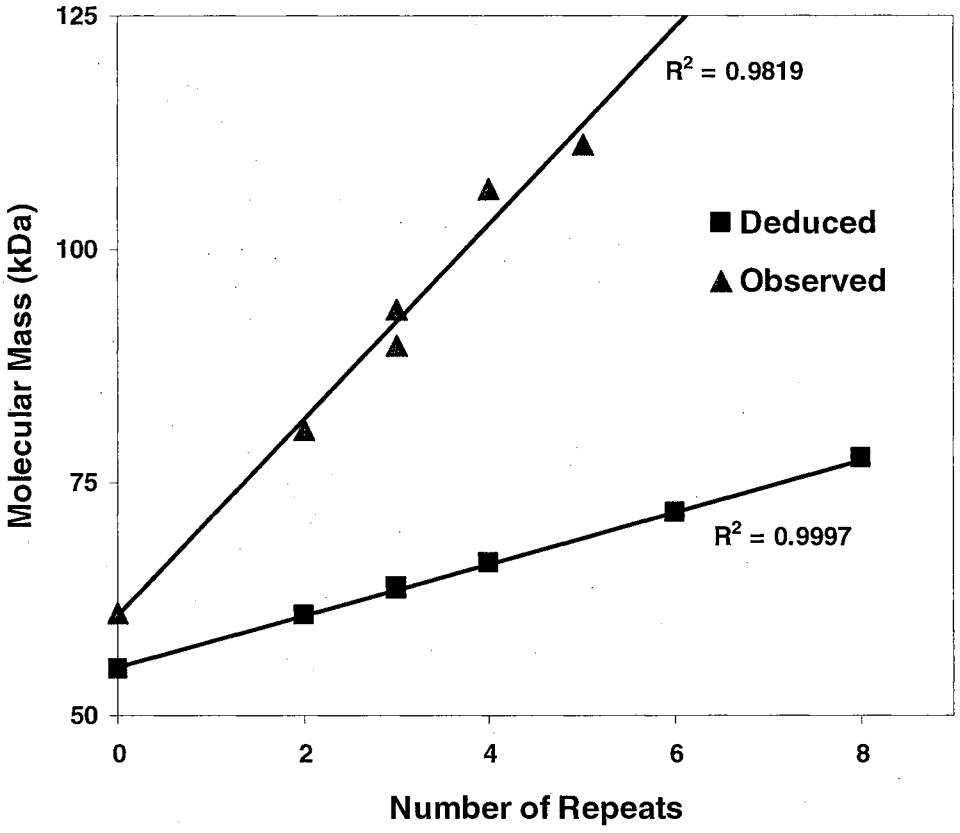

125
100
75
50
Molecular Mass (kDa)
0
2
4
6
8
Number of Repeats
R2 = 0.9819
R2 = 0.9997
Deduced
Observed

Figure 5. Analysis of MSP1a proteins from different *A. marginale* isolates expressed in *E. coli*. Recombinant *E. coli* cell extracts were separated by SDS-PAGE, transferred to a nitrocellulose membrane and (A) reacted with anti-MSP1a MAb ANA15D2 or (B) stained with carbohydrate-specific periodate oxidation and biotin hydrazide conjugation. Arrowheads indicate the recombinant MSP1a protein bands. Numbers on the left indicate molecular weights in kDa. Lane 1, Negative *E. coli* control. Lanes 2-7, protein extract of recombinant *E. coli* cells expressing MSP1a protein from *A. marginale* isolates from Virginia (lane 2), Oklahoma (lane 3), California (lane 4), St. Maries (lane 5), Texas (lane 6), and Okeechobee (lane 7).

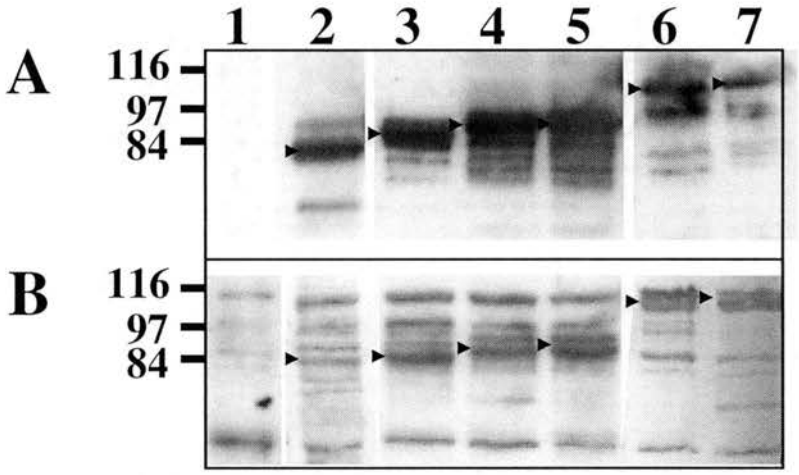

1
2
3
4
5
6
7
A
116
97
84
B
116
97
84

Figure 6. Analysis of MSP1b and mutant MSP1a proteins expressed in *E. coli*. Proteins were purified by FLAG-affinity chromatography, separated by SDS-PAGE, transferred to a nitrocellulose membrane and (A) reacted with anti-MSP1a MAb ANA15D2 (lane 1), anti-MSP1a MAb AFOR2.2F1 (lane 2, 3), or rabbit polyclonal anti-MSP1b serum (lane 4); or (B) stained specifically for carbohydrates. Lane 1, Oklahoma isolate MSP1a repeats; lane 2, MSP1a without the repeats; Oklahoma isolate MSP1a (lane 3), and MSP1b (lane 4). Arrowheads indicate recombinant protein bands. Numbers on the left indicate molecular weights in kDa.

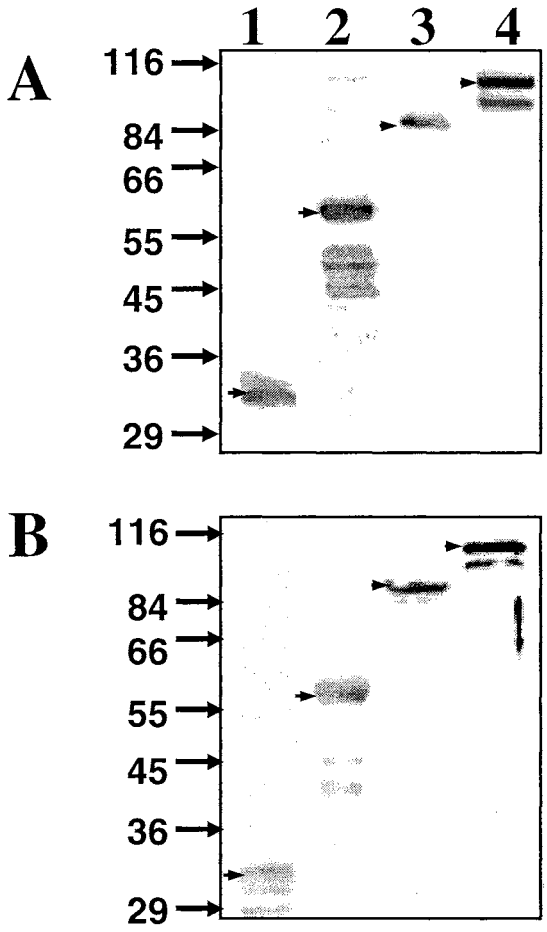

A
B
1
2
3
4
116
84
66
55
45
36
29

Figure 7. Chemical deglycosylation of MSP1a with TFMS. Native (lanes 1-3) and deglycosylated (lanes 4-6) MSP1a was transferred to a nitrocellulose membrane and reacted with anti-MSP1a MAb ANA15D2 (lanes 1, 4), anti-MSP1a MAb AFOR2.2F1 (lanes 2, 5), anti-FLAG M2 MAb (lanes 3, 6).

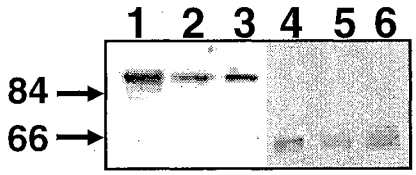
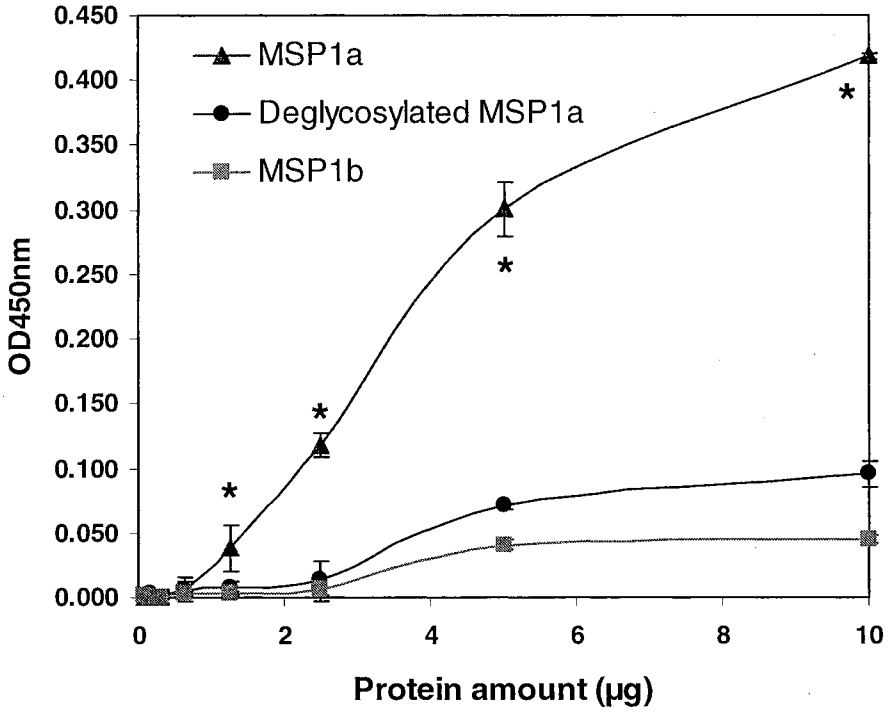

1 2 3 4 5 6
84
66

Figure 8. Binding of glycosylated and deglycosylated MSP1a to tick cells. Recombinant MSP1a, MSP1b and deglycosylated MSP1a were assayed in vitro for their ability to bind to tick cell proteins. Binding was expressed as the OD_{450nm} (mean ± S.D.) from three replicates. Asterisks denote statistically significant difference ($P<0.05$) between native and deglycosylated MSP1a determined using an ANOVA test.


0.450
0.400
0.350
0.300
0.250
0.200
0.150
0.100
0.050
0.000
OD450nm
MSP1a
Deglycosylated MSP1a
MSP1b
*
*
*
*
0
2
4
6
8
10
Protein amount (µg)

Chapter 5

SUMMARY

The major surface protein (MSP) 1a of the rickettsial tick-borne pathogen, *Anaplasma marginale*, is a functionally important surface protein. MSP1a in combination with MSP1b forms the MSP1 complex and both of these surface proteins were found to be structurally conserved on *A. marginale* derived from bovine erythrocytes and tick cells. The MSP1 complex has been shown to be involved in adhesion of the pathogen to host cells. MSP1a is an adhesin for tick cells and bovine erythrocytes, whereas MSP1b is an adhesin only for bovine erythrocytes. The N-terminal region of MSP1a, which contains tandemly repeated peptides, is necessary and sufficient to mediate adhesion of *A. marginale* to bovine erythrocytes and tick cells. MSP1a also contains a neutralization-sensitive epitope and is involved in immune protection against *A. marginale* infection.

Preliminary data that led to this research was the discovery that the antibody response of cattle immunized with *A. marginale* derived from bovine erythrocytes or tick cell culture differed. Cattle immunized with erythrocyte derived antigen had a preferential antibody response to MSP1a, whereas cattle immunized with tick cell culture-derived antigen developed a preferential antibody response to MSP1b. We also confirmed that the observed molecular weight of MSP1a was greater than the predicted molecular weight which led to our hypothesis that this difference may be due to the glycosylation of this protein. Both the regulation of the expression and the post-translational modifications of surface proteins may influence the ability of intracellular rickettsia to adhere to and infect both vertebrate and ticks cells during the parasite life cycle.

The research described in this thesis focuses on the characterization of the antigenic determinants, expression and glycosylation of the *A. marginale* MSP1a. We hypothesized that the regulation of the expression of MSP1a by *A. marginale* differs in bovine erythrocytes and tick cells and this differential expression influences the antibody response of cattle immunized with erythrocyte or tick cell-derived *A. marginale*. We further hypothesized that immunized cattle develop an antibody response against B-cell epitopes of MSP1a and that this antibody response is involved in protection against *A. marginale* infection. Finally, we hypothesized that MSP1a is glycosylated and that the glycosylation may influence the adhesive properties of the protein.

The molecular basis of the differential antibody response to *A. marginale* derived from bovine erythrocytes and tick cells was studied using Western blot, confocal microscopy and reverse transcriptase (RT)-PCR. Expression of MSP1b by *A. marginale* derived from bovine and tick host cells was similar at the protein and RNA levels, whereas expression of MSP1a by *A. marginale* in these cells differed. Low levels of MSP1a were observed in cultured tick cells and tick salivary glands, but high expression of MSP1a occurred on *A. marginale* derived from bovine erythrocytes. The analysis of the expression of the *msp1a* gene by RT-PCR suggested that the differential expression of MSP1a is regulated at the transcriptional level and may influence the infectivity of *A. marginale* for host cells. Variation in the expression of MSP1a may also contribute to phenotypic and antigenic changes in the pathogen.

We characterized the MSP1a antibody response of cattle using several immunogens, including rMSP1a protein, erythrocyte- or tick cell culture-derived *A. marginale*, or a combination of tick cell culture-derived *A. marginale* and rMSP1a. The MSP1a antibody response elicited by all these immunogens was directed primarily against the N-terminal region

of MSP1a, whereas low antibody levels were detected against the C-terminal portion of the protein. Linear B-cell epitopes of MSP1a were mapped using synthetic peptides representing the entire sequence of the protein. Only two peptides, both of which contained the linear epitope SSAGGQQQESS, were recognized by sera from immunized cattle. These epitopes were mapped to the N-terminal repeated peptides of MSP1a. The average differential of antibody titers against MSP1a minus those against MSP1b correlated with lower percent reductions in PCV. A preferential antibody response to MSP1a was observed in cattle immunized with erythrocyte-derived, cell culture-derived plus rMSP1a or rMSP1a, and the percent reduction PCV was significantly lower in these cattle as compared with the other immunization groups. Although we characterized the linear B-cell epitopes of MSP1a, the conformational or non-peptidic components of MSP1a may also be involved in protection against *A. marginale* invasion.

Since the observed molecular weight of MSP1a was greater than the deduced molecular mass, we determined whether the MSP1a protein was glycosylated. Native and *Escherichia coli*-derived recombinant MSP1a and MSP1b proteins were shown by gas chromatography to be glycosylated and to contain neutral sugars. Glycosylation of MSP1a appeared to be mainly O-linked to Ser/Thr residues in the N-terminal repeated peptides. Glycosylation may play a role in adhesion of *A. marginale* to tick cells because chemical deglycosylation of MSP1a significantly reduced its adhesive properties. Although the MSP1a polypeptide backbone alone was adherent to tick cell extract, the glycans in the N-terminal repeats appeared to enhance binding and may cooperatively interact with one or more surface molecules on host cells. Alternatively, glycosylation of the N-terminal repeats of MSP1a may increase the affinity of MSP1a for its host cell receptor.

The results of this research further confirm the importance of MSP1a in the adhesion and development of *A. marginale* in host cells. Furthermore, bovine immune response to MSP1a appears to be involved in the development of protection against *A. marginale* infection. The results of this research contribute to a better understanding of the expression, post-translational modifications and antigenic determinants of MSP1a and will be important in the development of more effective methods for the control of anaplasmosis and its transmission.

VITA 2

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Candidate for the Degree of
Doctor of Philosophy

Thesis: CHARACTERIZATION OF THE ANTIGENIC DETERMINANTS, GLYCOSYLATION AND EXPRESSION OF THE MAJOR SURFACE PROTEIN 1A OF *ANAPLASMA MARGINALE*

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Professional Memberships: American Association of Veterinary Parasitologists, American Society for Rickettsiology, Conference of Research Workers on Animal Diseases, Oklahoma Microscopy Society, Sigma Xi The Scientific Research Society, Society for Tropical Veterinary Medicine, The Protein Society.