Infection of Tick Cells and Bovine Erythrocytes with One Genotype of the Intracellular Ehrlichia *Anaplasma marginale* Excludes Infection with Other Genotypes

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*Anaplasma marginale*, a tick-borne rickettsial pathogen of cattle, is endemic in several areas of the United States. Many geographic isolates of *A. marginale* that occur in the United States are characterized by the major surface protein 1a, which varies in sequence and molecular weight due to different numbers of tandem repeats of 28 or 29 amino acids. Recent studies (G. H. Palmer, F. R. Rurangirwa, and T. F. McElwain, J. Clin. Microbiol. 39:631-635, 2001) of an *A. marginale*-infected herd of cattle in an area of endemicity demonstrated that multiple *msp1* 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. The present study was undertaken to confirm the phenomenon of infection exclusion of *A. marginale* genotypes in infected bovine erythrocytes and cultured tick cells. Two tick-transmissible isolates of *A. marginale*, one from Virginia and one from 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 with only the Virginia isolate of *A. marginale*. When cultures were inoculated with different ratios of the Oklahoma and Virginia isolates of *A. marginale*, the isolate inoculated in the higher ratio became established and excluded infection with the other. When cultures with established infections of one isolate were subsequently infected with the other, only the established isolate was detected. We documented infection exclusion during initial infection in cell culture by labeling each isolate with a different fluorescent dye. After 2 days in culture, only a single isolate was detected per cell by fluorescence microscopy. Finally, when *Anaplasma ovis* infections were established in cultures that were subsequently inoculated with the Virginia or Oklahoma isolate of *A. marginale*, *A. marginale* infection was excluded. These studies confirm that infection exclusion occurs with *A. marginale* in bovine erythrocytes and tick cells, resulting in the establishment of only one genotype, and appears to be the first report of infection exclusion for *Anaplasma* and *Ehrlichia* species.

Anaplasmosis, a tick-borne disease of cattle caused by the obligate intraerythrocytic bacterium *Anaplasma marginale* (*Rickettsiales; Anaplasmataceae*) is endemic in many tropical and subtropical areas, including several areas of the United States. Several geographic isolates of *A. marginale*, which differ in biology, morphology, sequence, and antigenic characteristics, have been identified in the United States (1, 11, 12, 37, 40). Feeding ticks effect biological transmission of *A. marginale*, whereas mechanical transmission occurs when infected blood is transferred to susceptible animals by biting flies or by blood-contaminated fomites. Cattle that recover from acute infection remain persistently infected and serve as reservoirs for mechanical transmission and infection of ticks (reviewed in reference 15).

*A. marginale* multiplies only within membrane-bound inclusions in the cytoplasms of host cells. The only known site of development of *A. marginale* in cattle is within erythrocytes (35), whereas *A. marginale* develops in several tissues in ticks and undergoes a development cycle that is complex and coordinated with the tick feeding cycle (16, 20–22).

*MSP1a* is one of six major surface proteins (MSPs) that have been described for *A. marginale* from bovine erythrocytes. MSP1a is encoded by a single gene, *msp1a*, which is conserved during the multiplication of the parasite in cattle and ticks, therefore resulting in a stable genetic marker of *A. marginale* geographic isolates (2, 3, 7). The molecular weight of MSP1a differs among geographic isolates because of a variable number of tandem repeats of 28 or 29 amino acids (1, 11).

Recently, *A. marginale* was propagated in continuous culture in a cell line, IDE8, derived from embryos of the tick *Ixodes scapularis* (6, 27). *A. marginale* organisms harvested from cell culture were infective for both cattle and ticks (6, 27). The MSPs characterized for *A. marginale* from bovine erythrocytes were found to be conserved on the cell culture-derived organisms, and the antigenic composition of *A. marginale* remained the same after successive passage in cell culture (3) or after passage through ticks (3, 4). The *A. marginale* isolate antigenic identity, as determined by the sequence and molecular weight of MSP1a, was retained in culture (3, 6, 7, 27).

A recent report by Palmer et al. (32) documented genetic heterogeneity in the structure of *msp1a* sequences recovered from infected animals in a herd of cattle in eastern Oregon.
where *A. marginale* is endemic. However, single *msp1α* genotypes were identified in individual cattle that were naturally or experimentally infected and sampled at different stages of infection (32). These findings suggest that exclusion of other *A. marginale* isolates may occur in infected cattle (14).

Infection (superinfection) exclusion or homologous interference is a phenomenon that was first described to occur in bacteriophages (29, 38) and later confirmed in animal viruses (9, 18, 24). Bacterial, vertebrate, or invertebrate cells infected with one virus did not become productively infected with the same or a closely related virus (18). Infection exclusion has been documented for *Rickettsia* spp. (8). The mechanism of infection exclusion is not well understood, and it has been suggested that a number of factors, including competition for host cell receptors or intracellular host factors, the production of interferon or interferon-like substances by the infected host cell, the production of defective interfering viral genomes from the first infecting virus, and the production of transacting protein by the first virus, have been posited to contribute to this phenomenon (18).

In this report, we used two tick-transmissible *A. marginale* isolates from Virginia and Oklahoma to test the hypothesis that exclusion of more than one genotype of *A. marginale* occurs in bovine erythrocytes and cultured tick cells. We also used a second species of *Anaplasma* in tick cell culture, *Anaplasma ovis*, to test whether infection exclusion occurs between two different *Anaplasma* species.

**MATERIALS AND METHODS**

**Isolates of *Anaplasma* spp.** The Virginia isolate of *A. marginale* used for these studies was originally obtained in 1978 from the USDA Animal Disease Research Laboratory, Beltsville, Md., and has been used for tick transmission and cell culture studies in our laboratory (5, 20–22, 27). The Oklahoma isolate of *A. marginale* was obtained from a naturally infected bovine from Wetumka, Okla., in 1997 (6, 11). Each isolate was inoculated into a splenectomized calf, and blood samples collected at the peak of infection were prepared as stablates and stored in liquid nitrogen or used to inoculate tick cell monolayers. The Idaho strain of *A. ovis* (28, 31) was obtained from Guy Palmer (Washington State University, Pullman) and used to infect a splenectomized sheep. Blood samples collected at the peak of infection were also used for preparation of blood stablates and for inoculation and propagation of *A. ovis* in cultured IDE8 tick cells.

**Coinfection studies in cattle.** (i) Cattle. Four mixed-breed cattle (8 to 10 months old) determined to be free of infection by an *A. marginale*-specific enzyme-linked immunosorbent assay (ELISA) (36) were used for the coinfection studies. Cattle were housed in the Anaplasmosis Research Barn and cared for by the Oklahoma State University Animal Research Unit with the approval of the Institutional Animal Care and Use Committee. Calves experimentally infected with the *A. marginale* isolates were monitored three times a week by examination of stained blood smears and determination of the packed cell volume. Thin smears were made on glass slides from cattle blood collected by venopuncture in EDTA-treated Vacutainer tubes and stained with Protocol Hema 3 stain (Biomedical Sciences, Inc., Swedesburg, N.J.). The percentage of infected erythrocytes out of 500 was determined and reported as the percentage of parasitized erythrocytes (PPE). Once infection was detected in blood smears, the calves were monitored daily.

(ii) **Trial 1: coinfection of *A. marginale* in cattle.** Bovine PA444 was inoculated intravenously (i.v.) with 8 ml of the Oklahoma *A. marginale* stablate made from blood collected from bovine PA417 with a PPE of 9.3% and 8 ml of the Virginia *A. marginale* stablate made from blood collected from bovine PA432 with a PPE of 9.8%. Blood samples (1 ml each) were collected daily during patent acute infection and stored at −70°C until used for PCR analysis for identification of the *A. marginale* isolate.

(iii) **Trial 2: coinfection of *A. marginale* in cattle.** Three cattle (PA465, PA466, and PA467) were used for trial 2. PA465 was inoculated i.v. with 4 ml of the Virginia *A. marginale*-infected blood stablate (PPE, 25.9%) made from blood collected from PA432. PA466 was inoculated i.v. with 4 ml of the Oklahoma *A. marginale*-infected blood stablate (PPE, 31.6%) made from blood collected from PA417. PA467 was inoculated i.v. with 4 ml of each of the same Oklahoma and Virginia *A. marginale*-infected blood stablates used for inoculation of PA465 and PA466. Blood samples (1 ml each) were collected daily from all cattle during patent infection and stored at −70°C until used for PCR analysis for identification of the *A. marginale* isolate.

**Coinfection studies of *A. marginale* and *A. ovis* in cultured tick IDE8 cells.** (i) Maintenance of infected and uninfected tick cells. Monolayers of IDE8 (ATCC CRL 1973) cells, originally derived from embryonic *I. scapularis* ticks, were maintained at 31°C in L-15B medium supplemented with 5% fetal bovine serum, tryptose phosphate broth, and bovine lipoprotein concentrate (ICN, Irvine, Calif.) as described by Munderloh et al. (26). Monolayers were subcultured at ratios of 1:5 to 1:10 (vol/vol) when monolayers reached a density of approximately 5 × 10⁶ cells/cm². *A. marginale*- and *A. ovis*-infected *I. scapularis* cells were maintained at 34°C in L-15B medium as described previously (27). The medium was replaced weekly until the desired infection levels were reached. Cultures were routinely harvested when infection levels reached 90% to 100% and detachment of infected host cells was apparent (terminal culture). Culture material was subinoculated onto uninfected tick cells at ratios of 1:5 to 1:10 (vol/vol) in 25-cm² flasks. The bacterial concentration was determined by comparison with an erythrocyte-based *A. marginale* standard in an antigen capture ELISA (36). Colony counts in infected tick cell monolayers were made by pelleting a sample of infected cells on a glass slide, staining the samples with Giemsa stain, and counting the number of colony-containing cells by light microscopy.

(ii) **Simultaneous infection of tick cells with the Virginia and Oklahoma *A. marginale* isolates.** In the first trial, a confluent monolayer of IDE8 cells was simultaneously inoculated with 1.4 × 10¹⁰ organisms each of the Virginia and Oklahoma isolates of *A. marginale*. Separate IDE8 monolayers received 1.4 × 10¹⁰ organisms of either the Virginia or Oklahoma isolate of *A. marginale*. Cultures received fresh L-15B medium and were maintained at 34°C as described above. At day 10 postinfection (p.i.), samples were collected from each flask for genotype identification and from flasklets receiving one isolate only for determination of bacterial concentration by antigen capture ELISA (36). Infected material from the dual-infection flask was passed onto an uninfected IDE8 monolayer and maintained as described above. A sample was collected from this flask at day 7 p.i. for genotype identification. In a second trial, IDE8 monolayers were simultaneously inoculated with the cultured Virginia and Oklahoma isolates of *A. marginale* at different ratios. Monolayers were inoculated, per flask, with 1.4
A. marginale organisms of either the Virginia or the Oklahoma isolate alone. Additional monolayers received the same infection dose but of both the Virginia and Oklahoma isolates at ratios of 1:3, 2:2, and 3:1. Samples were collected from each flask at day 13 p.i. for isolate identification. Each experimental treatment was done in duplicate.

(iii) Superinfection of tick cells with the Virginia and Oklahoma isolates of A. marginale, followed by heterologous infection. Monolayers of IDE8 tick cells were inoculated with either Virginia (flask 1) or Oklahoma (flask 2) isolate at a greatly increased density of $1.4 \times 10^{10}$ organisms per flask. After a 60-min incubation period, the inoculum was removed and the monolayers were washed with fresh L-15B medium. At 3 days p.i., flask 1 was inoculated with the Oklahoma isolate at a dose of $1.4 \times 10^{10}$ organisms. At 5 days p.i., flask 2 was inoculated with the Virginia isolate at a dose of $1.4 \times 10^{10}$ organisms per flask.

**FIG. 2.** Trial 1: exclusion of A. marginale isolates in cattle. Only the Virginia A. marginale isolate was present in bovine PA444 inoculated with equal amounts of erythrocytes infected with the Oklahoma or Virginia A. marginale isolate. DNA was extracted, and the msp1a genotype was analyzed after PvuII digestion of PCR products at the onset of infection (PPE, 1.6%; February 15) (A), the infection peak (PPE, 5.8%; February 17) (B), and the end of the acute phase of infection (PPE, 0.6%; February 28) (C). Samples were analyzed on a 1% EtBr-stained agarose gel.

**FIG. 3.** Trial 2: exclusion of A. marginale isolates in cattle. (A) Bovines PA465, PA466, and PA467 were inoculated with erythrocytes infected with the Virginia (VaAM) or Oklahoma (OkAM) isolate or both (VaAM+OkAM). (B) DNA was extracted, and the msp1a genotype was analyzed after PvuII digestion of PCR products at the onset of infection for PA467 (PPE, 0.6%) and at the infection peak for PA465, PA466, and PA467 (PPEs of 2.1, 5.1, and 16.5%, respectively). Samples were analyzed on a 1% EtBr-stained agarose gel. Lane MW, 1-kb Plus DNA ladder (Gibco BRL); C-, control sample with no DNA added before PCR. The Virginia A. marginale isolate was present in PA465, whereas PA466 and PA467 contained the Oklahoma isolate only.
inoculated with a similar dose of the Virginia isolate. Samples were collected from flasks 1 and 2 prior to infection with the second isolate of *A. marginale*, and colony counts were done by light microscopy. Flasks were maintained as described above until monolayers began detaching, and samples were collected for isolate identification. Each experimental treatment was done in duplicate.

(iv) Differential live staining of the Virginia and Oklahoma *A. marginale* isolates in tick cells. IDE8 monolayers were infected with $1.4 \times 10^{11}$ organisms of either the Virginia or the Oklahoma isolate and exposed to the inoculum for 60 min. Inoculum was removed, and the monolayers were washed in L-15B medium. After 24 h, fresh medium containing a 50-$\mu$g/ml concentration of CellTracker Green BODIPY dye (catalog number C-2102; Molecular Probes, Eugene, Oreg.) was added to the culture of the Virginia *A. marginale* isolate. Medium containing a 1-$\mu$g/ml concentration of CellTracker Orange CMTMR dye (catalog number C-2927; Molecular Probes) was added to the Oklahoma isolate culture. After 24 h incubation samples were collected from both cultures to verify the staining by each dye and the level of infection. Cell suspensions were dried on glass slides, washed three times in phosphate-buffered saline, fixed for 15 min in 2% glutaraldehyde (in 0.1 M sodium cacodylate buffer), and rinsed in phosphate-buffered saline. Samples were mounted with Mowiol-glycerol–1,4-diazabicyclo-(2,2,2)-octane (DAPCO; Sigma, St. Louis, Mo.) as the mounting medium (17) and with glass coverslips and examined under a Leica DM LB fluorescence microscope attached to a Spotcam (Diagnostic Inc., Sterling Heights, Mich.) ISA-bus 12-bit/channel RGB Peltier-cooled charge-coupled device camera. Cells labeled with CellTracker Green BODIPY and CellTracker Orange CMTMR were identified by using an A filter (450 to 490 nm band pass, long pass at 515 nm; Leica I3) or B filter (515 to 650 nm band pass, dichromatic at 580 nm, long pass at 590 nm; Leica N21), respectively. The stained cultures of the Virginia and Oklahoma isolates of *A. marginale* were then harvested and combined, and tick cells were mechanically disrupted by vigorously pipetting with a 10-$\mu$l syringe and a 19-gauge needle to release bacteria. Culture material containing the labeled Virginia and Oklahoma isolates was held for 15 min to allow intact host cells to settle, and 5 ml of supernatant was added to an uninfected monolayer of IDE8 cells. Samples were collected at 48 h p.i. and processed for examination by fluorescence microscopy as previously described.

(v) Infection of tick cells with *A. ovis* followed by infection with the Virginia and Oklahoma isolates of *A. marginale*. Monolayers of IDE8 cells were infected with terminal cultures of *A. ovis* from one T-25 flask and exposed to the inoculum for 60 min. Inoculum was removed, and monolayers were washed in L-15B medium. At day 1 p.i., samples were collected for *A. ovis* colony counts by light microscopy and flasks were then inoculated with 2.7 $\times$ $10^{10}$ organisms of either the Virginia or the Oklahoma isolate of *A. marginale*. At day 10 p.i. with *A. ovis*, cultures were harvested for isolate identification. Each experimental treatment was done in duplicate.

**Analysis of Anaplasma isolate identity.** (i) PCR. *A. marginale* cells from bovine erythrocytes or infected tick cell cultures were characterized by *Pvu*II restriction analysis of the *mfp1* gene amplified by PCR. *A. marginale* DNA was extracted from 0.5-ml stored blood samples containing infected bovine erythrocytes collected during different stages of acute infection or from infected IDE8 cells by using 500 $\mu$g of Trig (Sigma) as described in the manufacturer's recommendations. The *mfp1* gene, coding for MSP1a and containing 5' and 3' regulatory sequences, was amplified from 100 ng of DNA by PCR (13) with 10 pmol of each primer, *MSP1aP* (5'-GCATTACAACGCACACGCTGAG-3') and *MSP1a3* (5'-GCCTTACTGCAAGCCTGCGCCTG-3'), in a 50-$\mu$l-volume mixture (1.5 mM MgSO$_4$, 0.2 mM deoxyribonucleotide triphosphate, 1X avian myeloblastosis virus reverse transcriptase-thermophilus flavus reaction buffer, 5 U of *Thermus flavus* DNA polymerase) using the Access reverse transcription-PCR system (Promega, Madison, Wis.). Reactions were performed in an automated DNA thermocycler (Masterecyler personal; Eppendorf, Westbury, N.Y.) for 35 cycles. After an initial denaturation step of 30 s at 94°C, each cycle consisted of a denaturing step of 30 s at 94°C and an annealing-extension step of 2.5 min at 68°C. The program ended with the reaction mixtures being stored at 4°C. PCR products were electrophoresed on 1% agarose gels to check the sizes of the amplified fragments. Control samples with no DNA added were included in the experiments. Ten microliters of the PCR product were mixed with 0.5 $\mu$L of PvuII (Gibco BRL, Gaithersburg, Md.) and incubated for 30 min at 37°C. The reaction mixture was analyzed in a 1% ethidium bromide (EtBr)-stained agarose gel. The gene coding for MSP1a in *A. ovis* has not been cloned, but the *mfp1* gene has a sequence distinguishable from that of *A. marginale* isolates (J. de la Fuente, unpublished results). Therefore, in experiments with *A. ovis*, the *mfp1* gene was amplified by PCR and sequenced as previously reported (11).

(ii) Western blot. Expression of MSP1a in infected IDE8 cells was analyzed by Western blotting. Total proteins were extracted from infected cells with 500 $\mu$l of Trig (Sigma) in accordance with the manufacturer's recommendations after DNA precipitation. Proteins (10 $\mu$g/well) were separated by sodium dodecyl sulfate–7.5% polyacrylamide gel electrophoresis (23) and transferred to a nitrocellulose membrane for 1 h at 60-mA constant current in a Hoefer TE 70 (Amersham Pharmacia) semidy transfer unit. The membrane was blocked with 5% skim milk for 1 h at room temperature. The anti-MSP1a mouse monoclonal antibody Ana15D2 (25) was used. Antibodies were diluted to a concentration of 10 $\mu$g/ml in 3% bovine serum albumin in Tris-buffered saline (TBS). The membrane was incubated with the antibodies for 1 h at room temperature and washed three times with TBS. The membrane was then incu-
We demonstrated that both the Virginia and Oklahoma isolate gave three bands, of 997, 849, and 313 bp. The Virginia isolate gave two bands, of 1,071 and 997 bp, while the Oklahoma isolate gave three bands, of 997, 849, and 313 bp. These experiments confirmed that the two isolates could be detected by our procedure if they were present in the sample.

**Exclusion of *A. marginale* isolates in cattle.** When cattle were infected with equal amounts of erythrocytes infected with either the Virginia or Oklahoma isolate, only one of the isolates established infection (Fig. 2 and 3B). In the first trial, the *A. marginale* isolate from Oklahoma was the only isolate detected in bovine PA444 at the early stages of infection (1.6% infected erythrocytes) and thereafter until the end of the acute phase of infection (Fig. 2A and B). The *A. marginale* isolate from Virginia was also the only isolate detected in salivary glands of ticks that acquired infection after feeding on PA444 during acute infection (data not shown). In the second trial, the *A. marginale* isolates from the Oklahoma isolate was the only isolate detected in bovine PA467 (Fig. 3B) that was inoculated with equal amounts of blood stabilate infected with the Virginia isolate. The Oklahoma isolate that established infection was detected at very early stages of infection (0.6% infected erythrocytes) (Fig. 3). In this second trial, blood stabilates of each isolate caused infection when inoculated into separate susceptible cattle (bovine PA465 for the Virginia isolate and bovine PA466 for the Oklahoma isolate) (Fig. 3A).

**Exclusion of *A. marginale* isolates in tick cells.** Tick cell cultures that were infected simultaneously with 1.4 × 10^10 A. *marginale* organisms of each of the Virginia and Oklahoma isolates and analyzed 10 days p.i. contained only the Virginia isolate, and this was the only isolate detected 7 days after subpassage of the cultures (Fig. 4B). When cultures were infected with each individual isolate, the culture containing the Virginia isolate contained twice as many organisms as the Oklahoma isolate-infected culture, as detected by antigen detection ELISA (Fig. 4A).

Tick cell cultures were inoculated with different infection...
ratios of the Virginia and Oklahoma *A. marginale* isolates. When infected cultures were analyzed for *msp1α* DNA by PCR, the Virginia isolate established the predominant infection and excluded the Oklahoma isolate at infection ratios of 3:1 and 2:2 (Virginia/Oklahoma *A. marginale* isolates), whereas at a 1:3 infection ratio of the Virginia/Oklahoma isolates, the Oklahoma isolate established the predominant infection and excluded the Virginia isolate (Fig. 5A). When the same cultures were analyzed for MSP1α protein by Western blotting, the Virginia and Oklahoma isolates established the predominant infection at infection ratios of 3:1 and 1:3, respectively (Fig. 5B). However, at a 2:2 infection ratio, bands corresponding in size to the Virginia and Oklahoma isolates were observed (Fig. 5B). Although the results were essentially similar at the DNA and protein levels, the relative amounts of the Virginia and Oklahoma isolates in the sample infected with equal amounts of both isolates could have differed as the result of differences in the DNA and protein half-lives.

In a third series of experiments, when cell cultures that were first allowed to develop established infections with either the Virginia or Oklahoma isolate (Fig. 6A and B) were subsequently infected with the heterologous isolate and tested at 10 to 12 days following the second infection, each culture was infected with only the isolate used for establishing the initial infection (Fig. 6C).

The Virginia and Oklahoma isolates, labeled with the green and orange fluorescent CellTracker dyes (Molecular Probes C-2102 and C-2927, respectively), were used to coinfect cultured tick cells in order to document initial infection events (Fig. 7A through C). At 2 days p.i., cells infected with individual isolates were seen (Fig. 7D through F), although at an approximately 2.5-fold higher level for cells infected with the Virginia isolate of *A. marginale*. In monolayers infected with both labeled isolates, very few (<1%) cells with possible dual infections were seen (Fig. 7G through I).

**Exclusion of *A. marginale* by *A. ovis* infection in tick cells.** To assess the capacity of a second *Anaplasma* species, *A. ovis*, to exclude *A. marginale* infection, tick cell cultures with established infections of *A. ovis* (Fig. 8A and B) were infected with the Virginia or Oklahoma isolate of *A. marginale*. After 12 days of infection with the Virginia or Oklahoma isolate, only *A. ovis* was detected in cell monolayers, as demonstrated by the sequence of the amplified *msp1α* gene (Fig. 8C).

**DISCUSSION**

These studies were undertaken to test the hypothesis that a mechanism of infection exclusion occurs in host cells infected with *A. marginale* that results in the establishment of a single genotype and that excludes infection with other genotypes. In two trials, we demonstrated that cattle experimentally inocu-
FIG. 7. Infection of IDE8 tick cells with differentially labeled Virginia (VaAM) and Oklahoma (OkAM) A. marginale isolates in parasites. The Virginia and Oklahoma isolates were labeled with CellTracker Green BODIPY and Orange CMTMR dyes (Molecular Probes), respectively. (A through C) Culture material containing the labeled Virginia and Oklahoma isolates was added to an uninfected monolayer of IDE8 cells. (D through I) Samples were collected at 48 h p.i., mounted with Mowiol-glycerol-DAPCO and glass coverslips, and examined under a Leica DM LB fluorescence microscope attached to a Spotcam (Diagnostic Inc.) ISA-bus 12-bit/channel RGB Peltier-cooled charge-coupled device camera. Cells labeled with CellTracker BODIPY or CellTracker CMTMR were identified by using an A filter (515 to 560 nm excitation, dichromatic at 580 nm, long pass at 590 nm; Leica I3) (A, D, and G) or a B filter (450 to 490 nm excitation, dichromatic at 510 nm, band pass at 515 nm; Leica N21) (B, E, and H), respectively, or a combination of both (C, F, and I). Examples of cells showing infection with the Oklahoma and Virginia isolates are shown with orange and green arrows, respectively. Magnifications, ×1100 (A through C and G through I) and ×100 (A through F).
equal amounts of two *A. marginale* isolates in an artificial tick feeding system as described by Waladde et al. (39). The findings reported herein were similar to the results of Palmer et al. (32) from studies on a persistently infected reservoir herd within a region in Oregon where *A. marginale* is endemic. In herds in an area of *A. marginale* endemicity where more than one *A. marginale* genotype was detected, individual cattle were found to be infected with a single genotype. This mechanism of infection exclusion, which allows for infection of individual cattle with one genotype, would reduce competition and favor the survival of individual *A. marginale* genotypes.

Infection exclusion has been reported previously for bacteria of the genus *Rickettsia* only (8). Burgdorfer et al. documented exclusion of *Rickettsia rickettsii* from *Dermacentor andersoni* ovaries by the nonpathogenic *Rickettsia peacockii* (8). In a different study, Ridderhof and Barnes (34) demonstrated that

**FIG. 8.** Exclusion of *A. marginale* by *A. ovis* infection in tick cells. Only the sequence corresponding to *A. ovis msp4* was present in IDE8 cells infected with *A. ovis* and then reinfected with the Virginia or Oklahoma isolate. (A and B) Light micrographs of 1-μm-thick plastic sections of cultured tick cells at 1 day p.i. with *A. ovis* colonies (arrows) are shown. The cell culture in panel A was subsequently infected with the Oklahoma isolate, whereas the culture in panel B was subsequently infected with the Virginia isolate. Bars, 10 μm. (C) At day 9 p.i. with *A. marginale* isolates, cultures were harvested for isolate identification. The *msp4* gene was amplified by PCR and sequenced as previously reported (11). The *A. ovis msp4* coding sequence (AF393742) was translated and compared to those encoding the MSP4s from *A. marginale* isolates (identical for the Virginia and Oklahoma isolates) (11). Asterisks denote different amino acids.
infection exclusion did not occur with the intracellular bacterium *Chlamydia trachomatis*. In their experiments, more than 88% of HeLa cells were coinfected with two different *C. trachomatis* serovars (34). Phylogenetic studies have demonstrated that *Chlamydiae* are not closely related to *Anaplasmataceae* and may have evolved different mechanisms of survival that are host or host cell associated, whereas *Rickettsiae* are closely related to *Anaplasmataceae* (10, 30).

The epidemiology of anaplasmosis has been poorly understood. The mechanism of infection exclusion in *A. marginale* resulting in one genotype per animal would contribute to our understanding of anaplasmosis epidemiology. This mechanism would contribute to the geographic localization of *A. marginale* isolates with distinctive genotypes and antigenic characteristics (11). If cattle infected with another genotype were introduced into a herd in which *A. marginale* infection was endemic, these genotypes would be maintained and most likely also become endemic if they were transmitted to susceptible cattle. Both persistently infected cattle and ticks could serve as reservoirs of the introduced genotype.

Infection exclusion may contribute to the success of the live *Anaplasma centrale* vaccine used in Australia, Israel, Latin America, and Africa in which development of persistent infections would prevent cattle from being infected with *A. marginale*, as well as provide immunoprotection due to persistent infection. The protection afforded to cattle that are persistently infected with *A. marginale*, therefore, may be due to infection exclusion as well as to immunologic mechanisms.

The mechanism of infection exclusion which results in one genotype infection per animal may constrain the mobility and establishment of multiple *A. marginale* geographic 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.

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