

# *Anaplasma marginale* (Rickettsiales: Anaplasmataceae): recent advances in defining host–pathogen adaptations of a tick-borne rickettsia

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## SUMMARY

The tick-borne intracellular pathogen *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) develops persistent infections in cattle and tick hosts. While erythrocytes appear to be the only site of infection in cattle, *A. marginale* undergoes a complex developmental cycle in ticks and transmission occurs via salivary glands during feeding. Many geographic isolates occur that vary in genotype, antigenic composition, morphology and infectivity for ticks. In this chapter we review recent research on the host–vector–pathogen interactions of *A. marginale*. Major surface proteins (MSPs) play a crucial role in the interaction of *A. marginale* with host cells. The MSP1a protein, which is an adhesin for bovine erythrocytes and tick cells, is differentially regulated and affects infection and transmission of *A. marginale* by *Dermacentor* spp. ticks. MSP2 undergoes antigenic variation and selection in cattle and ticks, and contributes to the maintenance of persistent infections. Phylogenetic studies of *A. marginale* geographic isolates using *msp4* and *msp1a* provide information about the biogeography and evolution of *A. marginale*: *msp1a* genotypes evolve under positive selection pressure. Isolates of *A. marginale* are maintained by independent transmission events and a mechanism of infection exclusion in cattle and ticks allows for only the infection of one isolate per animal. Prospects for development of control strategies by use of pathogen and tick-derived antigens are discussed. The *A. marginale*/vector/host studies described herein could serve as a model for research on other tick-borne rickettsiae.

Key words: *Anaplasma marginale*, ticks, rickettsia, anaplasmosis.

## INTRODUCTION

*Anaplasma marginale* is a tick-borne pathogen that causes the disease anaplasmosis in cattle (Ristic, 1968; Bram, 1975). This pathogen is classified within the Order Rickettsiales which was recently reorganized into two families, Anaplasmataceae and Rickettsiaceae, based on genetic analyses of 16S rRNA, *groELS* and surface protein genes (Dumler *et al.* 2001) (Table 1). Organisms of the family Anaplasmataceae are obligate intracellular organisms that are found exclusively within membrane-bound vacuoles in the host cell cytoplasm. Within the family Anaplasmataceae, phylogenetic analyses consistently supported the formation of four distinct genetic groups of the organisms: (1) *Anaplasma* with a 96·1% similarity; (2) *Ehrlichia* (97·7% similarity); (3) *Wolbachia* with a minimum of 95·6% similarity; and (4) *Neorickettsia* with a minimum of 94·9% similarity (Dumler *et al.* 2001). The genus *Anaplasma* currently includes the three pathogens of ruminants, *A. marginale*, *A. centrale* which was originally described by Theiler

(1911) as a subspecies of *A. marginale* and *A. ovis*, as well as the addition of *A. bovis* (formerly *Ehrlichia bovis*), *A. phagocytophilum* (formerly *E. phagocytophilum*, *E. equi* and the HGE agent), and *A. platys* (formerly *E. platys*). *Aegyptianella*, also included in this genus, was retained as a *genus incertae sedis* due to lack of sequence information.

*A. marginale* is distributed worldwide in tropical and subtropical regions of the New World, Europe, Africa, Asia and Australia. Several geographic isolates of *A. marginale* have been identified in North and South America, which differ in morphology, protein sequence, antigenic characteristics and their ability to be transmitted by ticks (Smith *et al.* 1986; Wickwire *et al.* 1987; Allred *et al.* 1990; Rodriguez *et al.* 2000; de la Fuente *et al.* 2001*a,c*, 2003*a*; Palmer, Rurangirwa & McElwain, 2001).

*A. marginale* develops persistent infections in mammalian and tick hosts, both of which serve as reservoirs for infection of susceptible hosts. The only known site of replication of *A. marginale* in cattle is bovine erythrocytes (Richey, 1981) (Fig. 1). Within these cells membrane-bound inclusion bodies contain from 4–8 rickettsiae, and as many as 70% or more of the erythrocytes may become infected during acute infection and disease. Removal of infected cells by the bovine reticuloendothelial system results in mild to severe anaemia and icterus (Richey, 1981).

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Table 1. Current classification of the order Rickettsiales

|  |
|--|
| Order Rickettsiales  |
| Family Rickettsiaceae: Obligat intracellular bacteria that grow freely in the cytoplasm of their eukaryotic host cells                           |
| Genus <i>Rickettsia</i>  |
| Genus <i>Orientia</i>  |
| Family Anaplasmataceae: Obligat intracellular bacteria that replicate within membrane-derived vacuoles in the cytoplasm of eukaryotic host cells |
| Genus <i>Anaplasma</i>   |
| <i>Anaplasma marginale</i> (type species)  |
| <i>Anaplasma centrale</i>  |
| <i>Anaplasma ovis</i>  |
| <i>Anaplasma bovis</i> (formerly <i>Ehrlichia bovis</i> )  |
| <i>Anaplasma phagocytophilum</i> (formerly <i>Ehrlichia phagocytophilum</i> , <i>E. equi</i> , HGE agent)  |
| <i>Anaplasma platys</i> (formerly <i>Ehrlichia platys</i> )  |
| <i>Aegyptianella</i> (genus <i>incertae sedis</i> due to lack of sequence information)   |
| Genus <i>Ehrlichia</i>   |
| <i>Ehrlichia chaffeensis</i>   |
| <i>Ehrlichia ruminantium</i> (formerly <i>Cowdria ruminantium</i> )  |
| <i>Ehrlichia ewingii</i>   |
| <i>Ehrlichia ovis</i>  |
| <i>Ehrlichia canis</i>   |
| <i>Ehrlichia muris</i>   |
| Genus <i>Neorickettsia</i>   |
| <i>Neorickettsia helminthoeca</i>  |
| <i>Neorickettsia risticii</i> (formerly <i>Ehrlichia risticii</i> )  |
| <i>Neorickettsia sennetsu</i> (formerly <i>Ehrlichia sennetsu</i> )  |
| Genus <i>Wolbachia</i>   |
| <i>Wolbachia pipientis</i>   |

The acute phase of the disease is characterized by weight loss, fever, abortion, lowered milk production and often death.

Mechanical transmission of *A. marginale* occurs when infected blood is transferred to susceptible cattle via blood-contaminated fomites or mouthparts of biting flies (Ewing, 1981). Biological transmission of *A. marginale* is effected by ticks and approximately 20 species of ticks have been incriminated as vectors worldwide (Dikmans, 1950; as reviewed by Ewing, 1981). An updated listing of tick species tested as vectors of *A. marginale* (Table 2) lists conflicting reports on transmission of *A. marginale* by some tick species. These inconsistencies may have resulted because some *A. marginale* isolates (i.e. those from Illinois, Florida and California) have subsequently proven not to be transmissible by *Dermacentor* spp. ticks (Smith *et al.* 1986; Wickwire *et al.* 1987; de la Fuente *et al.* 2001*a*, 2003*a*). In general, tick vectors of *A. marginale* include *Boophilus* spp., selected *Dermacentor* spp., *Ixodes ricinus* and *Rhipicephalus* spp., while *Amblyomma* spp. do not appear to transmit *A. marginale*. Although transovarial transmission has been reported for *D. andersoni* (Howell, Stiles & Moe, 1941), others have not demonstrated this mode of transmission of *A. marginale*. However, transovarial transmission of *A. marginale* by the one-host *Boophilus* ticks has not been thoroughly investigated and may warrant further studies.

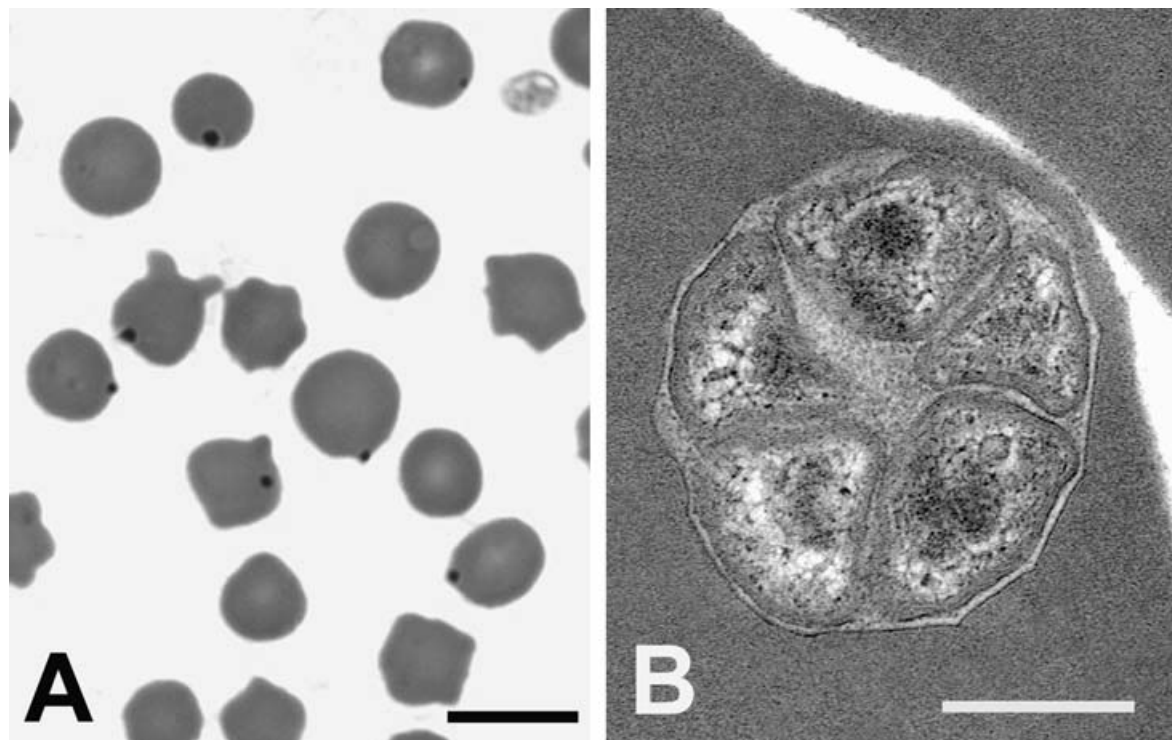


Fig. 1. Bovine erythrocytes infected with *Anaplasma marginale*. (A) Inclusion bodies are located at the periphery of the erythrocyte in a stained blood film. (B) An electron micrograph of an *A. marginale* inclusion that contains five organisms. A, bar = 10  $\mu\text{m}$ ; B, bar = 0.5  $\mu\text{m}$ .

Table 2. Studies in which Ixodid and Argasid ticks were tested as vectors of anaplasmosis

| Tick species                    | Reference                              | Transmission (+/-)*        |   |
|---------------------------------|--|----------------------------|---|
| <b>Ixodid Ticks</b>             |  |                            |   |
| <i>Amblyomma americanum</i>     | Piercy & Schmidt, 1941                 | -                          |   |
|                                 | Rees, 1934                             | -                          |   |
|                                 | Sanborn & Moe, 1934                    | -                          |   |
| <i>Amblyomma cajennense</i>     | Rees, 1934                             | -                          |   |
|                                 | Sanborn & Moe, 1934                    | -                          |   |
| <i>Amblyomma maculatum</i>      | Piercy, 1938                           | -                          |   |
|                                 | Piercy & Schmidt, 1941                 | -                          |   |
|                                 | Rees, 1934                             | -                          |   |
| <i>Boophilus annulatus</i>      | Rees, 1934                             | +/-                        |   |
|                                 | Samish, Pipano & Hadani, 1993          | +                          |   |
| <i>Boophilus calcaratus</i>     | Sergent <i>et al.</i> 1945             | +/-                        |   |
| <i>Boophilus decoloratus</i>    | Theiler, 1912                          | +                          |   |
| <i>Boophilus microplus</i>      | Bock & DeVos, 1999                     | +                          |   |
|                                 | Brumpt, 1931                           | +                          |   |
| <i>Dermacentor albipictus</i>   | Rosenbusch & Gonzalez, 1927            | +                          |   |
|                                 | Boynton <i>et al.</i> 1936             | +                          |   |
|                                 | Ewing <i>et al.</i> 1997               | +                          |   |
|                                 | Sanborn & Moe, 1934                    | -                          |   |
|                                 | Stiller, Leatch & Kuttler, 1981        | +                          |   |
|                                 | Stiller & Johnson, 1983                | +                          |   |
| <i>Dermacentor andersoni</i>    | Anthony & Roby, 1962                   | + (- transovarial)         |   |
|                                 | Anthony & Roby, 1966                   | +                          |   |
|                                 | Boynton <i>et al.</i> 1936             | +                          |   |
|                                 | Eriks, Stiller & Palmer, 1993          | +                          |   |
|                                 | Howell, Stiles & Moe, 1941             | + transovarial             |   |
|                                 | Kocan <i>et al.</i> 1981               | +                          |   |
|                                 | Kocan <i>et al.</i> 1992a, b           | +                          |   |
|                                 | Peterson <i>et al.</i> 1977            | +                          |   |
|                                 | Rees, 1933                             | +                          |   |
|                                 | Rees, 1934                             | +                          |   |
|                                 | Rozeboom, Stiles & Moe, 1940           | +/-                        |   |
|                                 | Sanborn, Stiles & Moe, 1938            | +                          |   |
|                                 | Wickwire <i>et al.</i> 1987            | +/-                        |   |
|                                 | Stiller <i>et al.</i> 1999             | +                          |   |
| <i>Dermacentor hunteri</i>      | Rees & Avery, 1939                     | -                          |   |
| <i>Dermacentor nitens</i>       | Sanborn & Moe, 1934                    | -                          |   |
|                                 | Anthony & Roby, 1966                   | +                          |   |
| <i>Dermacentor occidentalis</i> | Boynton <i>et al.</i> 1936             | +                          |   |
|                                 | Christensen & Howard, 1966             | +                          |   |
|                                 | Howarth & Roby, 1972                   | +                          |   |
|                                 | Howarth & Hokama, 1973                 | + (- transovarial)         |   |
|                                 | Stiller & Johnson, 1983                | +                          |   |
|                                 | Sanborn & Moe, 1934                    | -                          |   |
| <i>Dermacentor parumapertus</i> | Anthony & Roby, 1962                   | + (- transovarial)         |   |
|                                 | Anthony & Roby, 1966                   | +                          |   |
| <i>Dermacentor variabilis</i>   | Kocan <i>et al.</i> 1981 & 1992a, b    | +                          |   |
|                                 | Piercy, 1938                           | -                          |   |
|                                 | Rees, 1932                             | +                          |   |
|                                 | Rees, 1934                             | + (- transovarial)         |   |
|                                 | Rees & Avery, 1939                     | - transovarial             |   |
|                                 | Sanborn & Moe, 1934                    | -                          |   |
|                                 | Sanders, 1933                          | +                          |   |
|                                 | Schmidt & Piercy, 1937                 | -                          |   |
|                                 | Stich <i>et al.</i> 1989               | + (- transovarial)         |   |
|                                 | <i>Dermacentor venustus</i>            | Sanborn & Moe, 1934        | - |
|                                 | <i>Haemaphysalis leporis-palustris</i> | Sanborn & Moe, 1934        | - |
|                                 | <i>Hyalomma lusitanicum</i>            | Sergent <i>et al.</i> 1945 | - |
|                                 | <i>Hyalomma mauritanicum</i>           | Sergent <i>et al.</i> 1945 | - |
| <i>Hyalomma rufipes</i>         | Potgieter, 1979                        | +                          |   |
| <i>Ixodes pacificus</i>         | Howarth & Hokama, 1973                 | -                          |   |
| <i>Ixodes ricinus</i>           | Helm, 1924                             | +                          |   |
|                                 | Piercy, 1938                           | -                          |   |
|                                 | Sanborn & Moe, 1934                    | -                          |   |
|                                 | Zeller & Helm, 1923                    | +                          |   |

Table 2. (Cont.)

| Tick species                    | Reference                    | Transmission (+/-)* |
|---------------------------------|------------------------------|---------------------|
| <i>Ixodes scapularis</i>        | Rees, 1934                   | +/-                 |
|                                 | Sanborn & Moe, 1934          | -                   |
| <i>Ixodes sculptus</i>          | Rees, 1934                   | -                   |
|                                 | Sanborn & Moe, 1934          | -                   |
| <i>Rhipicephalus bursa</i>      | Brumpt, 1931                 | +                   |
|                                 | Sergent <i>et al.</i> 1945   | +/-                 |
| <i>Rhipicephalus evertsi</i>    | Potgieter, 1979              | +                   |
| <i>Rhipicephalus sanguineus</i> | Parker, 1982                 | +                   |
|                                 | Rees, 1930                   | +                   |
|                                 | Rees, 1934                   | +/-                 |
|                                 | Rees & Avery, 1939           | - transovarial      |
|                                 | Sanborn & Moe, 1934          | -                   |
| <i>Rhipicephalus simus</i>      | Potgieter, 1979              | +                   |
|                                 | Potgieter <i>et al.</i> 1983 | +                   |
|                                 | Theiler, 1912                | + transovarial      |
| Argasid Ticks                   |                              |                     |
| <i>Argas persicus</i>           | Howell, Stiles & Moe, 1941   | +/-                 |
| <i>Ornithodoros coriaceus</i>   | Howarth & Hokama, 1973       | -                   |
| <i>Ornithodoros megnini</i>     | Howell, Stiles & Moe, 1941   | -                   |
|                                 | Sanborn & Moe, 1934          | -                   |

\* Transmission of *A. marginale* to cattle by ticks was reported (+), did not occur (-) or both results were reported (+/-) from separate trials.

Intrastadial transmission of *A. marginale* is effected by male ticks. Recent studies have demonstrated that male *Dermacentor* ticks may play an important role in the biological transmission of *A. marginale* because they become persistently infected with *A. marginale* and can transmit *A. marginale* repeatedly when they transfer among cattle (Kocan *et al.* 1992*a, b*). Male ticks, therefore, also serve as a reservoir of *A. marginale*, along with persistently infected cattle (Kocan *et al.* 1992*a, b*; Ge *et al.* 1996; Kocan, Blouin & Barbet, 2000).

The developmental cycle of *A. marginale* was described in persistently infected male ticks infected as adults and this cycle is complex and coordinated with the tick feeding cycle (Kocan, 1986; Kocan *et al.* 1992*a, b*). Infected erythrocytes taken into ticks with the blood-meal provide the source of *A. marginale* infection for tick gut cells (Fig. 2A & B). After development of *A. marginale* in tick gut cells, many other tick tissues become infected, including the salivary glands (Fig. 2C & D) from where the rickettsiae are transmitted to vertebrates during feeding (Kocan, 1986; Kocan *et al.* 1992*a, b*; Ge *et al.* 1996). At each site of infection in ticks, *A. marginale* develops within membrane-bound vacuoles or colonies. The first form of *A. marginale* seen within the colony is the reticulated (vegetative) form that divides by binary fission (Fig. 3) forming large colonies that may contain hundreds of organisms. The reticulated form then changes into the dense form (Fig. 3) 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.

Six major surface proteins (MSPs) have been identified on *A. marginale* derived from bovine erythrocytes and found to be conserved on tick- and cell culture-derived organisms (as reviewed by Kocan, Blouin & Barbet, 2000). Three of these MSPs, namely MSP1a, MSP4 and MSP5, are from single genes and do not vary antigenically during the multiplication of the bacterium (Barbet *et al.* 1987; Allred *et al.* 1990; Bowie *et al.* 2002), while the other three, MSP1b, MSP2 and MSP3, are from multigene families and may vary antigenically, most notably in persistently infected cattle (French *et al.* 1998; French, Brown & Palmer, 1999; Barbet *et al.* 2000, 2001; Brayton *et al.* 2001, 2002; Meeus & Barbet, 2001; Bowie *et al.* 2002). However, recent results demonstrated selection of *msp2* sequence variants in persistently infected ticks (Rurangirwa *et al.* 1999; Rurangirwa, Stiller & Palmer, 2000; de la Fuente & Kocan, 2001).

MSP1 is a heterodimer composed of two structurally unrelated polypeptides: MSP1a and MSP1b. MSP1a is encoded by *msp1a* (Allred *et al.* 1990) and is involved in adhesion to bovine erythrocytes and tick cells, and transmission of *A. marginale* by *Dermacentor* spp. (de la Fuente *et al.* 2001*a, b*; McGarey & Allred, 1994; McGarey *et al.* 1994). MSP1b is encoded by at least two genes, *msp1β1* and *msp1β2* (Barbet *et al.* 1987; 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 proved not to be an adhesin for tick cells (McGarey *et al.* 1994; McGarey & Allred, 1994; de la Fuente *et al.* 2001*b*). In addition, MSP1 has a neutralization-sensitive epitope (Palmer *et al.* 1987). The

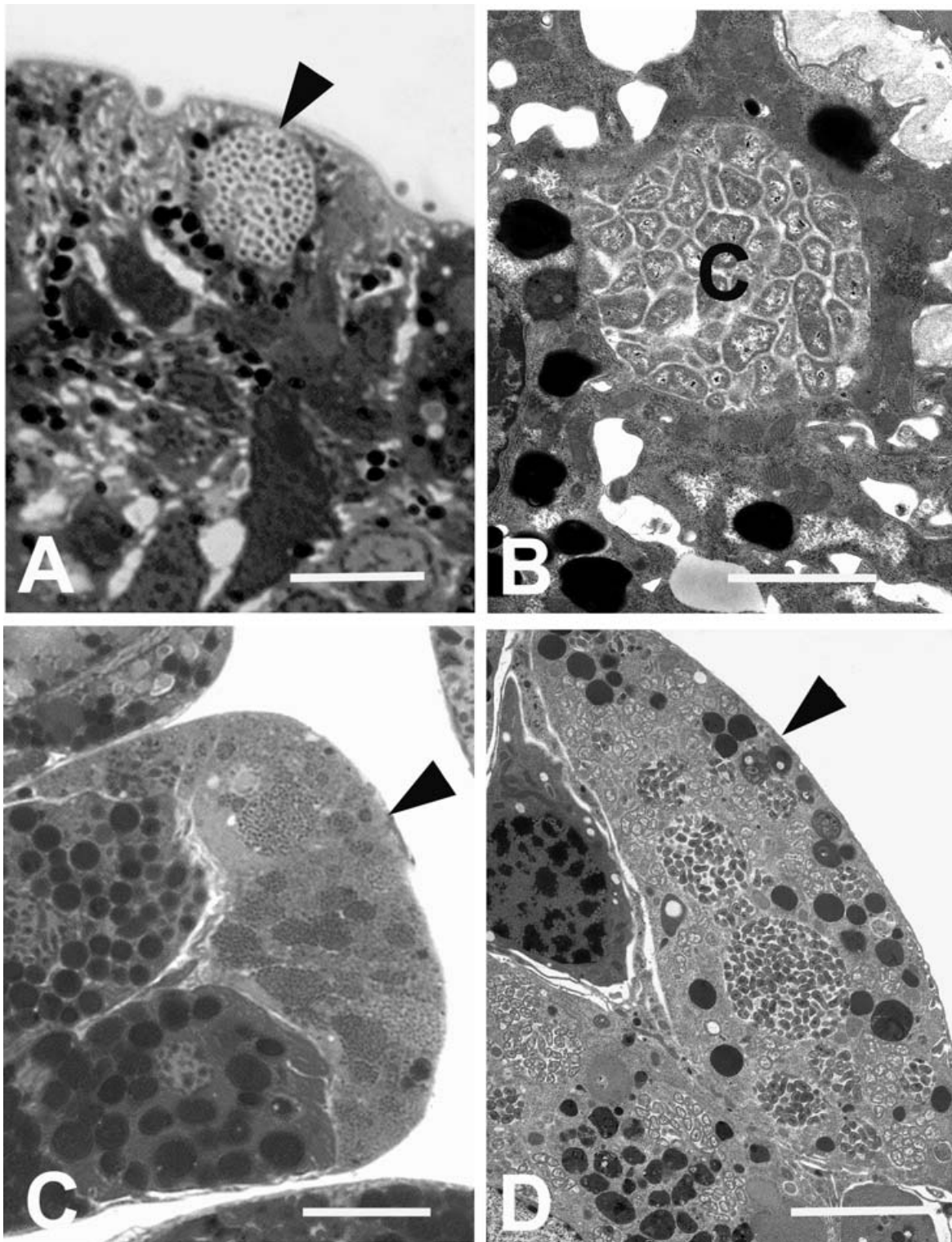


Fig. 2. Micrographs of colonies of *Anaplasma marginale* in tick gut and salivary gland cells. (A) A light micrograph of a large colony (arrow) in a tick gut cell. (B) An electron micrograph of a colony (C) in a tick gut cell. (C) A light micrograph of many colonies (arrow) in a salivary gland cell. (D) An electron micrograph of a tick salivary gland cell (arrow) that contains several *A. marginale* colonies. A, bar = 5  $\mu$ m; B, bar = 5  $\mu$ m; C, bar = 10  $\mu$ m; D, bar = 5  $\mu$ m.

molecular weight of MSP1a varies in size among strains of *A. marginale* because of different numbers of tandemly repeated 28–29 amino acid peptides (Allred *et al.* 1990; de la Fuente, Van Den Bussche & Kocan, 2001; de la Fuente *et al.* 2001c, 2002c,

2003a, b, c). *msp1a* is 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 (Bowie *et al.* 2002; Palmer, Rurangirwa & McElwain, 2001).

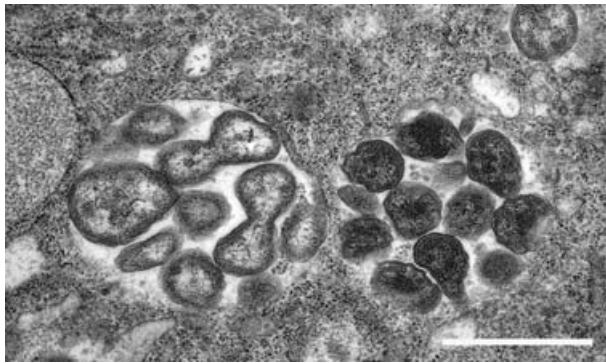


Fig. 3. Electron micrograph of the two developmental stages of *Anaplasma marginale* within colonies in tick cells. Reticulated forms within a colony on the left divide by binary fission and dense forms are within a second colony in a cultured tick cell. Bar = 1  $\mu$ m.

#### TICK CELL CULTURE: A MODEL FOR THE STUDY OF TICK-*A. MARGINALE* INTERACTIONS

##### *Development of the A. marginale/tick cell culture system*

A major impediment in anaplasmosis research has been the lack of a laboratory model or a cell culture system. Although cell culture systems were investigated using bovine erythrocytes and other arthropod cell lines (Marble & Hanks, 1972; Hildago, 1975; Mazzola, Amerault & Roby, 1976, 1979; Kessler & Ristic, 1979; Kessler *et al.* 1979; Mazzola & Kuttler, 1980; Samish, Pipano & Hana, 1988), none of these cell culture systems sustained the replication and continuous propagation of *A. marginale*. Recently our laboratory, in collaboration with Drs U. G. Munderloh and T. J. Kurtti at the University of Minnesota, developed methods for culturing *A. marginale* in an *Ixodes scapularis* tick cell culture which provides new research opportunities for studying the interaction of *A. marginale* and tick cells (Munderloh *et al.* 1996a; Blouin & Kocan, 1998; Blouin *et al.* 1999). A series of continuous tick cell lines were developed by Drs Munderloh and Kurtti (Munderloh & Kurtti, 1989; Munderloh *et al.* 1994). All cell lines have been characterized extensively by karyotyping and isoenzyme analysis (Munderloh *et al.* 1994). These analyses have also confirmed the species identity of the cell lines.

Bovine erythrocytes infected with *A. marginale* were used to inoculate monolayers of the tick cell line, IDE8, that was originally derived from embryos of *Ixodes scapularis*. We documented infection and transformation of *A. marginale* in tick cells from the erythrocyte inclusion body stage to development of large colonies in the cultured tick cells (Munderloh *et al.* 1996a; Blouin & Kocan, 1998; Blouin *et al.* 1999). The developmental cycle of *A. marginale* in cultured tick cells was similar to that described previously in naturally infected ticks (Blouin & Kocan, 1998) (Fig. 4). Cultured *A. marginale* retained its

antigenic composition and infectivity for cattle after successive passages (Blouin & Kocan, 1998; Barbet *et al.* 1999). Infection and development of *A. marginale* in cell culture was predictable, and cell monolayers became completely infected by 10–12 days post exposure (PE), after which cultures developed a cytopathic effect as cells began to detach (referred to as ‘terminal cultures’). The cell culture-derived organisms proved to be infective for cattle and *Dermacentor* spp. ticks which fed on those cattle. The six major surface proteins described on erythrocytic *A. marginale* were conserved on cell culture-derived organisms and the cell culture-derived *A. marginale* isolates retained their antigenic identity as determined by the molecular weight and sequence of MSP1a (Barbet *et al.* 1999). At present three geographic isolates originally derived from Virginia, Oklahoma and Oregon have been propagated in cell culture (Blouin & Kocan, 1998; Blouin *et al.* 2002a). *A. marginale* derived from cell culture proved to be a potent antigen for use in vaccine preparations (Kocan *et al.* 2001; de la Fuente *et al.* 2002b) and serologic tests (Saliki *et al.* 1998). Subsequently, this same IDE8 cell line was used in other laboratories to successfully propagate *A. phagocytophila* (Munderloh *et al.* 1996b, 1999), *E. canis* (Kocan, Munderloh & Ewing, 1998) and *E. (Cowdria) ruminantium* (Bell-Sakyi *et al.* 2000).

The cultured tick cells were adapted for use in experimental studies and assays (as reviewed by Blouin *et al.* 2002a). Infected cell cultures were frozen and stored in liquid nitrogen, and were then recovered and used to inoculate uninfected cell monolayers. Several pools of cell culture-derived *A. marginale* were frozen in liquid nitrogen to serve as seed stocks and as a permanent sample of the original isolate in the event of antigenic variation during subsequent passages. The *A. marginale*/tick cell culture system was adapted for short-term growth in 24-well and 96-well plate formats for use in the development of various assays. A competitive ELISA using a monoclonal antibody (MAb) directed against MSP5 and cell culture-derived *A. marginale* was developed in our laboratory for determination of serologic responses of cattle in vaccine trials (Saliki *et al.* 1998) similar to a cELISA developed for serodiagnosis of *A. marginale* in cattle (Visser *et al.* 1992; Knowles *et al.* 1996). A second indirect ELISA was developed in our laboratory for detection and quantification of *A. marginale* in cell cultures (Kocan *et al.* 2001).

##### *Infection and development of A. marginale in cultured tick cells*

The developmental cycle of *A. marginale* in tick cell culture has been described, including the entry and exit of the rickettsia from the IDE8 cells (Blouin & Kocan, 1998) (Fig. 4). Uninfected monolayers of IDE8 cells were inoculated with *A. marginale* culture

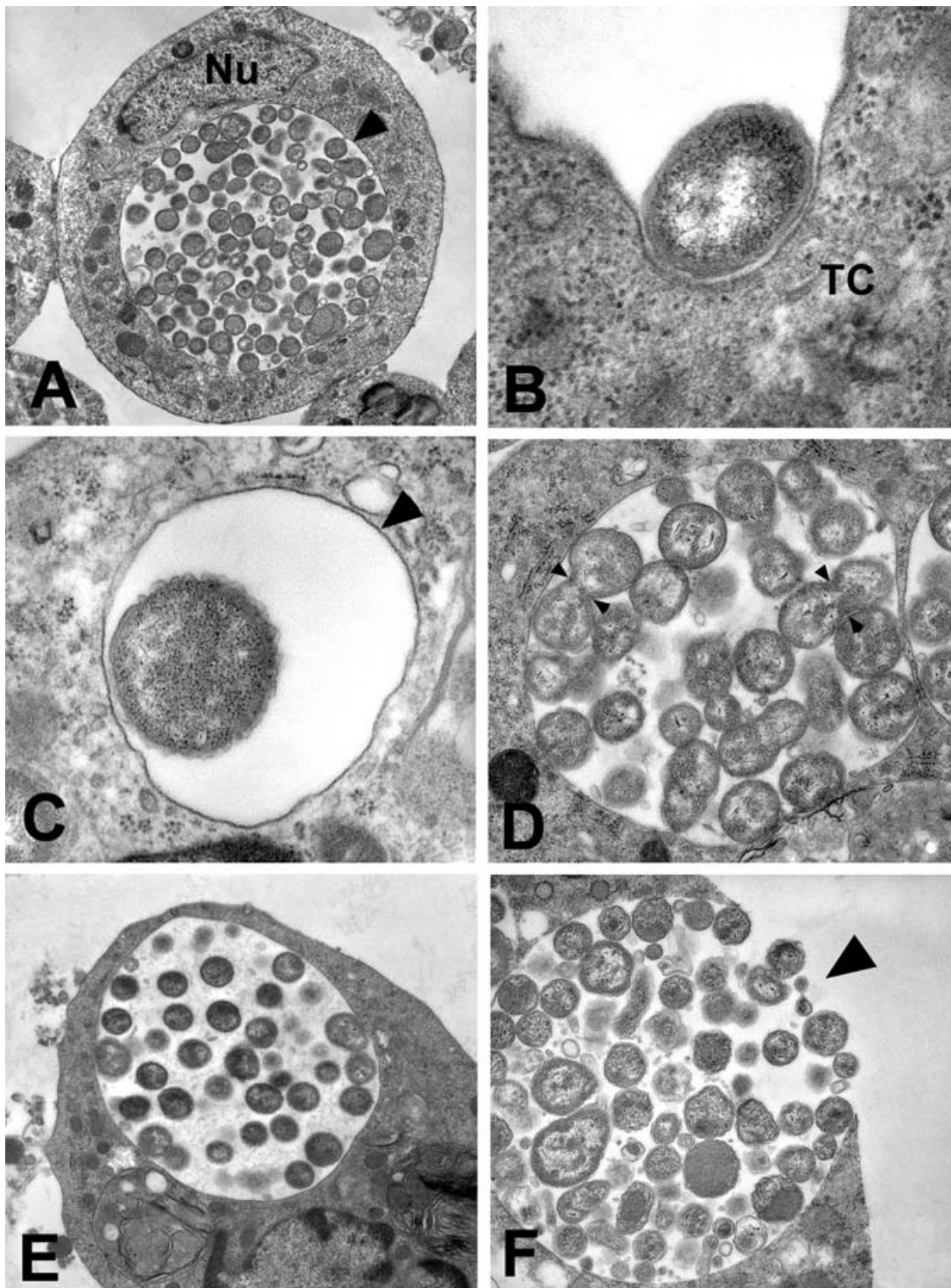


Fig. 4. Electron micrographs of *A. marginale* in cultured IDE8 tick cells. (A) A large colony of *A. marginale* adjacent to the nucleus (Nu) within a cultured tick cell. (B) An *A. marginale* adhered to the cell membrane of a cultured tick cell (TC). (C) A reticulated form of *A. marginale* within a membrane-bound colony. (D) A colony that contains reticulated forms of *A. marginale* that are dividing by binary fission (small arrows). (E) A colony that contains dense forms of *A. marginale*. (F) *A. marginale* being released (arrow) from a cultured tick cell. A, bar = 5  $\mu\text{m}$ ; B, bar = 0.5  $\mu\text{m}$ ; C, bar = 0.5  $\mu\text{m}$ ; D, bar = 5  $\mu\text{m}$ ; E, bar = 5  $\mu\text{m}$ ; F, bar = 1  $\mu\text{m}$ .

inoculum at a high multiplicity of infection and a short exposure period allowed for synchronization of development in the initial rickettsial cycles. Development of *A. marginale* in the cultured tick cells was documented using light and electron microscopy.

Infection of IDE8 cells occurred within 15 minutes post-exposure (PE). Host cell invasion was initiated by the adhesion of the dense form of *A. marginale* (diameter 0.5  $\mu\text{m}$  to 0.8  $\mu\text{m}$ ) to the host cell membrane. Distinct projections of the host cell plasmalemma

adhered along the outer membrane of *A. marginale*. The adhesion between the rickettsiae and tick cell membrane increased along a continuous section forming a depression in the host cell membrane. *A. marginale* was subsequently enclosed by the host cell membrane and internalized within a vacuole. *A. marginale* transformed into the reticulated (vegetative) form that divides by binary fission. Repeated division resulted in the formation of colonies by 48 hr PE that contained hundreds of rickettsiae. The reticulated forms of *A. marginale* subsequently transformed by day 3 PE into the infective or dense forms that were rounded and contained a denser and more uniform distribution of ribosomes and in which DNA fibrils were not readily apparent. On day 4 PE colony membranes were fused with the host cell plasma-membrane, followed by rupture of the membrane complex. A flap opened in the fused cell membranes that allowed for release of the dense forms from the parasitophorous vacuole without loss of host cell cytoplasm. The released rickettsiae then initiated a new series of infections resulting in host cells containing 5 or more colonies per cell (Blouin & Kocan, 1998). Tick cell death occurred after most of the cells became infected, resulting in detachment of tick cell monolayers and the cytopathic effect was then apparent with light microscopy. The mechanism of *A. marginale* entry into tick cells by endocytosis and exit by a process involving the fusion of the colony and host cell membranes, described herein for the first time, appears to be a mechanism controlled both by the cell and the parasite (Blouin & Kocan, 1998). The adherence of rickettsiae to the tick cell membrane prior to infection suggested the presence of adhesion molecules on the surface of *A. marginale* that were recognized by tick cell receptors. The sequence of infection and development of *A. marginale* in tick cell culture was predictable and easily followed, and the cell cultures provide a controlled system in which to study the effects of various agents on the infection and development of *A. marginale* in the cultured tick cells.

#### *Infectivity of A. marginale isolates for tick cells*

Many of the geographic isolates of *A. marginale* described in the US are infective and transmissible by ticks (i.e. Virginia, Oklahoma, Idaho and Oregon) while other isolates (i.e. Florida, Okeechobee, Illinois and California) do not appear to be infective for, or transmitted by, ticks (Smith *et al.* 1986; Wickwire *et al.* 1987; de la Fuente *et al.* 2001a, 2003a). Furthermore, the infectivity of *A. marginale* isolates appears to be directly related to the adhesive properties of the isolate MSP1a expressed in recombinant *E. coli* (de la Fuente *et al.* 2001a, 2003a).

We have used uniform techniques in an attempt to isolate and propagate several *A. marginale* isolates in the IDE8 tick cell line, including ones from Virginia,

Oklahoma, Florida, Okeechobee, California, and Oregon. Splenectomized calves were experimentally-infected with each of the isolates and blood was collected aseptically at peak parasitaemia from each of the calves and processed as described previously (Munderloh *et al.* 1996a; Blouin *et al.* 1999). Three *A. marginale* isolates (Virginia, Oklahoma and Oregon), also shown to be infective for ticks, proved to be infective for the cultured tick cells, while other isolates (Florida, Okeechobee and California), which were not infective for ticks, did not prove after repeated attempts to be infective for cultured tick cells (Blouin *et al.* 2002a). We have observed from these many studies that *A. marginale* isolates that are not infective for ticks are also not infective for cultured tick cells and, therefore, cannot be propagated in this cell culture system.

#### *Infection exclusion of A. marginale in cattle, ticks and tick cell culture*

Recent studies (de la Fuente *et al.* 2003c; Palmer *et al.* 2001) on *A. marginale*-infected cattle in endemic areas demonstrated that multiple *msp1a* genotypes were present, but that only one genotype was found per individual bovine. These findings suggested that infection of cattle with other genotypes was excluded.

Studies were undertaken to confirm infection exclusion of *A. marginale* genotypes in infected bovine erythrocytes, cultured tick cells and naturally infected ticks (de la Fuente *et al.* 2002a; de la Fuente, Blouin & Kocan, 2003). Two tick transmissible isolates of *A. marginale*, Virginia and Oklahoma, were used for these studies. In two separate trials, cattle inoculated with equal doses of the two isolates developed infection with only one genotype. Tick cell cultures inoculated with equal doses of the two isolates became infected only with the Virginia isolate. When cultures were inoculated with different ratios of the Oklahoma and Virginia isolates, the isolate inoculated in the higher ratio became established and excluded the other. When cultures with established infections of one isolate were subsequently infected with the other, only the established isolate was detected. Infection exclusion during initial infection in cell culture was documented by labeling each isolate with a different fluorescent dye. After 2 days in culture, only a single isolate was detected per cell by fluorescent microscopy. Finally, when *A. ovis* infections were established in cultures that were subsequently inoculated with the Virginia or Oklahoma isolates, *A. marginale* was excluded. These studies confirmed that infection exclusion occurs with *A. marginale* in bovine erythrocytes and tick cells, resulting in establishment of only one genotype. The phenomenon of infection exclusion was also recently demonstrated in ticks (de la Fuente, Blouin & Kocan, 2003). When ticks were allowed to feed on two cattle, first one infected with a Virginia isolate and the

second infected with an Oklahoma isolate, PCR studies on the tick salivary glands demonstrated that only the Virginia isolate became established in the ticks and apparently excluded the Oklahoma isolate.

These results point to the existence of some mechanisms common to tick cells and mammalian host cells during the multiplication of *Anaplasma* spp. The characterization of the mechanism of infection exclusion in *A. marginale* resulting in one genotype per animal would contribute to our understanding of anaplasmosis epidemiology. If cattle infected with a single *A. marginale* genotype were introduced into an endemic herd, these genotypes would be maintained and most likely also become endemic, if they are transmitted to susceptible cattle. Both persistently infected cattle and ticks could serve as reservoirs of the introduced genotype. The phenomenon of infection exclusion, which results in one genotype infection per animal, may constrain the mobility and establishment of multiple *A. marginale* isolates per geographic area and supports the need for novel vaccine preparations that are cross-protective against multiple genotypes that may be introduced from other areas via shipment of cattle.

#### *The tick cell culture as a biological assay system*

The development of the cell culture system for the propagation of *A. marginale* provided many new opportunities for research on this economically important pathogen of cattle (as reviewed by Blouin *et al.* 2002a). The *A. marginale*/tick cell culture system has been used to develop biological assays to determine the adhesive properties of selected *A. marginale* MSPs, the inhibition of *A. marginale* infection of tick cells by anti-*A. marginale* antibodies, the efficacy of drugs screened for affecting the multiplication of *A. marginale* in tick cells, and the effect of exogenous compounds in infection and transmission of *A. marginale*.

The role of MSP1a in adhesion to tick cells was confirmed using various adhesion assays, as well as by the demonstration of inhibition of infection by antibodies against the individual proteins (MSP1a and MSP1b) (de la Fuente *et al.* 2001a,b, 2003a; Blouin *et al.* 2003). The effect of tetracycline on *A. marginale* in cell culture demonstrated the utility of the tick cell culture system as a method of screening drugs (Blouin *et al.* 2002b). Additionally, tick saliva and bee venom phospholipase A<sub>2</sub> enhanced infection rates in ticks, supporting the concept that the vector itself contributes exogenous factors, which enhance infection rates of tick cells (Blouin *et al.* 2002a).

The ultimate goal of *A. marginale* research is to define host-pathogen relationships in order to discover a way to not only control disease but to prevent infection. The tick cell culture system has proved to be a source of antigens for the development of anti-*A. marginale* vaccines and a useful tool for

conducting research on the relationship between *A. marginale* and tick cells, and the information gained from these studies will most likely be applicable to other tick-borne pathogens.

#### ROLE OF MSP1A AND 1B IN INFECTION AND TRANSMISSION OF *A. MARGINALE*

MSP1a and MSP1b form the MSP1 complex. MSP1a is variable in molecular weight among geographic isolates because of different numbers of tandem 28 or 29 amino acid repeats located in the amino terminal portion of the protein (Allred *et al.* 1990; de la Fuente *et al.* 2001c,d, 2002c, 2003a,b,c). Because of the variation in the repeated portion of the *msp1a*, this gene has been used as a stable genetic marker for identification of *A. marginale* geographic isolates (Barbet *et al.* 1987, 1999; de la Fuente, Van Den Bussche & Kocan, 2001; de la Fuente *et al.* 2001c). The sequence of *msp1a* is conserved during the multiplication of the rickettsia in cattle and ticks (Palmer *et al.* 2001; Bowie *et al.* 2002). A neutralization-sensitive epitope was demonstrated on the MSP1a tandem repeats (Palmer *et al.* 1987; Oberle *et al.* 1988) and was found to be conserved among *A. marginale* isolates (Palmer *et al.* 1987; de la Fuente, Van Den Bussche & Kocan, 2001; de la Fuente *et al.* 2001c, 2002c, 2003a,b,c). MSP1a was shown to be an adhesin for bovine erythrocytes and both native and cultured tick cells using recombinant *E. coli* expressing MSP1a in microtitre haemagglutination and adhesion recovery assays and by microscopy (McGarey & Allred, 1994; McGarey *et al.* 1994; de la Fuente *et al.* 2001b). The portion of MSP1a with the tandem repeats was necessary and sufficient to effect adhesion to bovine erythrocytes and tick cells (de la Fuente *et al.* 2003a; as reviewed by Kocan *et al.* 2003b). MSP1a was shown to be involved in infection and transmission of *A. marginale* by *Dermacentor* spp. ticks (de la Fuente *et al.* 2001a) and to be involved in immunity to *A. marginale* infection in cattle (Palmer *et al.* 1987; Brown *et al.* 2001). Furthermore, a correlation was established between the adhesive properties of recombinant MSP1a and the transmissibility of the isolate by *Dermacentor* spp. ticks. Additional studies demonstrated the critical role of the 20th amino acid of the repeat in the interaction of MSP1a with host cell receptors (de la Fuente *et al.* 2003a). However, the 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 both the sequence and conformation of the repeated peptides influenced the adhesive properties of MSP1a (de la Fuente *et al.* 2003a).

MSP1b is polymorphic among geographic isolates of *A. marginale* (Barbet *et al.* 1987; Camacho-Nuez *et al.* 2000; Viseshakul *et al.* 2000; Bowie *et al.* 2002).

Although from a multigene family, only small variations in protein sequences were observed in MSP1b1 and MSP1b2 during the life cycle in cattle and ticks (Bowie *et al.* 2002). This protein, which forms a complex with MSP1a, has been shown to be an adhesin for bovine erythrocytes (McGarey & Allred, 1994; McGarey *et al.* 1994). However, MSP1b was recently demonstrated to be an adhesin only for bovine erythrocytes and did not prove to be an adhesin for tick cells (de la Fuente *et al.* 2001*b*).

Recent analyses conducted in our laboratory demonstrated that MSP1a and MSP1b are glycosylated. A similar observation was made by McBride, Xue-Jie Yu & Walker (2000) for *E. chaffeensis* and *E. canis* major surface proteins. The observed molecular weights of the two MSP1 proteins (90 kDa for MSP1a and 100 kDa for MSP1b) were greater than the predicted molecular weights (63 kDa and 79 kDa, respectively) (Garcia-Garcia *et al.* 2002*a*). The analysis of amino acid sequence showed that while MSP1b (Oklahoma isolate) contains only one potential O-glycosylation site and seven potential N-glycosylation sites, MSP1a (Oklahoma isolate) contains only one potential N-glycosylation site and 25 potential O-glycosylation sites, particularly in the N-terminal repeated peptides region where Ser/Thr accounts for 32% of the amino acids. Recombinant MSP1a and MSP1b proteins had the same molecular weights as the native proteins, suggesting that the post-translational modifications were similar in *E. coli*. Carbohydrates were found on both recombinant MSP1a and MSP1b using periodate oxidation and biotin-hydrazide conjugation. Only three sugar residues (glucose, galactose and xylose) were identified by gas chromatography, demonstrating the absence of conserved sugar moieties usually found in O-linked and N-linked oligosaccharides. Therefore, enzymes that recognize carbohydrate sequences containing amino sugars, such as PNGase F, NANase II, GALase III, HEXase I and O-glycosidase DS, were unable to deglycosylate these proteins. Although glycoproteins from both MSP1a and MSP1b contained the same sugars, the molar ratio varied between them. MSP1a had a higher content of glucose and only residual contents of xylose and galactose, while MSP1b had a higher content of xylose and galactose. These differences probably reflect variation in the nature of the glycosylation, since the oligosaccharides should be O-linked in MSP1a and N-linked in MSP1b. The molecular weights of the N-terminal portion of MSP1a containing the adhesive repeated peptides and the conserved C-terminal region of MSP1a were also larger than predicted. Carbohydrates were detected in both regions, although the degree of modification in the N-terminal region was higher. The significance and biological function of MSP1a and MSP1b glycosylation is unknown but the carbohydrate residues may contribute to the adhesive properties of the MSPs. Glycosylation of membrane

proteins may also function in protection of the protein against proteases during development, and could contribute to the generation of antigenic and phenotypic variation of the pathogen.

The expression of MSP1a is differentially regulated in erythrocytic and tick stages of *A. marginale*. Cattle immunized with *A. marginale* from tick cells or bovine erythrocytes produced antibodies against the *A. marginale* MSP5 but a differential antibody response to MSP1a and MSP1b was observed (Kocan *et al.* 2001; de la Fuente *et al.* 2002*b*; Garcia-Garcia *et al.* 2002*b*). Cattle immunized with erythrocyte-derived *A. marginale* elicited an antibody response mainly against MSP1a, while animals immunized with cell culture-derived antigen produced predominantly antibodies to MSP1b. The molecular bases of this differential antibody response were then studied by comparing the levels of MSP1a, MSP1b and MSP5 on *A. marginale* harvested from the two host cells. The amount of MSP1b and MSP5 was similar on *A. marginale* from both host cells, but the amount of MSP1a was higher in the erythrocyte-derived *A. marginale*. These differences were also found when RNA transcripts were analyzed by RT-PCR, demonstrating that the differential expression of MSP1a and MSP1b was regulated at the transcriptional level. Since MSP1a is an *A. marginale* adhesin for tick cells, biological transmission of the pathogen could be enhanced by increased levels of this surface protein. Differences in the level of surface exposed molecules may also contribute to phenotypic and antigenic variation of the pathogen.

#### EVOLUTION AND PHYLOGEOGRAPHY OF *A. MARGINALE*

The phylogenetic relationship and evolution of *A. marginale* isolates is important for understanding the biology and the possibilities for control of anaplasmosis. We chose two *A. marginale* MSPs, MSP1a and MSP4, for phylogenetic analysis. MSPs are involved in interactions with both vertebrate and invertebrate hosts (McGarey & Allred, 1994; McGarey *et al.* 1994; de la Fuente *et al.* 2001*b, c*) and, therefore, are also likely to evolve more rapidly than other nuclear genes because they are subjected to selective pressures exerted by host immune systems. For instance, the repeated sequence motifs in *A. marginale* MSP1a are important in host-pathogen interactions by serving as adhesins required for invasion and transmission of *A. marginale*. The role of MSP1a repeats is relevant to tick-pathogen interactions. Therefore, the function of MSP1a may have resulted from distinct evolutionary pressures, specifically from those exerted by ligand-receptor and host-pathogen interactions. Both *mSP1a* and *mSP4* have been found to be stable genetic markers during the multiplication of *A. marginale* (de la Fuente *et al.* 2001*c*; Bowie *et al.* 2002).

Phylogenetic analysis of *A. marginale* geographic isolates from the US was performed using *msp1a* and *msp4* gene and derived protein sequences (de la Fuente, Van Den Bussche & Kocan, 2001). Results of these analyses strongly supported a southeastern clade of *A. marginale* comprised of Virginia and Florida isolates. Furthermore, analysis of 16S rDNA fragment sequences from the tick vector of *A. marginale*, *D. variabilis*, from various areas of the US was performed and suggested co-evolution of the vector and pathogen (de la Fuente, Van Den Bussche & Kocan, 2001).

Phylogenetic studies were also executed using New World isolates of *A. marginale* from United States, Mexico, Brazil and Argentina. Seventeen isolates of *A. marginale*, plus two outgroup taxa (*A. centrale* and *A. ovis*), were included for the analysis of MSP4 sequences (de la Fuente *et al.* 2002c). Maximum-parsimony analysis of MSP4 sequences provided phylogenetic information on the evolution of *A. marginale* isolates. Strong bootstrap support was detected for a Latin American clade of *A. marginale* isolates. Moreover, within this Latin American clade, strong bootstrap support was detected for Mexican and South American clades. Isolates of *A. marginale* from the United States also grouped into two clades, a southern clade consisting of isolates from Florida, Mississippi, and Virginia and a west-central clade consisting of isolates from California, Idaho, Illinois, Oklahoma and Texas. Although little phylogeographic resolution was detected within any of these higher clades, *msp4* sequences appear to be good genetic markers for inferring phylogeographic patterns of isolates of *A. marginale* on a broad geographic scale. In contrast to the phylogeographic resolution provided by MSP4, DNA and protein sequence variation from MSP1a representing 20 New World isolates of *A. marginale* failed to provide phylogeographic resolution (de la Fuente *et al.* 2002c). Most variation in MSP1a sequences appeared unique to a given isolate. In fact, similar DNA sequence variation in *msp1a* was detected within isolates from Idaho and Florida and from Idaho and Argentina. These results suggested that *msp1a* sequences may be rapidly evolving and questioned the use of *msp1a* sequences for defining geographic isolates of *A. marginale*. Nevertheless, it was necessary to address the possibility that *msp1a* may provide phylogeographic information when numerous strains from a given area are included in the analysis.

Eleven *A. marginale* isolates from Oklahoma isolated from cattle with anaplasmosis during 2001, plus two previous isolates from Wetumka (Oklahoma isolate in de la Fuente, Van Den Bussche & Kocan, 2001; de la Fuente *et al.* 2001a,b,c, 2002c, 2003c) and Pawhuska identified in 1997 and 1960s, respectively, were then analyzed for *msp1a* and *msp4* gene and protein sequences. Maximum parsimony and maximum likelihood (ML) phylogenies of *msp4*

sequences of 13 strains from Oklahoma and in comparison with 7 Latin American and 12 strains from the US using as outgroups *A. centrale* and *A. ovis* sequences provided strong bootstrap support for a Latin American clade; within this clade, support was detected for Mexican and South American clades. Isolates of *A. marginale* from the US also grouped into two clades from the southern (isolates from Florida, Mississippi, and Virginia) and west-central (isolates from California, Idaho, Illinois, Oregon, Missouri, and Texas) states. Both clades contained strains from Oklahoma, suggesting extensive cattle movement in the past. Within Oklahoma, ML analysis of *msp4* sequences provided bootstrap support for east-central and north-central clades, both including strains from Stillwater, Oklahoma. ML analysis of codon and amino acid changes over the MSP4 phylogeny evidenced that *msp4* is not under positive selection pressure. In contrast, ML phylogeny of *msp1a* DNA and protein sequences of 13 strains from Oklahoma and in comparison with 7 Latin American and 13 strains from the US demonstrated no geographic clustering and showed that the gene is under positive selection pressure (de la Fuente *et al.* 2003c).

These results indicate that *msp1a* is not a marker for the characterization of geographic isolates of *A. marginale* and suggest that the genetic heterogeneity observed among strains of *A. marginale* within Oklahoma, and probably within other endemic regions, could be explained by cattle movement and maintenance of different genotypes by independent transmission events. Therefore, if cattle infected with a new *A. marginale* genotype were imported into a region, the new isolate could become established by mechanical and/or biological transmission to susceptible cattle. In regions with few cattle introductions, like Australia, little genotypic variation is found within *A. marginale* isolates (Bock & de Vos, 2001). In regions with extensive cattle movement, like Oklahoma, a highly heterogeneous *A. marginale* population would be expected. *msp4* gene sequences appear to be good genetic markers for evolutionary studies within the genus *Anaplasma* and for inferring phylogeographic patterns of *A. marginale* isolates.

#### PROSPECTS FOR CONTROL OF *A. MARGINALE* BY USE OF PATHOGEN- AND TICK-DERIVED ANTIGENS

The characterization of antigens identified on *A. marginale* and the vaccine preparations developed for the control of infections with *A. marginale* were recently reviewed by Kocan *et al.* (2003b) and will not be covered in this review. The recent report of vaccine preparations with antigens derived from *A. marginale* grown in cultured IDE8 cells has opened new possibilities for the development of safe, reproducible vaccine formulations against a broad

spectrum of *A. marginale* strains (Kocan *et al.* 2001; de la Fuente *et al.* 2002*b*). However, these vaccine formulations have only provided partial protection against infections with *A. marginale*, diminishing the severity and duration of the clinical signs.

These results stress the need for more efficacious vaccines against infections with *A. marginale*. The use of recombinant antigens for the development of subunit vaccines against anaplasmosis has provided promising results (Kocan *et al.* 2000, 2003*b*; reviewed by Palmer, 1989). However, the incorporation of recombinant antigens into vaccines for control of *A. marginale* infections will require a better understanding of the mechanisms governing bacterial infection and transmission and the nature of the protective response developed in persistently infected cattle. Furthermore, the differences in protein expression and structure between rickettsia grown in tick cells and bovine erythrocytes are crucial for the development of vaccine formulations capable of preventing infection and transmission of the pathogen, the ultimate goal of the vaccine against *A. marginale*. Current results suggest that differences exist between *A. marginale* in tick cells and bovine erythrocytes (Kocan *et al.* 2003*a, b*). Further research is needed to define the adaptations of *A. marginale* to cattle and ticks.

Finally, the inclusion of tick antigens in *A. marginale*-specific vaccines could enhance their protective effect and increase efficacy (Nuttall, 1999). This transmission-blocking approach is supported by evidence that host resistance to ticks provides some protection against tick-borne transmission of viruses and *B. burgdorferi* (Wikel *et al.* 1997). Furthermore, vaccination against *B. microplus* has been demonstrated to contribute to the control of tick-borne diseases (reviewed by de la Fuente & Kocan, 2003; de la Fuente *et al.* 1998). Therefore, the combination of anti-tick antigens with *A. marginale*-derived proteins may provide a means to control *A. marginale* infection and transmission through immunization of cattle.

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