IN VIVO ENDOTHELIAL CELL INFECTION BY *ANAPLASMA MARGINALE*

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IN VIVO ENDOTHELIAL CELL INFECTION BY *ANAPLASMA MARGINALE*

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IN VIVO ENDOTHELIAL CELL INFECTION BY *ANAPLASMA MARGINALE*

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Abigail Dickinson Carreño, daughter of Richard D. Carreño and Nancy D. Brown, was born on 20 April 1976 in Worcester, Massachusetts. She graduated from Tourtellotte Memorial High School in Thompson, Connecticut in 1994. She attended the University of Connecticut and graduated in May 1998 with a Bachelor of Science degree in Animal Science. After working for CuraGen Corporation as a Research Assistant for 3 years, she then worked as a Biological Laboratory Technician for the USDA, Plum Island Animal Disease Center for 2 years. She moved from Connecticut in January of 2004 to work in Auburn University College of Veterinary Medicine's Department of Pathobiology as a Research Assistant and pursued her Master's degree while retaining the title of Research Assistant.

THESIS ABSTRACT

IN VIVO ENDOTHELIAL CELL INFECTION BY *ANAPLASMA MARGINALE*

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Anaplasmosis is an arthropod-borne hemoparasitic disease of cattle and other ruminants. The causative agent is the gram negative bacterium, *Anaplasma marginale.* Infection of bovine erythrocytes by *A. marginale* has been well established *in vivo*, as well as *in vitro*. Recently, *A. marginale* has been propagated *in vitro* in bovine and primate vascular endothelial cell cultures. This finding provides evidence that infected endothelial cells may initiate MHC-Class-I restricted CTL responses in infected cattle. To determine the extent to which endothelial cells are susceptible to *A. marginale* infection *in vivo*, a dual staining technique was applied to tissues from a splenectomized calf experimentally inoculated with 10^9 organisms from the St. Maries strain of *A. marginale.* Sections of kidney, lung, and hemal lymph node were collected, embedded in freezing compound, frozen in isopentane/liquid nitrogen, and cryosectioned at 5 microns. Sections were co-labeled with monoclonal antibody ANAF16C1, recognizing *A. marginale* major surface protein 5 (MSP5) conjugated to fluorescein isothiocyanate (FITC) or Alexa Fluor 488

and a polyclonal rabbit antibody against human von Willebrand Factor (an endothelial cell marker) conjugated to tetramethylrhodamine isothiocyanate (TRITC) or Alexa Fluor 568. Nuclei were stained with 284nM 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI). Sections were evaluated by conventional wide field fluorescence microscopy using a Nikon Eclipse E800 and confocal fluorescence microscopy using a BioRad MRC 1024 Scanning Laser Confocal Microscope. As expected, non-endothelial cells within vascular lumens were the major reservoir for *A. marginale*. In addition, *A. marginale* fluorescence co-localized to capillary endothelial cells of the kidney, lung, and hemal lymph node. These results suggest that endothelial cells may serve as a cellular reservoir for *A. marginale in vivo,* and have implications for both pathogenesis and immune mechanisms.

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I. LITERATURE REVIEW

The importance of bovine Anaplasmosis is multifaceted and growing. There are several reasons for this, the most important being the severe losses in cattle due to the disease. In addition, its agent, *Anaplasma marginale* (*A. marginale*) recently has been reclassified within the order Rickettsiales. The order was reorganized into two families, Anaplasmataceae and Rickettsiaceae. With this reorganization, *A. marginale* has become the type species for the family Anaplasmataceae because of its strong homology of several characteristics between the genera within this family. Secondly, and more importantly, *A. marginale* has high homology to *Anaplasma phagocytophilum*, which causes human granulocytic ehrlichiosis, an important emerging disease in humans.

Table 1 summarizes the current classification of the order Rickettsiales.

Table 1. Current classification of the order Rickettsiales.

Order Rickettsiales
Family Rickettsiaceae: Obligate intracellular bacteria
that grow freely in the cytoplasm of their
eukaryotic host cells
Genus Rickettsia
Genus Orientia
Family Anaplasmataceae: Obligate intracellular bacteria
that replicate within membrane-derived vacuoles
in the cytoplasm of eukaryotic host cells
Genus Anaplasma
Anaplasma marginale (type species)

Anaplasma centrale
Anaplasma ovis
Anaplasma bovis (formerly Ehrlichia bovis)
Anaplasma phagocytophilum (formerly Ehrlichia phagocytophilum, E. equi, HGE agent)
Anaplasma platys (formerly Ehrlichia platys)
Aegyptianella (genus incertae sedis due to lack of sequence information)
Genus Ehrlichia
Ehrlichia chaffeensis
Ehrlichia ruminantium (formerly Cowdria ruminantium)
Ehrlichia ewingii
Ehrlichia ovis
Ehrlichia canis
Ehrlichia muris
Genus Neorickettsia
Neorickettsia helminthoeca
Neorickettsia risticii (formerly Ehrlichia risticii)
Neorickettsia sennetsu (formerly Ehrlichia sennetsu)
Genus Wolbachia
Wolbachia pipientis

Bovine Anaplasmosis:

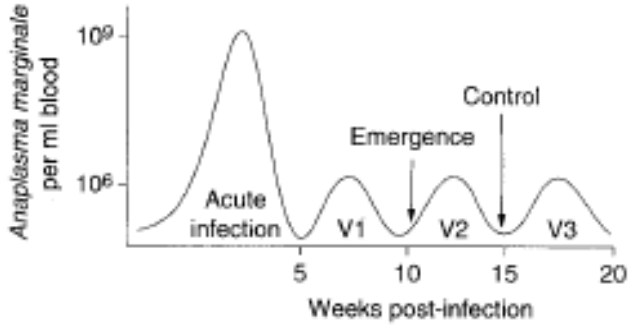
Anaplasmosis is an arthropod-borne hemoparasitic disease of cattle and other ruminants caused by the gram-negative bacterium, *Anaplasma marginale,* belonging to the order Rickettsiales, and the family Anaplasmataceae.[42,43] *A. marginale* is the most prevalent tick-borne pathogen of animals worldwide, occurring in six continents and responsible for severe morbidity and mortality in temperate, subtropical, and tropical regions.[49,57] Cattle have the most notable clinical disease, but other ruminants including water buffalo, bison, African antelopes, and mule deer can become persistently infected with *A. marginale.*[44] The disease is characterized by severe anemia associated with

intraerythocytic parasitism and hemolysis, resulting in depression, weakness, high body temperature, dehydration, jaundice, and low milk production.[43] Lactating cows have decreased milk production[55] and infected bulls show a lack of libido, and abnormal sperm morphology which is occasionally accompanied by transitory testicular degeneration during acute infection.[70] Cattle that recover remain chronic carriers of the parasite for life, and may serve as a reservoir for uninfected cattle.[47] Anaplasmosis severely reduces the production of meat, milk, and fiber in tropical and subtropical areas of the world.[43] Enzootic regions are found in Africa, Asia, Australia, Southern Europe, South America, the former Soviet Union, and 40 states of the United States. Based on 1981 estimates, infection of cattle may result in death (36% of clinical cases), abortion (24% of clinical cases in pregnant cows), weight loss (average of 86kg per clinical case), and increased veterinary and management costs.[2] Yet, it is hard to calculate losses due to anaplasmosis in many regions because of inadequate records, inability to quantify production losses, or concurrent infection with other hemoparasitic and tick-borne diseases.

A. marginale infection was first described by Sir Arthur Theiler when it was seen in infected erythrocytes of South African cattle as "marginal points."[44] In 1896, a similar report was described by Salmon and Smith in the United States that described the presence of a point-like pathogen in blood smears of cattle as "very minute roundish body....The body as a rule is situated near the edge of the corpuscle."[44] The scientific name is based on its staining characteristics and location within the host cell. "Anaplasma" refers to the lack of a stained cytoplasm and "marginale" refers to the peripheral location of the organism in the host erythrocyte.[41]

As of 2006, the only known site of infection of *A. marginale* in cattle was erythrocytes. Within these cells the membrane-bound inclusions (also called initial bodies) contain four to eight rickettsia and 70% or more of the erythrocytes may become infected during acute infection. [43] Removal of the infected cells by the mononuclear-phagocyte system results in mild to severe anemia and icterus.[43] The incubation period of infection (prepatent period) varies with the number of organisms in the infective dose and ranges from 7 to 60 days with an average of 28 days. After the erythrocytic infection is detected, the number of parasitized erythrocytes increases geometrically. [44] Cattle that survive acute infection develop persistent infections characterized by cyclic low level rickettsemia (Figure 1).[44]

Figure 1. Cyclic rickettsemia seen in persistently infected cattle.



(Courtesy KM Kocan, et *al.*, Alternatives for Control of *Anaplasma marginale* Infection in Cattle, *Clinical Microbiology Reviews*, 2003)

Persistence is characterized by sequential rickettsemic cycles, occurring at approximately 5 week intervals, in which new MSP2 variants replicate to a peak of greater than 10^6 organisms per milliliter of blood and then are controlled by a variant-

specific immune response.[44] Between the peaks in the cyclic rickettsemia, the levels are often undetectable by conventional microscopic examination of blood smears at fewer than 10^7 per milliliter.[57] Persistently infected or "carrier" cattle have lifelong immunity and are resistant to clinical disease on challenge exposure and are reservoirs of *A. marginale* because they provide a source of infective blood for both mechanical and biological transmission by ticks.[44]

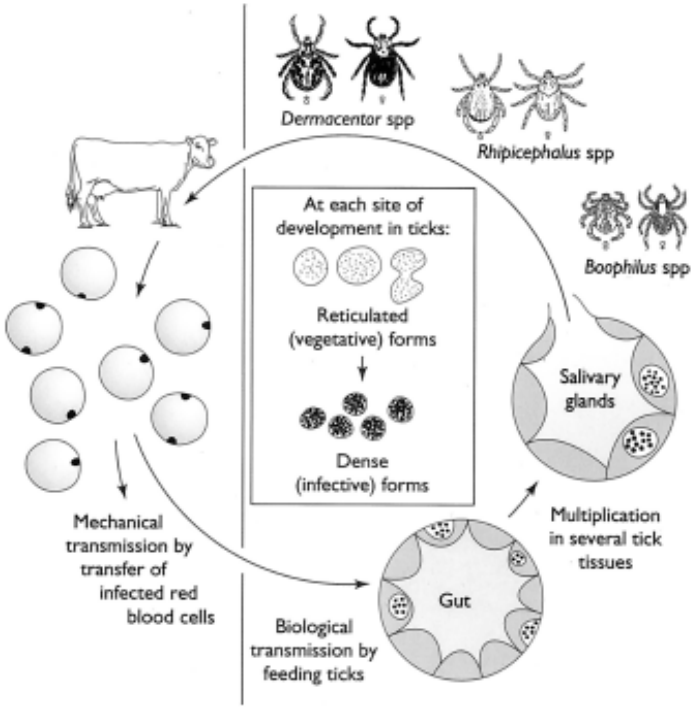
Calves are less susceptible to infection with *A. marginale* and when infected are less susceptible to clinical disease. This phenomenon is not well understood, but removal of the spleen renders calves fully susceptible to infection, and disease is often more severe than in older cattle.[44]

Transmission of *A. marginale* can occur either mechanically or biologically. Mechanical transmission most often occurs through the transfer of infected blood by castration devices, dehorning devices, needles, or ear tagging devices.[41] Biological transmission occurs through the transmission of infected blood by ticks. It has been shown that *A. marginale* can be transmitted transplacentally as well and it is thought that this route may contribute to the epidemiology of this disease in some regions.[44]

The developmental cycle of *A. marginale* in ticks is complex and coordinated with the tick feeding cycle. Infected erythrocytes taken into ticks with the blood meal provide a 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. At each site of infection in ticks, *A. marginale* develops within membrane-bound vacuoles or colonies (Figure 2). The first form of *A. marginale* within the colony is a reticulated

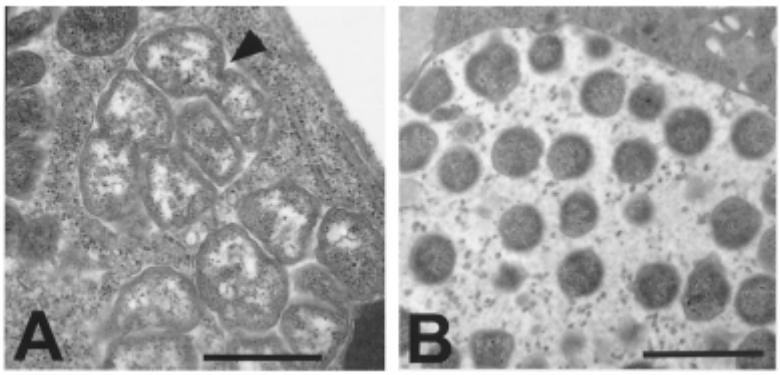
(vegetative) form (Figure 3A), which divides by binary fission, forming large colonies that may contain hundreds of organisms. The reticulated form then changes to the dense form (Figure 3B), which is the infective form and can survive outside the host cells. Cattle are infected when the dense form is transmitted during tick feeding via the salivary glands (summarized in Figure 2).[44]

Figure 2. Schematic of the development of A. marginale in ticks and cattle.



(Courtesy KM Kocan, et *al.*, Alternatives for Control of *Anaplasma marginale* Infection in Cattle, *Clinical Microbiology Reviews*, 2003)

Figure 3. (A) Reticulated forms of A. marginale within colonies in tick cells. (B) Dense forms with a colony in an infected tick cell.



(Courtesy KM Kocan, et *al.*, Alternatives for Control of *Anaplasma marginale* Infection in Cattle, *Clinical Microbiology Reviews*, 2003)

Vector:

Persistently infected cattle are the main reservoir for *A. marginale* in enzootic regions, and the main arthropod vector is the tick. Approximately 20 species of ticks are thought to be vectors of transmission of *A. marginale* worldwide.[44] Tick transmission can occur from stage to stage (transstadial) or within a stage (intrastadial), while transovarial transmission from one tick generation to another does not appear to occur.[69] Intrastadial transmission by male ticks is believed to be an important mechanism of transmission because male ticks can become infected after a short feeding period on an infected cow and then transmit infection during repeated feeding on multiple susceptible cattle. Male ticks, therefore, serve as a reservoir of infection.[41] In addition, the percentage of infected ticks is directly related to the level of parasitemia during feeding, but once ticks become infected, the level of infection in individual ticks is similar because of extensive multiplication in tick cells.[41]

The Ixodid tick family is the main vector with a majority of the genera proven to transmit *A. marginale*.[43] Species include *Boophilus* spp., selected *Dermacentor* spp.,

Ixodes ricinus and *Rhipicephalus* spp., while *Amblyomma* spp. do not appear to transmit *A. marginale*.[43] In addition, transovarial transmission has been reported for *D. andersoni* by Howell, Stiles, & Moe (1941), but others have not been able to demonstrate this mode of transmission of *A. marginale*[43] and transovarial transmission of *A. marginale* by *Boophilus* ticks has not been thoroughly investigated and needs further study.[43] In North America, *D. variablilis* (American dog tick) is the primary vector for transmission of *A. marginale*.

Persistent infection in cattle and tick-borne transmission:

Persistently infected or "carrier" cattle serve as the main reservoir for new infection of uninfected cattle. Thus, persistence is very important for the transmission of *A. marginale* in a tick-borne manner.

Acquisition of infection by ticks feeding on persistently rickettsemic cattle appears to be relatively efficient with over 50% of feeding *Dermacentor andersoni* adult male ticks being infected in a study by Eriks, et *al*. (1993).[30] The same study also found that tick infection rates correlate with the level of rickettsemia during acquisition feeding, but even during the lowest levels of the cyclic persistent rickettsemia, 27% of ticks became infected.[30] But regardless of whether ticks fed during high or low points in the cyclic persistent rickettsemia, the same number of *A. marginale* develop within the tick salivary gland.[57] Efficient infection of feeding ticks, development of high levels of *A. marginale* in the salivary gland, and longevity of rickettsemia all support an important role for persistent infection in continuous transmission.

The mechanism in which this persistent infection continues seems to involve a mechanism of escape from the immune response.[57] Fully immunocompetent hosts can

harbor persistent *A. marginale* infection indefinitely. Persistently infected cattle are protected against both high-level rickettsemia and clinical disease upon challenge with the homologous strain.[49,59] The paradox, therefore, is that cattle can harbor a low level persistent infection, but can effectively control a challenge dose of greater than 10^8 ID_{100}.[57] A potential mechanism for protection of the organism from immune recognition is the continual emergence and reinfection of new erythrocytes as a means to maintain infection.[57] Cyclic rickettsemia has been hypothesized to reflect a sequential emergence and immune control of antigenic variants.[40] *A. marginale* variants that compose the acute rickettsemia are completely cleared simultaneously with the onset of a primary immune response and distinctly different variant types emerge in persistent infection.[12,57] Furthermore, the variants of the major surface protein-2 (MSP2) are completely cleared ,while new variants of MSP2 emerge.[57] Up to six MSP2 variants are expressed in each rickettsemic cycle, which occur every 4 to 8 weeks in infected cattle.[31,32,40] *A. marginale* has been shown to persist for at least 7 years, indicating that over 500 variants may be expressed in that time period.[7] This supports the hypothesis that it is antigenic variation, rather than ineffective immune response, that is responsible for persistent infection.[57]

Control of the rickettsemic cycles during persistent infection is associated with an IgG_2 response to B cell epitopes predominantly in the hypervariable region[1,31] and also a CD4+ T cell response to the multiple CD4+ T cell epitopes in the N and C terminal regions, and the variant-specific hypervariable region of MSP2.[1,15]

Major Surface Proteins:

The genome of *Anaplasma marginale* is a small circular DNA of only 1.2 to 1.6 Mb.[44,53] After 20 years of research six major surface proteins (MSPs) have been identified. MSP1a (60-105 kDa), MSP1b (100 kDa), MSP2 (36 kDa), MSP3 (86 kDa), MSP4 (31 kDa), and MSP5 (19 kDa) have been identified on erythrocyte-derived organisms, and information about the gene sequences, recombinant protein, monospecific and monoclonal antibodies, isolate variability, and potential value in diagnostic assays and vaccines is available. MSP1a, MSP4, and MSP5 are encoded by single genes, while MSP1b, MSP2, and MSP3 are encoded by multigene families.[42,44]

MSP1a and MSP1b form the MSP1 complex which is a heterodimer composed of two structurally unrelated polypeptides: MSP1a, which is encoded by a single gene *msp1α*, and MSP1b, which is encoded by at least two genes, *msp1β1* and *msp1β2*.[8] MSP1a is variable in molecular weight among geographic isolates because of the different numbers of tandem 28 or 29 amino acid repeats located in the amino-terminal portion of the protein.[21,27] Because of the variation in the repeated portion of the MSP1a gene, it has been used as a stable genetic marker for identification of *A. marginale* geographic isolates.[27] The gene, *msp1α*, that encodes MSP1a is conserved during the multiplication of the rickettsia in cattle and ticks.[10] A neutralization-sensitive epitope was demonstrated on the MSP1a tandem repeats[60] and was found to be conserved among *A. marginale* isolates.[21,26,27] MSP1a was shown to be an adhesin for bovine erythrocytes and both native and cultured tick cells by using recombinant *E. coli* expressing MSP1a in microtiter hemagglutination and adhesion recovery assays and by microscopy.[20,21] The portion of MSP1a with the tandem repeats was found to be necessary and sufficient to

effect adhesion to bovine erythrocytes and tick cells.[21] In addition, MSP1a has been shown to be involved in infection and transmission of *A. marginale* by *Dermacentor* spp. ticks and to be involved in immunity to *A. marginale* infection in cattle.[60] MSP1a was found to be differentially expressed in tick cells and erythrocytes.[37] The amount of MSP1a was higher in erythrocyte derived *A. marginale* which could be linked to the fact that MSP1a is an adhesin for erythrocytes and increased levels would enhance the transmission.[43]

Cattle immunized with *A. marginale* from erythrocytes produce an antibody response against MSP1a, while cattle immunized with *A. marginale* from tick cells produce an antibody response mainly against MSP1b.[37] MSP1a induces a strong T cell response recognizing epitopes in the C terminus region[16], while B cell epitopes are located within the repeated region the variable N terminus end.[38]

MSP1b, which is encoded by the genes *msp1β1* and *msp1β2*, is polymorphic between geographic isolates of *A. marginale*.[10,18,73] There are only small variations in the protein sequences $MSP1b_1$ and $MSP1b_2$ during the life cycle of the rickettsia in cattle and ticks even though MSP1b is encoded by a multigene family.[10,58] MSP1b has also been shown to be an adhesin for bovine erythrocytes,[51,52] but not for tick cells.[22]

MSP3 is encoded by a large polymorphic, multigene family.[4] MSP3 varies in antigenic properties and structure between geographic isolates.[3] MSP3 is also involved in the induction of protective bovine immune response to *A. marginale*.[59] MSP4 and MSP5 are encoded by single copy genes. MSP4 is highly conserved, but its function is still unknown.[26-28] MSP5 is a 19 kDa protein that is also a highly conserved protein with no known function, but it is used as a diagnostic antigen and used in a competitive enzyme

linked immunosorbent assay (ELISA) that is available in the United States commercially.[71,74]

Of all the *A. marginale* MSPs, MSP2 is the most studied and significant contributor to antigenic variation and immunologic evasion by *A. marginale* which leads to persistence in cattle.[31,57] It has been shown that it is variants of MSP2 that are involved in the cyclic rickettsemia seen in *A. marginale* infection and persistence associated with this cyclic rickettsemia.[32,57] Analysis of variants in sequential rickettsemic cycles indicates that MSP2 sequence heterogeneity increases over time during persistence.[65] Ticks that acquisition-feed on cattle with persistent infections ingest a heterogeneous population of variants that differ over time and within different cattle in a herd. Interestingly though, the heterogeneity of the variants is lost as *A. marginale* passes transtadially within the tick. A restricted set of MSP2 variants is expressed in the tick salivary gland, which will be transmitted to cattle in new infection.[65] This is important because this limits the heterogeneity in subsequent acute rickettsemia in uninfected cattle.[65]

The MSP2 multigene family includes 10 or more variable genes widely dispersed throughout the genome.[58] One operon with four open reading frames (ORFs) containing the *msp2* gene at the 3' terminus has been identified.[7] Nine to twenty other truncated pseudogenes for *msp2* have been identified within the operon and these pseudogenes recombine into the operon expression site to generate new hypervariable sequences.[11] Antigenic variants arise from a mechanism in which a single population of *A. marginale* expresses multiple forms of MSP2 pseudogenes, each with conserved amino- and carboxyl termini, but a central hypervariable region (HVR) of about 100 amino acids.[53]

There are at least four different variants of the central hypervariable region in each rickettsemia cycle of persistent infection. The variants differ from one another by a combination of substitutions, deletions, and insertions.[53] The hypervariable region contains exposed surface epitopes that induce antibody after the rickettsemia cycle resulting in a delay in immunity to the new variant.[31,32]

The MSP2 protein is encoded on a polycistronic mRNA that is transcribed from a single genomic expression site.[7] The diversity is so great that up to twenty pseudogenes are insufficient to produce the number of variants that can be seen during lifelong persistence. The currently accepted mechanism for the generation of the diversity is recombination of a whole pseudogene into the expression site[11], followed by a second level of variation which involves small segments of pseudogenes recombining into the expression site by gene conversion.[14]

Vaccine development for anaplasmosis:

The ideal vaccine for anaplasmosis is one that induces protective immunity against *A. marginale*, and thereby prevents infection of ticks and transmission of the agent to susceptible cattle.[34] At present, vaccines do not prevent infection, but do control clinical anaplasmosis. Because of the lack of infection prevention, cattle are persistently infected and are reservoirs for new infections.[44] Control measures have not changed dramatically in the last half century. Current control measures vary with geographic location and include arthropod control by application of acaricides, administration of antibiotics, and premunization with live vaccines, immunization with killed vaccines, and maintenance of *A. marginale*-free cattle herds.[41]

Arthropod control is labor intensive and expensive. It is mainly used in Africa and other areas other than the United States because of the toxicity of some acaricides and the potential development of resistant tick populations due to repeated use.[41]

The most widely used control method in the United States is antibiotic therapy by use of tetracycline drugs. This form of control is directed at the prevention of clinical anaplasmosis and does not prevent persistent infection in cattle. The disadvantages are that cattle may not be cleared of infection, it is expensive, and the requirement that the antibiotic must be continuously fed to cattle.[41]

The two types of vaccines for anaplasmosis are a live vaccine and a killed vaccine. Both use antigen that is derived from infected red blood cells and both are designed to prevent morbidity and mortality, but do not prevent cattle from becoming infected upon challenge exposure.[41] This leaves these vaccinated cattle persistently infected and reservoirs for *A. marginale* transmission by either mechanical or biological means.[41] The other important consideration is that geographic isolates of *A. marginale* often do not cross protect and vaccines are then limited to a geographic area.[47]

Live vaccination has been used for the control of anaplasmosis has been used since Sir Arthur Theiler initiated this strategy in the early 1900's, and is widely used today. Cattle are inoculated with erythrocytes infected with less pathogenic isolates of *A. marginale* or *Anaplasma centrale* (*A. centrale*).[44] Vaccinated cattle develop persistent infections which are protective for life, so the need for revaccination is not necessary.[63] Live vaccines have not been licensed in the United States because of their use of blood from *A. marginale*-infected cattle which may pose a risk of transmitting other blood-borne pathogens.[13,59]

To use the attenuated live *A. marginale* vaccine, two methods have been reported to make a less pathogenic strain. Attenuation was attempted by passage in deer with successful results ,[46] but it has also been reported not to be successful by others.[64] The vaccine was tested and found to be effective and safe for all ages, breeds, and sexes, but there were some reports of post vaccination reactions.[44] Different results were seen in other studies where the vaccinated cattle experienced acute disease and death in the most severe cases.[44] More recently, in 2000, an experiment was carried out where a live, but less pathogenic, *A. marginale* vaccine was given to cattle, but it was not proven to induce protective immunity against anaplasmosis and clinical disease was seen in some cattle.[9]

A. centrale was isolated in the early 1900's by Sir Arthur Theiler, and is the most widely used live vaccine worldwide. Theiler also observed that *A. centrale* is less pathogenic for cattle than *A. marginale* and that cattle infected with *A. centrale* were protected from *A. marginale* infection.[44] Africa, Australia, and Latin America are among some of the areas that still use this vaccination protocol.[44] Several factors contribute to *A. centrale*'s cross protective nature with regard to *A. marginale*. *A. centrale* shares the same MSP2 antigenic variation and persistence that *A. marginale* produces and T cell epitopes have been found to be conserved between *A. centrale* and *A. marginale*.[66] In addition, it has been shown that using MSP1a as a marker, *A. centrale* identity can be determined and differentiated from natural *A. marginale* infection.[67] On the other hand, there have been reports of the *A. centrale* vaccine causing severe anaplasmosis in cattle.[61] Nevertheless, even with the contradictory results, the *A. centrale* vaccine has proven to be worthwhile in protecting against anaplasmosis in many study protocols.[44]

As with the live vaccines, the killed *A. marginale* vaccine has been shown to be effective in preventing anaplasmosis[54], but also ineffective in some studies.[47] These protective failures are caused by some *A. marginale* isolates' lack of cross-protection. The vaccines are most effective when local isolates are used to make them.[47]

Killed vaccines were developed in the United States in the 1960's and were used until 1999 when they were taken off the US market due to company restructuring, but they are still used in some areas.[44] Currently, there are no killed vaccines on the market in the U.S. due to the high production costs and inconsistency in efficacy caused by batch-to-batch variation.[13] The first killed vaccine developed in the 1960's was contaminated with bovine cell membranes and caused hemolytic anemia in some calves after ingesting colostrum from dams that were vaccinated. Since then the vaccines have been purified to remove all host cells.[41]

There are several advantages and disadvantages with the killed vaccines. Advantages include a low risk of contamination with other infectious agents, inexpensive storage, and limited post inoculation reactions of clinical relevance. Disadvantages include the need for yearly boosters, a higher cost of purification of *A. marginale* from erythrocytes, and the lack of cross protection between geographic isolates.[44]

In addition to vaccinating cattle for control of anaplasmosis, the infection-treatment method is used. It involves infecting cattle with *A. marginale*-infected erythrocytes and then treating with antibiotics during patent infection.[44] The cattle do not experience acute anaplasmosis and are persistently infected.[63] However, this type of vaccination is unfavorable because of its requirement for veterinary care which increases costs to farmers, and the monitoring needed to treat at the appropriate time.[44]

There are several new approaches to vaccine development being explored at this time. Of the 6 MSPs, MSP1a seems to be the best candidate for vaccine development.[38] Although MSP2 induces a strong T cell response, its antigenic variability reduces its effectiveness and potential as a vaccine candidate.[25] Limited recombinant vaccine trials with recombinant MSPs or with naked DNA have been have been conducted. Only partial protection has been achieved with recombinant antigens, indicating that multiple antigens will have to be used to gain a protective immune response.[44]

MSP1a is being examined as a vaccine candidate because it induces a strong T cell response[37] and has conserved B cell epitopes that are recognized by immunized and protected cattle.[38] In addition, MSP1a also plays an important role in infection and transmission because it is an adhesin for both tick cells and bovine erythrocytes.[20, 21,60] One factor to be considered regarding MSP1a as a vaccine candidate is the fact that when infected cattle mount an immune response to MSP1a, it is likely that is it actually the MSP1 complex against which the response is directed, which may hide some of the MSP1a epitopes that would be delivered in the recombinant form.[25] In support of this, Garcia-Garcia, et *al*. found that after immunizing cattle with *A. marginale* from infected erythrocytes, MSP1a was up regulated with respect to MSP1b and was probably not coupled with MSP1b to form the MSP1 complex The level of protection obtained with this strategy was comparable to that of cattle immunized with recombinant MSP1a.

Another strategy that has been considered is the identification of a functionally similar protein to the *A. marginale* MSP1a in *A. centrale*. Multiple attempts at cloning have not identified a similar protein yet, but both *A. marginale* and *A. centrale* share immunodominant epitopes that may play a role in protection against *A. marginale*.[66,67]

A second approach to develop an effective *A. marginale* vaccine is the inclusion of tick antigens in addition to *A. marginale* antigens. A vaccine using tick antigens has already been developed against the tick species *Boophilus spp*., which has been proven to transmit *A. marginale* as well as other species in the Ixodid family.[23] This approach is aimed at controlling tick populations and reduction of tick-borne pathogen transmission.[34] The antigen, 4D8 (subolesin)[19], has been identified as a tick antigen expressed in the tick gut and salivary glands and mRNA expression is detected in all *Ixodes scapularis* developmental stages (eggs, larvae, nymphs, and adults).[5] Tick infestations are also reduced with vaccination with 4D8 tick antigen.[6] Using an antigen expressed in the salivary glands may affect the tick's ability to properly feed by impacting the salivary gland development and therefore the ability to transmit *A. marginale*.[19] The mode of action is both antibody dependent with ingestion of blood from a vaccinated host, and complement dependent with the destruction of the tick gut.[76] Also, rickettsemia may be reduced in ticks that feed off vaccinated hosts.[19]

The third and final approach to making a better *A. marginale* vaccine is a cell culture system that will allow for propagation of *A. marginale* for vaccines. There are many advantages to using a cell culture system to propagate *A. marginale*, as opposed to using the current method of isolating A. *marginale* from infected bovine erythrocytes. The cell culture system provides a much higher percentage of infected material per host cell than an equivalent volume of infected erythrocytes and *A. marginale* can be harvested continually. This system also provides a method of propagating *A. marginale* without inadvertently including contaminating pathogens in the antigen preparation. Furthermore, the need for cattle is eliminated, thus reducing the cost to make a vaccine.[41]

In the last ten years, a cell culture system for propagation of *A. marginale* has been established using the tick cell line, IDE8 that is derived from *Ixodes scapularis* embryos. It has been determined that the developmental cycle of *A. marginale* is the same in the tick culture system as in naturally infected ticks and the *A. marginale* isolated was infective for both cattle and ticks.[44] All six MSPs are conserved in the cell culture-derived *A. marginale*, the antigenic composition remained the same through successive passage in cell culture and ticks, and the antigenic identity, determined by MSP1a molecular weight, was retained in cell culture.[44] Cattle immunized with cell culture-derived *A. marginale* developed protective immunity and did not develop clinical anaplasmosis when challenged. The benefits of tick cell culture-derived vaccines resembled those imparted by vaccines derived from erythrocytes infected with *A. marginale*.[24]

Interestingly, there was a different immune response to the cell culture derived *A. marginale* and erythrocyte derived *A. marginale* in immunized cattle. Cattle immunized with erythrocyte derived *A. marginale* had a preferential antibody response to MSP1a, while cattle immunized with cell culture derived *A. marginale* had a preferential antibody response to MSP1b.[24] This difference probably correlates with the different functions of MSP1a and MSP1b in bovine erythrocytes and tick cells. MSP1a is an adhesin for both bovine erythrocytes and tick cells, while MSP1b is an adhesin only for bovine erythrocytes.[37]

An improvement to a cell culture derived vaccine would be to include isolates from the two clades recently identified: a southeastern U.S. clade and a central and western US clade.[27] This would enhance the efficacy of the vaccine by protecting against

a more diverse selection of isolates. To date, a cell culture derived vaccine has not been marketed, but efforts continue to develop an effective one.

A. marginale immunity is characterized by both B and T cell responses, but the key is a cytotoxic T lymphocyte (CTL) response , as is elicited by natural infection.[37, 38] Mature erythrocytes are susceptible to infection, but lack nuclei; therefore, they cannot process and present *A. marginale* peptides in the context of MHC. One must conclude that other nucleated cells in infected cattle are infected by *A. marginale* and can present foreign antigen in the context of MHC I to elicit a CTL response. One possible cell type is the endothelial cell, as has been shown recently to be susceptible to infection by *A. marginale in vitro*[56]. Efforts in vaccine development for *A. marginale* might be best focused on using endothelial cell culture to generate protective B and T cell responses. This approach would capitalize on the many advantages of cell culture-derived vaccines and the proven susceptibility of endothelial cells to infection *in vitro* with *A. marginale*. The purpose of this study was to define the susceptibility of endothelial cells to infection by *A. marginale* in an attempt to determine the initiators of strong cell-mediated immunity in infected cattle. If endothelial cells are shown to be susceptible *in vivo* to *A. marginale* infection, then this would provide further support for the development of endothelial-derived cell culture vaccines against anaplasmosis.

II. *IN VIVO* CELL INFECTION BY *ANAPLASMA MARGINALE*

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Abstract

Anaplasmosis is an arthropod-borne hemoparasitic disease of cattle and other ruminants. The causative agent is the gram negative bacterium, *Anaplasma marginale.* Infection of bovine erythrocytes by *A. marginale* has been well established *in vivo*, as well as *in vitro*. Recently, *A. marginale* has been propagated *in vitro* in bovine and primate vascular endothelial cell cultures. This finding provides evidence that infected endothelial cells may initiate MHC-Class-I restricted CTL responses in infected cattle. To determine the extent to which endothelial cells are susceptible to *A. marginale* infection *in vivo*, a dual staining technique was applied to tissues from a splenectomized calf experimentally inoculated with 10^9 organisms from the St. Maries strain of *A. marginale.* Sections of kidney, lung, and hemal lymph node were collected, embedded in freezing compound, frozen in isopentane/liquid nitrogen, and cryosectioned at 5 microns. Sections were co-labeled with monoclonal antibody ANAF16C1, recognizing *A. marginale* major surface protein 5 (MSP5) conjugated to fluorescein isothiocyanate (FITC) or Alexa Fluor 488 and a polyclonal rabbit antibody against human von Willebrand Factor (an endothelial cell marker) conjugated to Tetramethylrhodamine isothiocyanate (TRITC) or Alexa Fluor 568. Nuclei were stained with 284nM 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI). Sections were evaluated by conventional wide field fluorescence microscopy using a Nikon Eclipse E800 and confocal fluorescence microscopy using a BioRad MRC

1024 Scanning Laser Confocal Microscope. As expected, non-endothelial cells within vascular lumens were the major reservoir for *A. marginale*. In addition, *A. marginale* fluorescence co-localized to capillary endothelial cells of the kidney, lung, and hemal lymph node. These results suggest that endothelial cells may serve as a cellular reservoir for *A. marginale in vivo* has implications for both pathogenesis and immune mechanisms.

Key Words: *Anaplasma marginale*, CTL, endothelial cells, fluorescent microscopy

Anaplasmosis is an arthropod-borne hemoparasitic disease of cattle and other ruminants. The causative agent is the gram negative bacterium, *Anaplasma marginale* belonging to the order Rickettsiales, and the family of the Anaplasmataceae.[42,43] The disease is characterized by severe anemia associated with intraerythocytic parasitism. Clinical signs include depression, weakness, high body temperature, dehydration, jaundice, and low milk production, which are mainly due to severe anemia caused by erythrocyte disruption and removal.[43] Cattle that recover remain chronic carriers of the parasite for life, and serve as a reservoir for uninfected cattle.[47]

The disease is devastating to meat, milk, and fiber production in many tropical and subtropical areas.[43] Enzootic regions include Africa, Asia, Australia, Southern Europe, South America, the former Soviet Union, and is believed to be endemic to 40 states of the United States. Bovine anaplasmosis losses include death (36% of clinical cases), abortion (24% of clinical cases in pregnant cows), weight loss (86kg per clinical case), and increased veterinary and management costs.[2] Yet, it is hard to calculate losses due to anaplasmosis in many regions because of inadequate records, inability to quantify

production losses, or concurrent infection with other hemoparasitic and tick-borne diseases.

After many reclassifications, *A. marginale* it is now classified in the order of the Rickettsiales, family of the Anaplasmataceae.[28] The family includes the genera *Anaplasma, Ehrlichia, Neorickettsia,* and *Wolbachia* encompassing a group of obligate intracellular bacteria that reside in eukaryotic cells. They all reside within a cytoplasmic vacuole inside host cells that include erythrocytes, reticuloendothelial cells, endothelial cells, and cells of insect, helminth, and arthropod reproductive tissues.[28] Most often, these pathogens are detected in cells of hematopoietic origin and ticks ensure transmission. It is thought that the only site of development of *A. marginale* is the erythrocyte.[45]

The closely related bovine-infecting agent, *Ehrlichia (Cowdria) ruminantium*, is known to infect and develop in endothelial cells after initial development in macrophages and neutrophils.[48,62] A similar mode of infection may be present in *A. marginale* infected hosts. *A. marginale* enters red blood cells by endocytosis after which it divides by binary fission.[33,68]

Presently, *A. marginale* has only been shown to infect mature, circulating erythrocytes *in vivo*.[45] In contrast, the organism invades and replicates in the nucleated cells of the midgut and salivary gland epithelium of the ixodid tick vectors, including *Rhipicephalus (Boophilus) microplus*, *R. annulatus, Dermacentor andersoni*, and *D. variabilis*.

Immature circulating erythrocytes and bone marrow precursor cells appear to be resistant to infection by the organism.[39] Recently, Munderloh *et al*. used tick cell cultures of the Virginia isolate of A. *marginale*, Am291, to inoculate, *in vitro,* the cell line, BCE

C/D1-b, from bovine vascular endothelial cells, primate Vero cells, and RF/6A, from rhesus monkey microvascular endothelium.[56] At first, large intracellular inclusions were seen by phase contrast microscopy, then tiny granules developed, and within a week, the monolayers were completely destroyed, and Giemsa-stained culture samples confirmed massive invasion, endothelial cell rupture, and release of *A. marginale* initial bodies.[56]

Immunity to *A. marginale* is correlated with the emergence of neutralizing IgG_2 antibodies and the activation of CD4+ T cells, which in turn secrete cytokines such as IFN gamma that activate macrophages to phagocytize and kill the organism.[17,35] However, neutralization of IFN-gamma or inhibition of nitric oxide synthase fails to significantly influence the course of disease [36,72], suggesting that other effector mechanisms, such as cytotoxic T-lymphocytes, might contribute to immunity through the recognition of *A. marginale* peptides in the context of MHC Class I. Mature erythrocytes are the only known cellular reservoir for *A. marginale* in vivo, but because they lack a nucleus, they would be unable to present foreign peptides in the context of MHC I molecules. Therefore, there must be another cell type that is causing this CTL immune response. Because it has been shown that endothelial cells *in vitro* can be infected with *A. marginale*, it is plausible that they are also infected *in vivo*. The present study was conducted to show that endothelial cells are, indeed, infected *in vivo*.

Materials and Methods

Tissue preparation and fluorescent labeling

Tissue sections and RF/6A cells were first blocked with 5% normal rabbit serum (NRS) (Biomeda, Foster City, CA) for 30 minutes at room temperature, rinsed in PBS, and then co-incubated with the *A. marginale* monoclonal antibody, anti-ANAF16C1[74] (100 µg/ml) and polyclonal rabbit anti-human von Willebrand Factor (vWF, Dako, Carpinteria, CA) at the concentration recommended by the manufacturer for 30 minutes at room temperature, then rinsed for 3 minutes in PBS. Next, the tissue sections were co-incubated with 200µl of Alexa Fluor 488 (or fluorescein isothiocyanate (FITC)) -conjugated goat anti-mouse IgG and Alexa Fluor 568 (Tetramethylrhodamine isothiocyanate (TRITC)) -conjugated goat anti-rabbit IgG (Molecular Probes, Carlsbad, CA), both at a concentration of 2µg/ml for 30 minutes at room temperature. Sections were then rinsed for 3 minutes in 1X PBS. RF/6A cells infected with *A. marginale* were used as a positive control for organism staining.[56] In addition, RF/6A cells and tissue sections were labeled with the lectin, FITC-conjugated *Ulex europaeus* Agglutinin I (UEAI) (Vector Laboratories, Burlingame, CA), in the same manner as the primary antibodies. UEAI is reported to label bovine endothelial cells.[39] All procedures after the application of fluorochromes were performed in a dark chamber. Tissue sections were then incubated with 200µl of 4', 6-diamidino-2-phenylindole, dihydrochloride (DAPI) (Molecular Probes, Carlsbad, CA) at a concentration of 284nM, for 30 minutes at room temperature. Negative control sections were incubated with secondary antibodies alone and counterstained with DAPI. Tissue sections were coverslipped with Citifluor (Electron

Microscopy Sciences, Hatfield, PA) and maintained in the dark at 4° C prior to examination.

Wide field fluorescent analysis

Tissue sections and the RF/6A cells were analyzed with a Nikon Eclipse E800 microscope fitted with 3 Nikon filter cubes for wide field fluorescence capture. The UV2A filter cube (330-380 nm excitation) was used for DAPI capture, the G2A filter cube (510-570 nm) for Alexa 568 capture, and the B3A filter cube (420-495 nm) for Alexa 488 capture. Images were captured using the Spot Advanced™ software (Diagnostic Instruments, Sterling Heights, MI).

Confocal fluorescent analysis

Prior to analyzing the tissue sections with the BioRad MRC 1024 Confocal Scanning Laser Microscope (Carl Zeiss, Inc., Thornwood, NY), the co-localization capability of the microscopy was assessed with Tetraspeck fluorescent microspheres (Molecular Probes, Invitrogen, Carlsbad, CA). Microspheres of 1µm size were used because they are about the same size of one *A. marginale* bacterium.

Tissue sections were analyzed with a BioRad MRC 1024 Confocal Scanning Laser Microscope (Carl Zeiss, Inc., Thornwood, NY). The 488nm laser was used for FITC capture, and the 568nm laser was used for the TRITC capture. Images were captured and processed using the LaserSharp 2000 Software (Carl Zeiss, Inc., Thornwood, NY).

Differential Interference Contrast (DIC) analysis

Tissue sections were analyzed with a Nikon Eclipse E800 microscope. The analyzer and Wollaston prisms were employed to capture the DIC images.

Results

Wide field fluorescent microscopy

Wide field fluorescence microscopy of kidney sections revealed co-localization of *A. marginale* and vWF along vascular endothelium in a granular pattern of fluorescence (Figure 4).

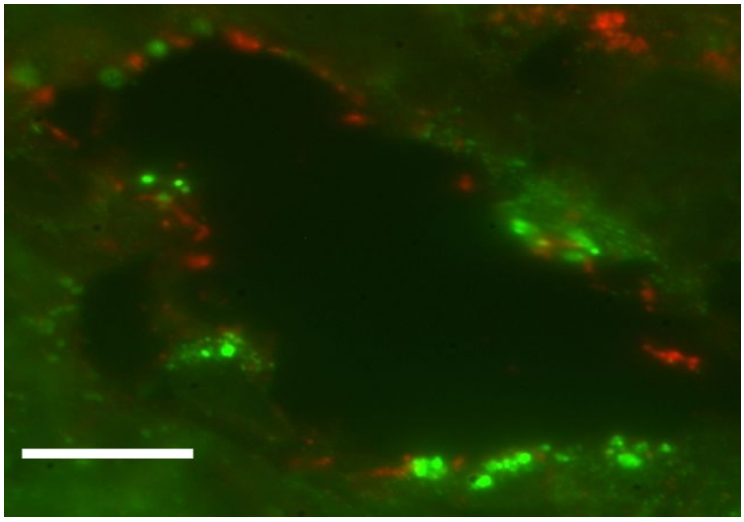

Figure 4. Kidney; calf #C1058. Green fluorescence shows the localization of A. marginale (antibody ANAF16C1) along the endothelial surface of a vein. Indirect immunofluorescence method with Alexa 488 fluorochrome. Red fluorescence shows endothelial cells as identified by vWF expression in the same section using indirect immunofluorescence method with Alexa 568 fluorochrome. Bar =20µm.

Similar patterns of fluorescence were identified in the lung and hemal lymph nodes (data not shown). The granular pattern was attributable to the localization of vWF in Weibel-Palade bodies, which are reported to occur at a lower density in endothelial cells of capillaries when compared with endothelial cells of small arteries.[75] As a confirmation of endothelial labeling, the kidney sections were also labeled with ANAF16C1 and the lectin, UEAI (Figure 5).

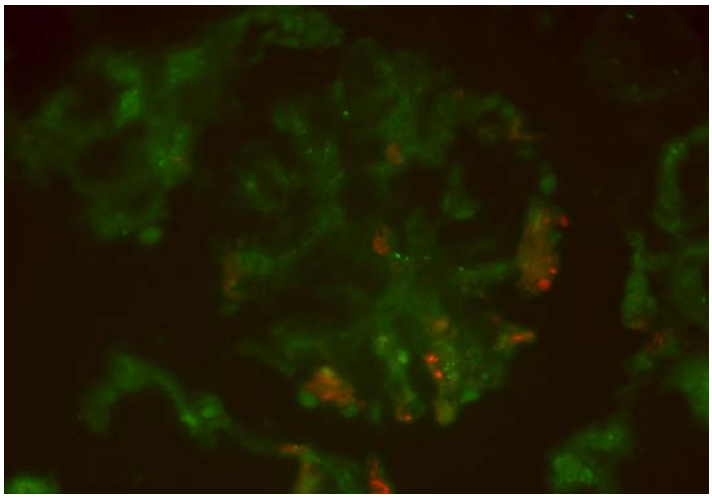

Figure 5. Kidney; calf #C1058. Green fluorescence with Ulex europaeus Agglutinin I (UEAI) shows endothelial labeling within a glomerulus. Red fluorescence with A. marginale antibody ANAF16C1 and indirect immunofluorescence with Alexa 568. Direct lectin-FITC fluorescence.

Negative controls, consisting of sections from the same tissue without the application of primary antibody or lectin, were devoid of specific staining of both *A. marginale* and vWF. RF/6A cells expressed low levels of vWF, as reported[50] and therefore UEAI (expressed highly in these cells) was used as an endothelial cell marker. These cells demonstrated co-labeling with the endothelial cell-lectin UEAI-FITC (as provided by the manufacturer) and mAb ANAF16C1 conjugated to Alexa 568 (counterstained with DAPI) (Figure 6).

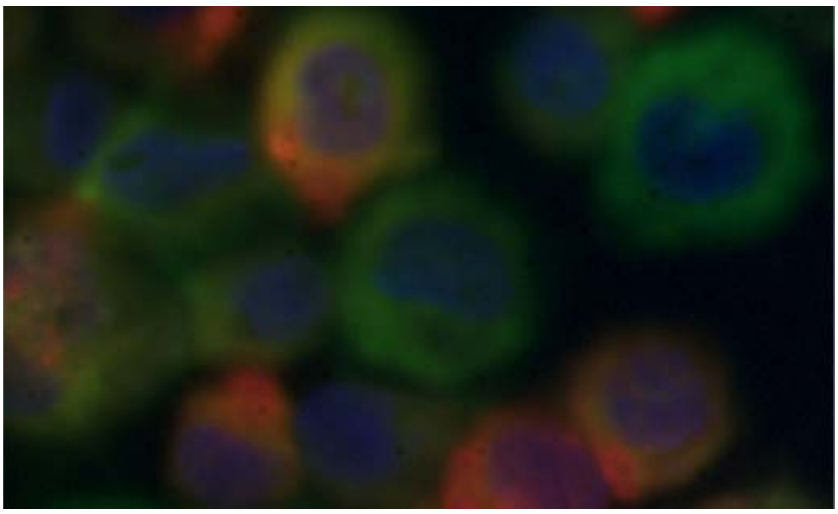

Figure 6. RF/6A cell culture. Green fluorescence with UEAI identifies the endothelial cell line RF/6A by direct lectin-FITC labeling and DAPI counterstaining. Red fluorescence shows A marginale labeling of RF/6A cells incubated with A. marginale antibody ANAF16C1 and detected by indirect immunofluorescence method using Alexa 568 fluorochrome.

Confocal fluorescent microscopy

Analysis of the co-localization capability of the BioRad MRC 1024 Confocal Scanning Laser Microscope (Carl Zeiss, Inc., Thornwood, NY) with the Tetraspeck fluorescent microspheres (Molecular Probes, Invitrogen, Carlsbad, CA) revealed that the microscope was capable of co-localizing different fluorochromes (Figures 7-9).

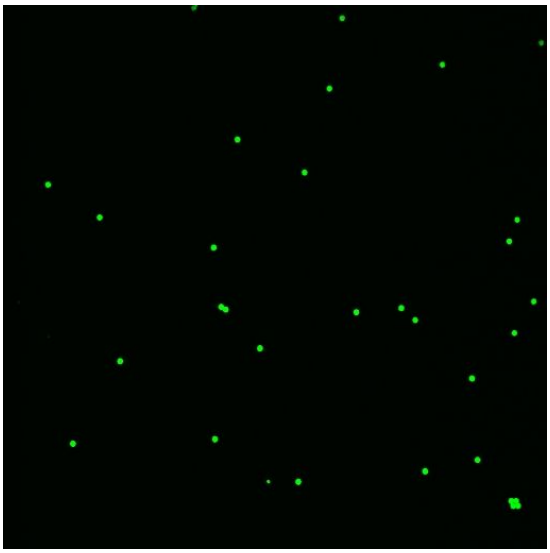

Figure 7. Confocal analysis of Tetraspeck fluorescent microspheres using the 488 nm laser.

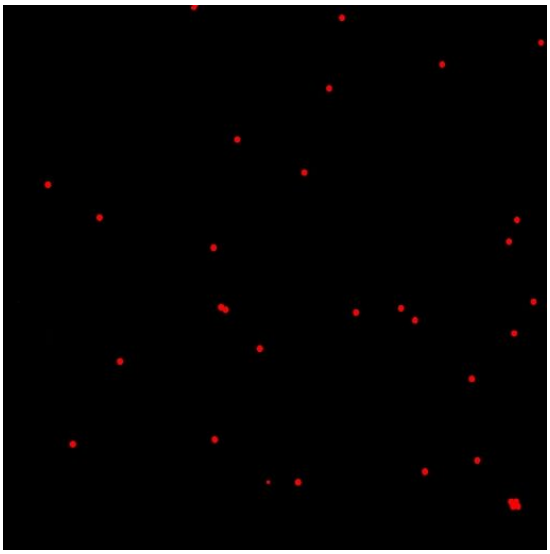

Figure 8. Confocal analysis of Tetraspeck fluorescent microspheres using the 568 nm laser.

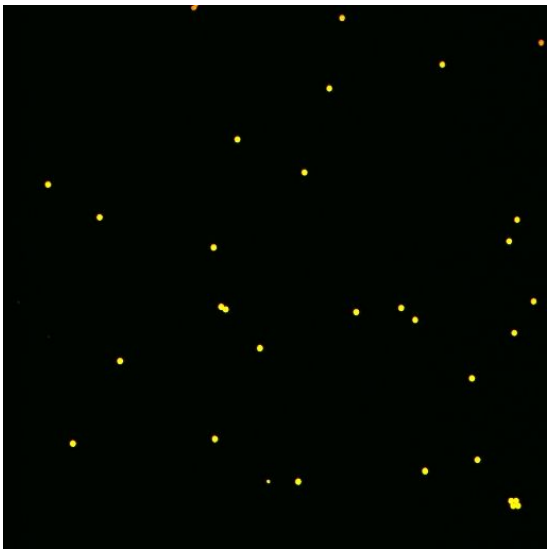

Figure 9. Merged image of Figures 4 and 5 showing co-localization of the fluorescent microspheres.

Confocal fluorescent microscopy was used because of its capabilities to enhance the image quality by capturing very sharp images. It accomplishes this in two ways. First, it captures images through a pinhole aperture as opposed to a wide, open aperture with conventional fluorescent microscopy which allows for only the in-focus layer of tissue to be captured. Secondly, confocal fluorescent microscopy enhances image quality through the use of a Z stack. A Z stack is a series of images taken at pre-set intervals throughout the thickness of the tissue section. This series of images is then compressed to make one image combining the fluorescence throughout the tissue having an additive effect on the total fluorescence of the tissue section. The confocal fluorescent images of the hemal lymph node, kidney, and lung show a marked enhancement of the image sharpness and overall quality (Figures 10-12).

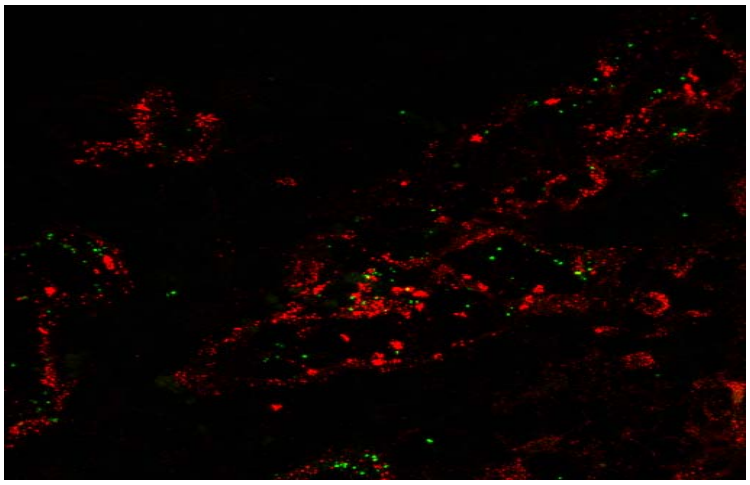

Figure 10. Confocal Laser Scanning Microscopy. Hemal lymph node tissue section labeled with A. marginale antibody ANAF16C1 and Alexa Fluor 488 (green fluorescence). Endothelial cell labeling with vWF and indirect limmunofluorescent labeling with Alexa 568 (red fluorescence).

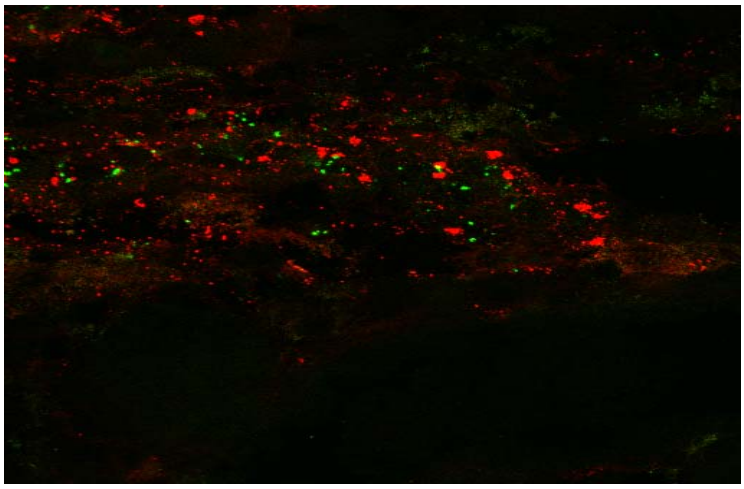

Figure 11. Confocal Laser Scanning Microscopy. Kidney tissue section labeled with *A. marginale* antibody ANAF16C1 and Alexa Fluor 488 (green fluorescence). Endothelial cell labeling with vWF and indirect limmunofluorescent labeling with Alexa 568 (red fluorescence).

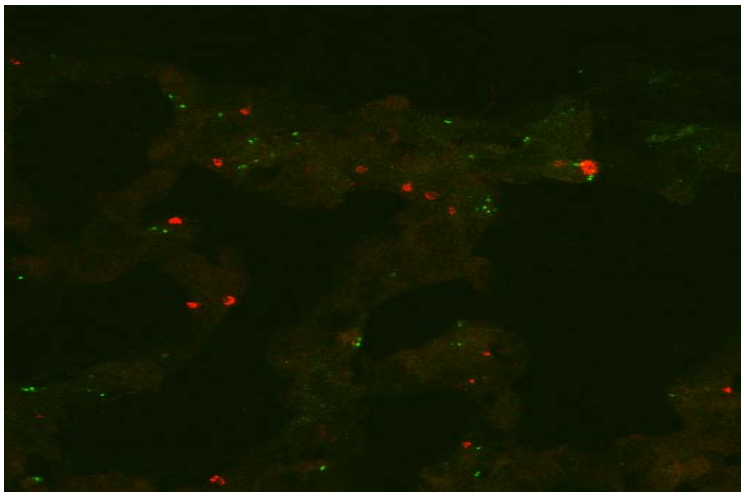

Figure 12. Confocal Laser Scanning Microscopy tissue labeling. Lung tissue section labeled with A. marginale antibody ANAF16C1 and Alexa Fluor 488 (green fluorescence). Endothelial cell labeling with vWF and indirect limmunofluorescent labeling with Alexa 568 (red fluorescence).

Differential Interference Contrast microscopy

DIC microscopy is a very useful tool when analyzing tissue sections by immunofluorescence because it allows for the visualization of the tissue architecture which is lost using fluorescent analyzation. DIC uses two beam splitting prisms, called Wollaston prisms, in addition to two polarizers, called a polarizer and an analyzer. The first Wollaston splits the beam of light causing the light to take two different paths through the object. Differential interference between the two light paths causes the contrast seen in the object. After the light passes through the object, the second prism, called a Wollaston prism, recombines the two light paths which are what is seen through the objective. The glomerulus captured in the kidney section by conventional wide field fluorescent microscopy was also analyzed using DIC microscopy. The images show both the kidney architecture, and the fluorescence of *A. marginale* and vWF (Figures 13-15).

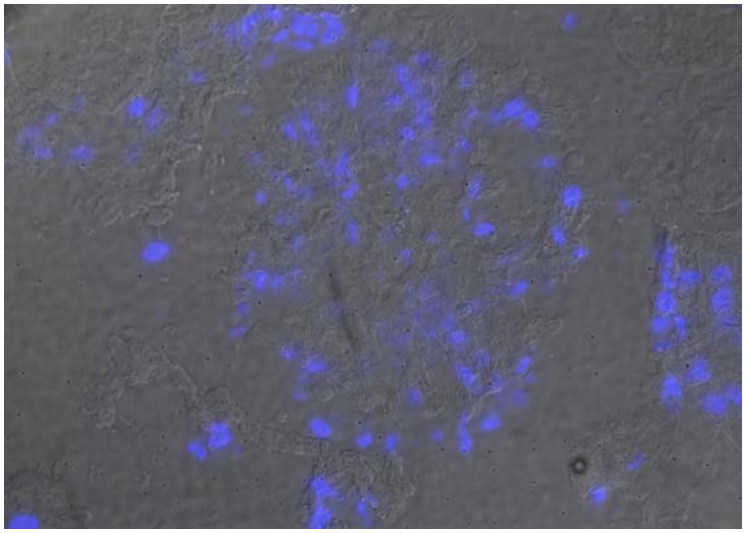

Figure 13. DIC microscopy of the kidney. DAPI counterstaining.

Figure 14. DIC microscopy of the kidney. Indirect immunofluorescence with Alexa 568 staining and A. marginale antibody ANAF16C1.

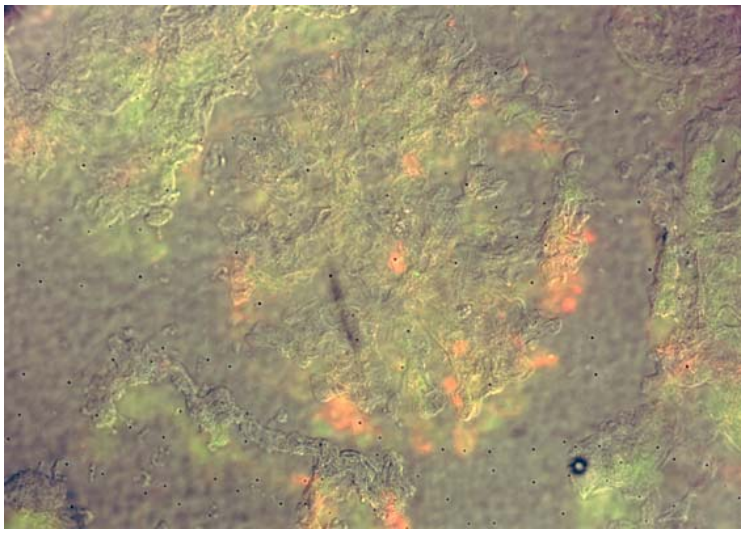

Figure 15. DIC microscopy of the kidney. vWF with indirect immunofluorescence with Alexa 488 (green fluorescence). Red fluorescence with A. marginale antibody ANAF16C1 and indirect immunofluorescence with Alexa 568.

Discussion

It is well known that *A. marginale* infects erythrocytes readily both *in vivo* and *in vitro*, but given the lack of MHC-I expression, it is unknown how a CTL response in the host is initiated. Activated CD4+ T cells secrete cytokines such as IFN-gamma that activate macrophages to phagocytize and kill intracellular pathogens, but neutralization of IFN-gamma fails to influence the clearance of infection by *A. marginale*. This finding suggests that other cell-mediated effector mechanisms, such as CTL, may contribute to the immune response to *A. marginale.* *A. marginale* major surface proteins (MSP) MSP1a and MSP2 both induce a strong T cell response[25,37], including cytotoxic T lymphocytes (CTL), that is necessary for an adequate immune response. Since there is a T cell response, there must be a cell type that recognizes CTL epitopes of *A. marginale* in the context of MHC I. This leads to the conclusion that there are one or more cell types

other than erythrocytes that are susceptible to *A. marginale* infection in infected cattle. T cell responses could be important in the clearance of infected nucleated cells that serve as reservoirs for *A. marginale* infection. These data demonstrate endothelial cell infection *in vivo* after experimental infection of a calf with *A. marginale* and extend the findings that endothelial cells are susceptible to infection after *in vitro* challenge.[56] Endothelial cells may serve as an early reservoir for *A. marginale* infection at the site of tick attachment, and through their expression of MHC Class-I, could be important in the initiation of a CTL response during the early stages of infection. Further studies are needed to define the role of endothelial cells in these key components of *A. marginale* infection and pathogenesis.

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