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Factors influencing in vitro infectivity and growth of *Rickettsia peacockii* (Rickettsiales: Rickettsiaceae), an endosymbiont of the Rocky Mountain wood tick, *Dermacentor andersoni* (Acari, Ixodidae)

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Abstract

Rickettsia peacockii, a spotted fever group rickettsia, is a transovarially transmitted endosymbiont of Rocky Mountain wood ticks, *Dermacentor andersoni*. This rickettsia, formerly known as the East Side Agent and restricted to female ticks, was detected in a chronically infected embryonic cell line, DAE100, from *D. andersoni*. We examined infectivity, ability to induce cytopathic effect (CPE) and host cell specificity of *R. peacockii* using cultured arthropod and mammalian cells. Aposymbiotic DAE100 cells were obtained using oxytetracycline or incubation at 37 °C. Uninfected DAE100 sublines grew faster than the parent line, indicating *R. peacockii* regulation of host cell growth. Nevertheless, DAE100 cellular defenses exerted partial control over *R. peacockii* growth. Rickettsiae existed free in the cytosol of DAE100 cells or within autophagolysosomes. Exocytosed rickettsiae accumulated in the medium and were occasionally contained within host membranes. *R. peacockii* multiplied in other cell lines from the hard ticks *D. andersoni*, *Dermacentor albipictus*, *Ixodes scapularis*, and *Ixodes ricinus*; the soft tick *Carios capensis*; and the lepidopteran *Trichoplusia ni*. Lines from the tick *Amblyomma americanum*, the mosquito *Aedes albopictus*, and two mammalian cell lines were non-permissive to *R. peacockii*. High cell densities facilitated rickettsial spread within permissive cell cultures, and an inoculum of one infected to nine uninfected cells resulted in the greatest yield of infected tick cells. Cell-free *R. peacockii* also were infectious for tick cells and centrifugation onto cell layers enhanced infectivity approximately 100-fold. The ability of *R. peacockii* to cause mild CPE suggests that its pathogenicity is not completely muted. An analysis of *R. peacockii*–cell interactions in comparison to pathogenic rickettsiae will provide insights into host cell colonization mechanisms.

Keywords

Rickettsia peacockii; East Side Agent; *Dermacentor andersoni*; Cultivation; Rocky Mountain wood tick; Endosymbiont; Rickettsia; Host cell range

1. Introduction

The spotted fever group rickettsia (SFGR) *Rickettsia peacockii*, formerly known as the East Side Agent (Burgdorfer et al., 1981), is an endosymbiont of the Rocky Mountain wood tick, *Dermacentor andersoni* (Burgdorfer et al., 1981; Niebylski et al., 1997). *R. peacockii* has been detected in female wood ticks collected in the Bitterroot valley (Montana) and in Colorado

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(Baldrige et al., 2004; Niebylski et al., 1997; Simser et al., 2001), but not in males collected in the field or reared in the laboratory (Niebylski et al., 1997). *R. peacockii* is mainly confined to oocytes and interstitial cells of the ovarian tissues (Munderloh et al., 2005; Niebylski et al., 1997) and does not infect the hypodermis, Malpighian tubules, midgut, salivary glands or hemocytes (Niebylski et al., 1997). Though oocytes are heavily infected (Munderloh et al., 2005; Niebylski et al., 1997), *R. peacockii* does not affect fecundity or the number of viable larvae per female (Niebylski et al., 1999). Based on sequences of the *ompA* gene, *R. peacockii* is most closely related to *Rickettsia rickettsii*, the causative agent of Rocky Mountain spotted fever (Baldrige et al., 2004). *R. peacockii* does not express OmpA because of three premature stop codons in its *ompA* gene (Baldrige et al., 2004; Niebylski et al., 1997). Additionally, high sequence similarity exists between the 16S rRNA, citrate synthase, and 17 kDa antigen genes of these two rickettsiae. Unlike pathogenic SFGR that shuttle between mammalian and tick hosts (Azad and Beard, 1998), *R. peacockii* has been found only in *D. andersoni* and is maintained in nature by transstadial and transovarial transmission within tick populations (Burgdorfer et al., 1981). The lack of a functional OmpA, a putative adhesin (Li and Walker, 1998), might limit *R. peacockii* distribution to the ovarian tissue of *D. andersoni*.

Unlike virulent strains of *R. rickettsii* that are highly pathogenic for the tick (Burgdorfer and Brinton, 1975; Niebylski et al., 1999), *R. peacockii* does not cause a reduction in tick fecundity and may be beneficial for tick hosts by antagonizing super-infection of ovarian tissues by *R. rickettsii*. Nevertheless, the critical experiments demonstrating whether or not *R. peacockii* is pathogenic for ticks remain to be done. In this report, we use endosymbiosis in the broad sense, meaning an endosymbiont and its host living together and without any implication of mutualism.

We have previously isolated a *D. andersoni* embryonic cell line DAE100 that is persistently infected with *R. peacockii* (Simser et al., 2001). The availability of this culture system made it possible for us to explore the interactions between *R. peacockii* and host tick cells and provided an opportunity to evaluate the infectivity and pathogenicity of *R. peacockii* for various host cells in vitro. The slow growth of *R. peacockii* and infected DAE100 cells led us to develop culture strategies that differ from those used to maintain all other isolated, and more virulent, strains of SFGR. In this study, we evaluated whether *R. peacockii* infection was obligatory for DAE100 cell survival by eliminating rickettsiae by heat or antibiotic treatment. Additionally, we compared growth and ultrastructure of infected with uninfected cells and examined *R. peacockii* infectivity for selected arthropod and mammalian cells.

2. Materials and methods

2.1. Maintenance of *R. peacockii*

Rickettsia peacockii, strain DaE100R, originally isolated from the *D. andersoni* embryonic cell line DAE100 (Simser et al., 2001), was used for these analyses. The status of strain DaE100R was established on the basis of its molecular and serological identity with *R. peacockii* strain Skalkaho^T (Baldrige et al., 2004; Niebylski et al., 1997; Simser et al., 2001). DAE100 cells persistently infected with *R. peacockii* were maintained in antibiotic-free L-15B medium supplemented with fetal bovine serum (5%), tryptose phosphate broth (5%), and bovine lipoprotein concentrate (0.1%), pH 6.8–7.0 (Munderloh and Kurtti, 1989). Subcultures were made every 3–4 weeks with a seeding density of approximately 5×10^5 cells/ml, incubated at 34 °C, and fed weekly by replacing spent with fresh medium.

2.2. Development of aposymbiotic DAE-100 cell lines

Rickettsia peacockii-free DAE100 sublines were developed by two methods, incubation of infected cells at 37 °C for a month (DAE100H) or addition of oxytetracycline (0.25 or 0.5 µg/ml) to the medium for 2 months (DAE100A). The DAE100 sublines (H and A) were confirmed to be free of rickettsiae by transmission electron microscopy, Giemsa staining, PCR, and IFA analyses (Simser et al., 2001). Oxytetracycline or heat treatment was discontinued after 60 days when the cells were completely free of rickettsiae.

2.3. Transmission electron microscopy of cells

Rickettsia peacockii infected and uninfected cultures were prepared for transmission electron microscopy to evaluate effects of *R. peacockii* on host cell ultrastructure (Munderloh et al., 1998). Extracellular rickettsiae released by infected cells were recovered by differential centrifugation and likewise prepared for TEM.

2.4. Cell–cell transfer of *R. peacockii*

We measured transfer of *R. peacockii* from infected to uninfected cells by inoculating uninfected replicate host cell cultures with infected cells at proportions of infected cells ranging from 7 to 50% and determining the proportion of infected cells at subsequent time points. The initial seeding density (infected plus uninfected cells) was held constant. DAE100 cultures had an initial seeding density of 8×10^5 cells/ml and the smaller ISE6 cells were seeded at 1×10^6 cells/ml. At the end of 2 weeks, the cultures were suspended and the number of cells per ml determined using a hemocytometer. Samples were taken 1 and 2 weeks post-seeding and processed for Giemsa staining to determine the proportion of cells that were infected.

2.5. Preparation of cell-free rickettsiae for infecting cell cultures

To inoculate *R. peacockii* into tick and mammalian cell lines as host-cell-free suspensions, rickettsiae were first released from infected cells by passage through a 27-gauge needle. Large debris was removed by centrifugation (275g for 10 min) followed by filtration of the supernatant first through a 5.0-µm pore size syringe filter (Millex-SV; Milipore, Bedford, MA) and finally through a 2.7 µm filter (GD/X glass microfiber, Whatman, Clifton, NJ). Rickettsiae were counted using a Petroff–Hausser counting chamber.

Heavily infected DAE100 cultures yielded 100–200 *R. peacockii* per infected cell or 1.9×10^9 *R. peacockii* per 5 ml culture. The mammalian cell lines were centrifuged (10 min at 2000g, and 4 °C) with rickettsiae to promote rickettsial adherence to the cells. Cell pellets were resuspended in fresh medium and inoculated into 25-cm² culture flasks.

2.6. Evaluation of infectivity of cell-free *R. peacockii*

To evaluate infectivity of rickettsiae freshly isolated from host cells, heavily infected ISE6 cultures were suspended by pipetting and centrifuged (2500 rcf, 4 °C, 10 min) to separate extracellular rickettsiae in the medium (supernatant fluid) from intracellular rickettsiae (pellet). The pellet of infected cells was resuspended in ice-cold SPG buffer (220 mM sucrose, 3.8 mM KH₂PO₄, 8 mM K₂HPO₄, 5 mM potassium glutamate, and 10 mM MgCl₂) and lysed by vortexing with 3 mm glass beads (Rachek et al., 1998). The lysate was centrifuged (2500g, 4 °C, 10 min) and the supernatant was subsequently filtered through 5 µm and 2.7 µm syringe-driven filters to remove any remaining host cells. The filtrate was centrifuged (18,000g, 4 °C, 10 min) and sedimented rickettsiae were resuspended in complete culture medium. Serial 10-fold dilutions of the rickettsial suspension were inoculated into wells of a 96-well tissue culture plate seeded with ISE6 cells ($2\text{--}3 \times 10^5$ cells per well). The number of rickettsiae inoculated into each well was determined by counting rickettsiae stained with the *BacLight* viability stain kit (Molecular Probes, Eugene, Oregon) in a Petroff–Hausser bacterial counting chamber and

examined with a Nikon E400 microscope equipped for epifluorescence. To enhance rickettsia–host cell contact in some experiments, host-cell-free rickettsiae were added to ISE6 cell layers established in tissue culture plates and centrifuged at 1800g for 20 min at 4 °C. Plates were incubated at 34 °C in humidified candle jars for 2–4 weeks, with weekly changes of medium. Replicate wells (3) for each rickettsial dilution were resuspended and prepared for Giemsa staining using a cytocentrifuge. Approximately 300 cells per replicate were examined to determine the percentage of cells infected with rickettsiae.

2.7. Challenge of arthropod and mammalian cell lines

We examined the infectivity of *R. peacockii* for selected arthropod and mammalian cell lines (Table 1). Tick cell lines tested had been previously isolated in our laboratory from embryonated eggs of *D. andersoni* (DAE100H, DAE3, and DAE15), *Dermacentor albipictus* DALBE3 (Policastro et al., 1997), *Dermacentor variabilis* (DVE1) (Munderloh and Kurtti, unpublished), *Ixodes scapularis* (ISE6, IDE2, and IDE8) (Munderloh et al., 1994, 1996), *Ixodes ricinus* (IRE11) (Simser et al., 2002), and *Carios capensis* (Mattila et al., in preparation). A lepidopteran, *Trichoplusia ni* (High Five) was purchased from Invitrogen (Carlsbad, CA) and the mosquito, *Aedes albopictus*, cell line C7-10 was obtained from Dr. Ann Fallon (University of Minnesota, St. Paul, MN). All arthropod cell lines were grown in complete L-15B (see above) and incubated at 31 or 34 °C. The mammalian L-929 (ATCC CCL-1) and Vero (ATCC CCL-81) cell lines were grown in L-15B300 (Munderloh et al., 1999), prepared from basal L-15B medium diluted 25% with sterile tissue culture grade water and further supplemented with NaHCO₃ (0.25%), FBS (5%), bovine lipoprotein concentrate (0.1%), TPB (5%) and buffered with 3-(*N*-morpholino)-propanesulfonic acid (MOPS, 10mM) or 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes, 10 mM). The mammalian cell lines, maintained at 37 °C, were incubated at 34 °C after challenge with *R. peacockii* prepared by forcing infected cell suspensions through a 27-gauge needle three times, centrifugation (275g for 10 min) to remove debris and filtration of the supernatant through a 5.0-μm pore size syringe filter. For the mammalian lines, host cells were centrifuged with *R. peacockii* for 10 min at 2000g, to promote rickettsial adherence prior to transfer to 25-cm² culture flasks. Inoculated cultures were observed for over 2 months and evaluations of rickettsial infection were determined by light microscopy of Giemsa stained cell spreads.

3. Results

3.1. Growth characteristics of *R. peacockii* infected and uninfected DAE100 lines

The *D. andersoni* cells, line DAE100, replicated slowly which made it necessary to measure cell growth in terms of the period of time between serial transfers (Fig. 1). The persistently infected DAE100 line was heavily infected with *R. peacockii* (70–100% of cells infected) but harbored vastly different numbers of rickettsiae per cell. The average time for a subculture of infected cells (using subculture dilutions of 1:3) to reach confluency was 45 days, equivalent to an approximate population doubling time of 28 days. To evaluate the effect of rickettsiae on growth of DAE100 cells, we treated the DAE100 line with oxytetracycline or incubation at 37 °C during the 22nd passage. Either treatment effectively eliminated *R. peacockii* from the cells within 1–2 months. The uninfected sublines grew more rapidly than the infected line and were maintained using a subculture ratio of 1:5. The time interval between transfers in the uninfected sublines was 14–28 days (estimated population doubling time of 8–12 days). The mitotic indices for infected DAE100 cells (range: 1–8 mitotic figures per 1000 cells) were lower than those for uninfected cells (range: 2–17 mitotic figures per 1000 cells).

3.2. Ultrastructural comparison of infected and uninfected DAE 100 cells

Rickettsia peacockii infected DAE100 cells generally contained a single excentric nucleus with heterochromatin clustered near the inner nuclear membrane (Fig. 2A). The cells were heavily

vacuolated with thin stretches of cytoplasm. Membrane-bound vacuoles containing parallel or concentric thin electron-dense membrane lamellae (multilamellar bodies) occupied the bulk of the cytoplasm. Coccobacillary rickettsiae were clustered free within the cytoplasm or enclosed within vacuoles. *R. peacockii* were sometimes surrounded by endoplasmic reticulum that appeared to fuse with lysosomes to form putative auto-phagosomes (Fig. 2B). Rickettsiae within vacuoles were electron-dense with condensed inner bodies and conspicuous cell walls. The peripheral layer of host cell cytoplasm was periodically interrupted by ruptured or exocytosed vacuoles (Fig. 2A, arrows).

Cultures of infected DAE100 cells accumulated copious extracellular debris (Fig. 3A). We did not observe cellular endocytosis/phagocytosis or adherence of rickettsiae. Extracellular rickettsiae were somewhat swollen and pleomorphic and most were free of host cell membranes (Fig. 3B). Some had electron-dense cytoplasm while others had the normal uniformly granular cytoplasm. Degenerating electron-dense rickettsiae were also found within extracellular, membrane-lined inclusions along with membrane lamellae. The images suggested that both cytosolic and membrane enclosed rickettsiae were exocytosed into the medium.

Uninfected DAE100 cells were smaller (Fig. 4A) indicating that the rickettsiae caused cellular hypertrophy. The ultrastructure resembled that of chronically infected cells (Fig. 4B). Nuclei were surrounded by heavily vacuolated cytoplasm containing mitochondria, endoplasmic reticula, and Golgi complexes similar to those found in infected cells. Vacuoles and inclusions contained multi-layered pleated and circular membranes. PCR using rickettsia-specific primers and IFA with polyclonal anti-rickettsia antisera confirmed the absence of rickettsiae (data not shown) in both aposymbiotic DAE100 sublines, which have remained free of *R. peacockii* for over 40 transfers. We examined the infectivity of *R. peacockii* for DAE100A. A suspension of 10^9 rickettsiae freshly released from infected cells was inoculated into a flask of DAE100A (approximate multiplicity of infection of 100). Five days after inoculation, 25.4% of the cells were infected with low numbers of rickettsiae. At 12 days, 54% of the cells were infected and by 18 days 99.1% of the cells were infected, indicating an approximate increase in apparent infection of 5% per day.

3.3. Host cell specificity of *R. peacockii*

Rickettsia peacockii, released from DAE100 cells, were infectious for most of the tick cell lines tested (Table 1). The *Boophilus microplus* BME26 and *I. scapularis* ISE6 lines along with the lepidopteran High Five cells were most permissive for *R. peacockii*. Cultures of ISE6 or BME26 when inoculated with cell-free rickettsiae were completely infected within 1–2 weeks and could not be maintained as chronically infected lines; instead *R. peacockii* were maintained in these lines by transferring suspensions of infected cells and rickettsiae to uninfected cultures. Several other lines could be maintained as chronically infected sublines, including those from *D. andersoni* (DAE3, DAE15, and DAE100), *D. albipictus* (DALBE3), *I. scapularis* (IDE2, IDE12, and IDE8), and *I. ricinus* (IRE11). However, in some cases to promote spread of rickettsiae, it was necessary to maintain cultures at high cell densities without subculturing for up to 8 weeks. Once infection was established, sublines persistently infected with *R. peacockii* could be passaged every 3–4 weeks. A young cell line (CCE3) isolated from embryos of the soft tick *C. capensis* (Mattila et al., in preparation) could also be infected but was not used to propagate *R. peacockii* because of its cellular heterogeneity and differential permissiveness of the different cell types. On the other hand, we were unable to infect a cell line, AAE2, from the lone star tick, *A. americanum*. Cell-free *R. peacockii* were not infectious for the mosquito (C7-10 from *A. albopictus*) cell lines or mammalian (L929 and Vero) cells.

The *I. scapularis* cell lines proved useful for propagation of *R. peacockii* to high numbers (Fig. 5A) and, as in infected DAE100 cells, rickettsiae were found both free in the cytosol and undergoing degradation within putative autophagolysosomes (Fig. 5B). The *I. scapularis* cell

lines also accumulated massive amounts of extracellular debris. With continued passage, maintenance of persistently infected tick cell lines was erratic and the yield of rickettsiae unpredictable, whereas transfer of infected, lysed host cells to fresh uninfected cell layers was possible at regular intervals.

3.4. Cross-infection of host cells by mixing *R. peacockii* infected with uninfected cells

To facilitate the in vitro maintenance of *R. peacockii*, we examined the influence of inoculum size on yield of infected cells. We measured the rate of *R. peacockii* transfer from infected to uninfected DAE100A cells. Infected cells (*R. peacockii* passage 38) were mixed with uninfected DAE100A (passage 44) to give initial proportions of infected cells (10, 33, and 50%) in replicated cultures (25 cm² with 5 ml per culture; 8×10^5 cells per ml). The proportions of cells infected 14 days after inoculation are given in Table 2. The yield of cells (infected and uninfected) was highest in cultures seeded at the lowest initial proportion of infected cells (10%). However, the rate of cross-infection was similar for all three initial levels of infection at $3.7 \pm 0.8\%$ per day. Cultures initiated at an infection level of 10% contained the fewest extracellular rickettsiae. Similar results were obtained with ISE6 cells (Table 2). Again, the highest yield of cells, infected and uninfected, was obtained with cultures seeded at the lowest level of infection and the rate of cross-infection was similar for all four initial levels of infection and at $3.6 \pm 0.3\%$ per day. Thus, with both cell lines, a low initial level of infection yielded the most cells (infected and uninfected), consistent with the negative effects of *R. peacockii* on cell growth. Lower initial infection levels also reduced the accumulation of extracellular *R. peacockii* in the medium. Cultures initiated with infection rates of more than 20–30% accumulated massive numbers of extracellular rickettsiae, indicative of low ability of *R. peacockii* to adhere to and re-invade tick cells.

3.5. Infectivity of cell-free *R. peacockii*

We evaluated the infectivity of *R. peacockii* physically released from their host cells. Previously accumulated extracellular rickettsiae were eliminated by recovering host cells with low speed centrifugation that were then ruptured mechanically to release intracellular rickettsiae. Infectivity of host cell-free *R. peacockii* was estimated by inoculating serial 10-fold dilutions of rickettsiae into 96-well culture plates containing ISE6 cells at doses ranging from 0.1 to 1×10^5 rickettsiae per well. The effect of centrifuging the rickettsiae onto the cell layer was examined as well. Without centrifugation, infected cells were clearly observed at 15 days post-inoculation in wells seeded with 10^3 or more rickettsiae (infection rate of 13.8%) (Fig. 6, solid circles and solid line). Centrifugation enhanced infection rates approximately 100-fold (Fig. 6, solid line, open squares) with infected cells apparent in wells inoculated with as few as 10 rickettsiae (15.2% infection rate). Incubation of the cultures for an additional 2 weeks (total of 27 days) further improved the detection of cells infected without and with centrifugation (Fig. 6; dotted line, solid circles). Centrifugation enhanced infection and the detection of infected cells in wells inoculated with a calculated single *R. peacockii* (Fig. 6, dotted line, open squares; approximate infection rate of 6.2%).

4. Discussion

Rickettsia peacockii was originally detected in wood ticks collected on the East side of the Bitterroot valley in Montana where ticks infected with *R. rickettsii* and human cases of Rocky Mountain spotted fever were rare (Burgdorfer et al., 1981). Unlike *R. rickettsii*, it was uncultivable in mammalian tissue culture or embryonated chicken eggs, persisted as localized infections in ovarian tissues, but not hemocytes, of infected ticks, and did not induce fever in laboratory animals. *R. peacockii* did not affect the fecundity of female *D. andersoni* (Niebylski et al., 1999) but appeared to interfere with *R. rickettsii* infection of ovaries (Burgdorfer et al., 1981). These observations gave rise to the postulate that *R. peacockii* is a non-pathogenic

endosymbiotic rickettsia of the wood tick (Burgdorfer et al., 1981). We serendipitously isolated *R. peacockii* while developing embryonic cell lines from *D. andersoni* colonized in the laboratory (Simser et al., 2001). The availability of cell line DAE100 persistently infected with *R. peacockii* enabled us to test the hypothesis of non-pathogenicity by examining the infectivity and host cell specificity of *R. peacockii* and its effects on tick cell ultrastructure and growth.

Several features of the slow growing, persistently infected, DAE100 cell line have been described in a previous report where we noted that even heavily infected DAE100 cells were mitotically active (Simser et al., 2001). Slow growing obligate intracellular symbionts possess mechanisms to couple their replication with that of host cells to dampen their virulence and prevent elimination (Ruang-areerate et al., 2004). We examined the influence of *R. peacockii* on DAE100 cell growth by eliminating the rickettsia from the DAE100 cells by incubation at 37 °C or addition of oxytetracycline to the medium. Curing the DAE100 cells of their rickettsial endosymbiont resulted in more rapid DAE100 cell growth. This result could be taken as empirical evidence for rickettsial regulation of host cell replication, or alternatively slower growth due to the metabolic demands the rickettsiae placed on the host cells or virulence factors retained by *R. peacockii*.

Other than the absence or presence of rickettsiae, the ultrastructure of the nuclei and cytoplasm of uninfected and infected DAE100 cells was similar, consistent with a non-pathogenic phenotype. *R. peacockii* did not cause the CPE induced by *R. rickettsii* which invades and dilates the rough endoplasmic reticulum and nuclear membranes leading to eventual cytolysis (Silverman, 1984; Silverman and Wisseman, 1979). In contrast, tick cells have been found to be more resistant than mammalian cells to CPE induced by *R. rickettsii* and display reduced lysis (Policastro et al., 1997). The DAE100 cells bore little ultrastructural similarity to the ovarian interstitial cells that harbor *R. peacockii* in adult *D. andersoni* (Niebylski et al., 1997) nor did they resemble hemocytes of *D. andersoni* (Brinton and Burgdorfer, 1971). The persistently infected DAE100 line was isolated from a culture seeded with a mixture of embryonic tissues, and the tissue origin of DAE100 cells is unknown.

Our electron microscopic observations showed that the DAE100 cells mounted active cellular defense responses and regulated *R. peacockii* numbers by degradation of rickettsiae within vacuoles and by exocytosis. The appearance of extracellular rickettsiae suggested that both viable rickettsiae free in the cytoplasm and those degraded within phagosomes were exocytosed from infected cells. There was little or no adherence to tick cells and we did not observe evidence of phagocytosis of the extracellular rickettsiae. *R. peacockii* numbers in host cells were apparently regulated by autophagy. Autophagic mechanisms and pathways are evolutionarily conserved in eukaryotic cells and are intended for cell survival when extracellular nutrients are limited or when cells are invaded by intracellular bacteria (Levine, 2005). Autophagic (xenophagic) pathways are also a component of the innate immune system used to regulate and degrade intracellular microbes (Kirkegaard et al., 2004; Levine, 2005). Intracellular nitric oxide concentration in mouse endothelial cells has been correlated with the presence of *Rickettsia conorii* within autophagolysosomes (Walker et al., 1997). The presence of double membrane-bound and electron-dense rickettsiae apparently undergoing degradation within vacuoles and multivesicular bodies containing cytosolic material was suggestive of active xenophagic mechanisms in the DAE100 cells. Such processes are highly regulated and further studies using biochemical analyses and autophagosomal markers are needed to demonstrate their role in regulating rickettsial numbers in infected cells.

Pathogenic spotted fever rickettsiae infect tick cells that are capable of robust immune responses, most notably the hemocytes (Burgdorfer, 1970; Johns et al., 2001; Simser et al., 2004a,b). The DAE100 cells express innate immune factors found in ticks, including immune responsive factors induced by bacterial (*Escherichia coli*) challenge such as D-like serine

proteinase (Simser et al., 2004a) and c-type lysozyme (Simser et al., 2004b). The steady-state levels of lysozyme and factor D-like serine proteinase levels were high in DAE100 cells and similar to that from hemocytes of *D. variabilis*. The effect of these DAE100 immune responses on *R. peacockii* and the mechanisms used to evade the tick's defense responses are unknown. The effect of the antimicrobials cecropin A, ceratotoxin A, and lysozyme on extracellular *R. peacockii* has been examined (Baldrige et al., 2005). Cecropin A was lethal for *R. peacockii* and the killing was synergized by egg white lysozyme. However, *R. peacockii* was less sensitive to these antimicrobial peptides than most bacteria that grow extracellularly.

We demonstrated that *R. peacockii* could be propagated in several tick cell lines, but did not infect mosquito or mammalian cell lines that have been found permissive for growth of pathogenic *R. rickettsii* (Cory and Yunker, 1974). Tick cells infected with *R. peacockii* remained viable, capable of replication and able to support rickettsial growth. In most of the tick cell lines, *R. peacockii* behaved as expected of an endosymbiont, leading to a persistent infection without the cytolysis and killing commonly associated with the pathogenic rickettsiae (Policastro et al., 1997; Silverman and Wisseman, 1979). Either infected cells or cell-free rickettsiae could be used to transfer *R. peacockii* to uninfected cell cultures. *R. peacockii* cross-infected tick cells in vitro and transferred between cells in the absence of host cell replication. When initial infection rates were high (>30%) population growth slowed in both the DAE100 and ISE6 cultures and yield of cells declined. Infected cells still divided but at rates lower than those of uninfected cells. Long-term experience has shown that propagation of *R. peacockii* in a chronically infected tick cell line is erratic and slow and we therefore routinely passage *R. peacockii* by transferring infected cells to confluent cultures of uninfected cells with an inoculum that is generally 2% of the volume of the recipient tick cell culture (e.g., 0.1 ml per 5 ml of culture). Cell-free *R. peacockii* retained their infectivity and ability to invade uninfected tick cells and infectivity was enhanced by the centrifugation of rickettsiae onto tick cells. To infect new cell lines and avoid cross-mixing of tick cell lines, we centrifuge cell-free suspensions of rickettsiae onto cell layers.

Clues about the mechanisms of virulence and pathogenicity of SFG rickettsiae are emerging. *R. peacockii* is more closely related to highly pathogenic *R. rickettsii* than to other non-pathogenic rickettsiae such as *R. montanensis* or *R. rhipicephali* (Baldrige et al., 2004). *R. peacockii* does not express the major outer membrane protein, OmpA (Baldrige et al., 2004) known to play a role in adhesion of *R. rickettsii* to mammalian cells (Li and Walker, 1998). Our observations that *R. peacockii* does not adhere to tick cells and that invasion of tick cells could be enhanced 100-fold by centrifuging *R. peacockii* onto the cell layer may reflect the absence of OmpA on the rickettsial surface. SFG rickettsiae polymerize eukaryotic host cell actin for intra- and intercellular movement (Heinzen et al., 1993; Teyssie et al., 1992) but *R. peacockii* is unable to induce actin tails (Simser et al., 2001). Rickettsial movement disrupts the actin scaffolding and facilitates rickettsial penetration of the internal and external membranes of the host cell (Heinzen et al., 1993, 1999). A WASP-like (RICKA) protein is involved in the *R. rickettsii* and *R. conorii* polymerization of actin tails in host cells (Gouin et al., 2004; Jeng et al., 2004). The *rickA* gene also is defective in *R. peacockii* because of an insertion sequence or transposable element present in the coding sequence (Simser et al., 2005). The lack of *R. peacockii* adherence to tick cells and its inability to form actin tails are consistent with the known roles of OmpA and RickA in rickettsial infectivity and pathogenesis, supporting the hypothesis that defective adhesion (OmpA) and host actin polymerization capabilities (RickA) may be involved in the reduced pathogenicity of *R. peacockii* virulence for host cells. The defective *ompA* and *rickA* genes partially explain the slow growth of *R. peacockii* in tick cell cultures, but do not render *R. peacockii* non-infectious or unable to spread from cell to cell. The infectivity of host-cell-free *R. peacockii* also indicates that this non-pathogenic rickettsia has retained mechanism(s) of infectivity that allow it to evade or escape endosomes and lysosomes. Taken together, results of the present and previous reports

(Baldrige et al., 2004; Burgdorfer et al., 1981; Niebylski et al., 1997; Simser et al., 2001) indicate that *R. peacockii* is an endosymbiotic rickettsia with muted virulence mechanisms.

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References

- Azad AF, Beard CB. Rickettsial pathogens and their arthropod vectors. *Emerg. Infect. Dis* 1998;4:179–186. [PubMed: 9621188]
- Baldrige GD, Burkhardt NY, Simser JA, Kurtti TJ, Munderloh UG. Sequence and expression analysis of the *ompA* gene of *Rickettsia peacockii*, an endosymbiont of the Rocky Mountain wood tick, *Dermacentor andersoni*. *Appl. Environ. Microbiol* 2004;70:6628–6636. [PubMed: 15528527]
- Baldrige GD, Kurtti TJ, Munderloh UG. Susceptibility of *Rickettsia monacensis* and *Rickettsia peacockii* to cecropin A, ceratotoxin A and lysozyme. *Curr. Microbiol* 2005;51:233–238. [PubMed: 16132458]
- Brinton LP, Burgdorfer W. Fine structure of normal hemocytes in *Dermacentor andersoni* Stiles (Acari: Ixodidae). *J. Parasitol* 1971;57:1110–1127. [PubMed: 4332374]
- Burgdorfer W. Hemolymph test: a technique for detection of rickettsiae in ticks. *Am. J. Trop. Med. Hyg* 1970;19:1010–1014. [PubMed: 4992724]
- Burgdorfer W, Brinton LP. Mechanisms of transovarial infection of spotted fever rickettsiae in ticks. *N. Y. Acad. Sci* 1975;266:61–72.
- Burgdorfer, W.; Hayes, SF.; Mavros, AJ. Nonpathogenic rickettsiae in *Dermacentor andersoni*: a limiting factor for the distribution of *Rickettsia rickettsii*. In: Burgdorfer, W.; Anacker, RL., editors. *Rickettsiae and Rickettsial Diseases*. Academic Press; New York: 1981. p. 585–594.
- Cory J, Yunker CE. Rickettsial plaques in mosquito cell monolayers. *Acta Virol* 1974;18:512–513. [PubMed: 4156160]
- Fallon, AM.; Stollar, V. The biochemistry and genetics of mosquito cells in culture. In: Maramorosch, K., editor. *Adv Cell Culture*. Academic Press; New York: 1987. p. 97–137.
- Gouin E, Egile C, Dehoux P, Villiers V, Adams J, Gertler F, Li R, Cossart P. The RickA protein of *Rickettsia conorii* activates the Arp2/3 complex. *Nature* 2004;427:457–461. [PubMed: 14749835]
- Heinzen RA, Grieshaber SS, Van Kirk LS, Devin CJ. Dynamics of actin-based movement by *Rickettsia rickettsii* in vero cells. *Infect. Immun* 1999;67:4201–4207. [PubMed: 10417192]
- Heinzen RA, Hayes SF, Peacock MG, Hackstadt T. Directional actin polymerization associated with spotted fever group rickettsia infection of Vero cells. *Infect. Immun* 1993;61:1926–1935. [PubMed: 8478082]
- Jeng RL, Goley ED, D'Alessio JA, Chaga OY, Svitkina TM, Borisy GG, Heinzen RA, Welch MD. A rickettsia WASP-like protein activates the Arp2/3 complex and mediates actin-based motility. *Cell. Microbiol* 2004;6:761–769. [PubMed: 15236643]
- Johns R, Sonenshine DE, Hynes WL. Identification of a defensin from the hemolymph of the American dog tick, *Dermacentor variabilis*. *Insect Biochem. Mol. Biol* 2001;31:857–865. [PubMed: 11439245]
- Kirkegaard K, Taylor MP, Jackson WT. Cellular autophagy: surrender, avoidance and subversion by microorganisms. *Nat. Rev. Microbiol* 2004;2:301–314. [PubMed: 15031729]
- Kurtti, TJ.; Munderloh, UG.; Ahlstrand, GG. Tick tissue and cell culture in vector research. In: Harris, KF., editor. *Adv. Dis. Vector Res.* Springer-Verlag; New York: 1988. p. 87–109.
- Levine B. Eating oneself and uninvited guests: autophagy-related pathways in cellular defense. *Cell* 2005;120:159–162. [PubMed: 15680321]
- Li H, Walker DH. rOmpA is a critical protein for the adhesion of *Rickettsia rickettsii* to host cells. *Microb. Pathog* 1998;24:289–298. [PubMed: 9600861]
- Mattila J, Hutcheson J, Kurtti TJ. in preparation

- Munderloh UG. unpublished data
- Munderloh UG, Hayes SF, Cummings J, Kurtti TJ. Microscopy of spotted fever rickettsia movement through tick cells. *Microsc. Microanal* 1998;4:115–121.
- Munderloh UG, Jauron SD, Fingerle V, Leitritz L, Hayes SF, Hautman JM, Nelson CM, Huberty BW, Kurtti TJ, Ahlstrand GG, Greig B, Mellencamp MA, Goodman JL. Invasion and intracellular development of the human granulocytic ehrlichiosis agent in tick cell culture. *J. Clin. Microbiol* 1999;37:2518–2524. [PubMed: 10405394]
- Munderloh, UG.; Jauron, SD.; Kurtti, TJ. The tick: a different kind of host for human pathogens. In: Goodman, JL.; Dennis, D.; Sonenshine, DE., editors. *Tick-Borne Diseases of Humans*. ASM Press; Washington, DC: 2005. p. 37-64.
- Munderloh UG, Kurtti TJ. Formulation of medium for tick cell culture. *Exp. Appl. Acarol* 1989;7:219–229. [PubMed: 2766897]
- Munderloh UG, Kurtti TJ. unpublished
- Munderloh UG, Liu Y, Wang M, Chen C, Kurtti TJ. Establishment, maintenance and description of cell lines from the tick *Ixodes scapularis*. *J. Parasitol* 1994;80:533–543. [PubMed: 8064520]
- Munderloh UG, Madigan JE, Dumler JS, Goodman JL, Hayes SF, Barlough JE, Nelson CM, Kurtti TJ. Isolation of the equine granulocytic ehrlichiosis agent, *Ehrlichia equi*, in tick cell culture. *J. Clin. Microbiol* 1996;34:664–670. [PubMed: 8904434]
- Niebylski ML, Peacock MG, Schwan TG. Lethal effect of *Rickettsia rickettsii* on its tick vector (*Dermacentor andersoni*). *Appl. Environ. Microbiol* 1999;65:773–778. [PubMed: 9925615]
- Niebylski ML, Schrupf ME, Burgdorfer W, Fischer ER, Gage KL, Schwan TG. *Rickettsia peacockii* sp. nov., a new species infecting wood ticks, *Dermacentor andersoni*, in western Montana. *Int. J. Syst. Bacteriol* 1997;47:446–452. [PubMed: 9103635]
- Policastro P, Munderloh UG, Fischer ER, Hackstadt T. *Rickettsia rickettsii* growth and temperature-inducible protein expression in embryonic tick cell lines. *J. Med. Microbiol* 1997;46:839–845. [PubMed: 9364140]
- Rachek LI, Tucker AM, Winkler HH, Wood DO. Transformation of *Rickettsia prowazekii* to rifampin resistance. *J. Bacteriol* 1998;180:2118–2124. [PubMed: 9555894]
- Ruang-areerate T, Kittayapong P, McGraw EA, Baimai V, O'Neill SL. *Wolbachia* replication and host cell division in *Aedes albopictus*. *Curr. Microbiol* 2004;49:10–12. [PubMed: 15297923]
- Silverman DJ. *Rickettsia rickettsii*-induced cellular injury of human vascular endothelium in vitro. *Infect. Immun* 1984;44:545–553. [PubMed: 6724689]
- Silverman DJ, Wisseman CLJ. In vitro studies of rickettsia–host cell interactions: ultrastructural changes induced by *Rickettsia rickettsii* infection of chicken embryo fibroblasts. *Infect. Immun* 1979;26:714–727. [PubMed: 121115]
- Simser JA, Mulenga A, Macaluso KR, Azad AF. An immune responsive factor D-like serine proteinase homologue identified from the American dog tick, *Dermacentor variabilis*. *Insect Mol. Biol* 2004a; 13:25–35. [PubMed: 14728664]
- Simser JA, Mulenga A, Macaluso KR, Azad AF. Immune-responsive lysozymes from hemocytes of the American dog tick, *Dermacentor variabilis* and an embryonic cell line of the Rocky Mountain wood tick, *D. andersoni*. *Insect Biochem. Mol. Biol* 2004b;34:1235–1246. [PubMed: 15544937]
- Simser JA, Palmer AT, Fingerle V, Wilske B, Kurtti TJ, Munderloh UG. *Rickettsia monacensis* sp. nov., a spotted fever group rickettsia, from ticks (*Ixodes ricinus*) collected in a European city park. *Appl. Environ. Microbiol* 2002;68:4559–4566. [PubMed: 12200314]
- Simser JA, Palmer AT, Munderloh UG, Kurtti TJ. Isolation and characterization of *Rickettsia peacockii* maintained in tick cell culture. *Am. J. Trop. Med. Hyg* 1999;61(Suppl):359.
- Simser JA, Palmer AT, Munderloh UG, Kurtti TJ. Isolation of a spotted fever group rickettsia, *Rickettsia peacockii*, in a Rocky Mountain wood tick, *Dermacentor andersoni*, cell line. *Appl. Environ. Microbiol* 2001;67:546–552. [PubMed: 11157215]
- Simser JA, Rahman MS, Dreher-Lesnick SM, Azad AF. A novel and naturally occurring transposon, *ISRpe1* in the *Rickettsia peacockii* genome disrupting the *rickA* gene involved in actin-based motility. *Mol. Microbiol* 2005;58:71–79. [PubMed: 16164550]
- Teyssiere N, Chiche-Portiche C, Raoult D. Intracellular movements of *Rickettsia conorii* and *R. typhi* based on actin polymerization. *Res. Microbiol* 1992;143:821–829. [PubMed: 1299836]

Walker DH, Popov VL, Crocquetvaldes PA, Welsh CJR, Feng HM. Cytokine-induced, nitric oxide-dependent, intracellular antirickettsial activity of mouse endothelial cells. *Lab. Invest* 1997;76:129–138. [PubMed: 9010456]

Fig. 1.

Comparison of growth (transfer frequency) of uninfected *D. andersoni*, DAE100 cultures with cultures of DAE100 cells persistently infected with *R. peacockii*. The slower growing persistently infected line is shown with the plot having open circles. During the 22nd passage, selected cultures were cured of the endosymbiont by incubation at 37 °C or by the addition of oxytetracycline to the medium. The faster growing aposymbiotic oxytetracycline-treated subline is depicted in the plot having closed circles.

Fig. 2.

Transmission electron photomicrograph (TEM) of a *D. andersoni* DAE100 cell infected with *R. peacockii*. (A) Note cluster of rickettsiae in the cytoplasm (small arrowheads) and degenerating rickettsiae within putative lysosomes (large arrowheads). The cell contains numerous vacuoles filled with residual bodies. Some of the vacuoles in the cell periphery appear to be releasing their contents (large arrows). Bar=2 μ m. (B) TEM of *R. peacockii* in direct contact with host cell cytoplasm (arrows) and within a putative lysosome (arrowhead). The area shown here is from the rectangle outlined in (A). Bar=0.2 μ m.

Fig. 3. Rickettsiae and cellular debris released by DAE100 cells persistently infected with *R. peacockii*. (A) Phase contrast photomicrograph of cells in culture. Note abundance of extracellular particles in the spaces between the cells. Bar=50 μ m. (B) TEM micrograph of extracellular rickettsiae and cellular debris released from persistently infected DAE100 cells. Arrowheads point to rickettsiae that are free of host provided membranes. Arrow points to a rickettsia within an extracellular multivesicular body. Bar=0.2 μ m.

Fig. 4. Aposymbiotic DAE100A line cleared of *R. peacockii* by addition of oxytetracycline to the medium. (A) Phase contrast photomicrograph of uninfected DAE100 cells. Note absence of extracellular debris and *R. peacockii*. (B) TEM of a cell from the subline DAE100A. Note numerous vacuoles with residual bodies and overall similarity to cell shown in Fig. 2A. Bar = 3 μm.

Fig. 5.

TEM of *R. peacockii* in the *I. scapularis* embryonic cell line IDE2. (A) Photomicrograph of cell heavily infected with *R. peacockii*. Cell was infected using cell-free rickettsiae released from DAE100 cells. Arrowheads point to rickettsiae free within the cytoplasm and arrows to rickettsiae within putative autophagolysosomes. Bar = 2 μm . (B) TEM of *R. peacockii* in direct contact with host cell cytoplasm (arrowheads) and within a putative lysosome (arrows). The area shown here is from the rectangle outlined in (A). Bar = 0.2 μm .

Fig. 6.

Infectivity of cell-free *R. peacockii* for *I. scapularis* embryonic cell line ISE6. Rickettsiae were released from infected cells, separated from host cell debris, and 10-fold serial dilutions were made of the resultant rickettsial suspension. ISE6 cells in multiwell plates were inoculated with the suspensions. In one set of plates, the rickettsial suspension was centrifuged onto the cell layer (open squares) and with the other sets the rickettsiae were allowed to settle onto the cell layer by gravity (solid circles). Wells were harvested after 15 (solid lines) or 27 (dotted lines) days of incubation, prepared for Giemsa staining, and the proportion of cell infected with rickettsiae was determined by light microscopy. Values shown are means $\pm$ SD.

Table 1

In vitro host cell range of *R. peacockii*

Species	Common name	Line code	Reference for source of line
Highly permissive lines—propagation by mixing infected with uninfected cells			
<i>Ixodes scapularis</i>	Hard tick	ISE6	Munderloh et al. (1999)
<i>Boophilus microplus</i>	Hard tick	BME26	Kurtti et al. (1988)
<i>Dermacentor variabilis</i>	Hard tick	DVE1	Munderloh (unpublished)
<i>Trichoplusia ni</i>	Lepidoptera	High 5	Invitrogen, Carlsbad, CA
Permissive lines—propagation in chronically infected line			
<i>Dermacentor andersoni</i>	Hard tick	DAE100	Simser et al. (1999)
	Hard tick	DAE3	Munderloh (unpublished)
	Hard tick	DAE15	Munderloh (unpublished)
<i>Ixodes scapularis</i>	Hard tick	IDE12	Munderloh et al. (1994)
	Hard tick	IDE2	Munderloh et al. (1994)
	Hard tick	IDE8	Munderloh et al. (1994)
<i>Ixodes ricinus</i>	Hard tick	IRE11	Simser et al. (2002)
Lines infected but limited <i>R. peacockii</i> replication			
<i>Carios capensis</i>	Soft tick	CCE3	Mattila et al. (in preparation)
<i>Amblyomma americanum</i>	Hard tick	AAE2	Munderloh (unpublished)
Lines not supportive of <i>R. peacockii</i> replication			
<i>Aedes albopictus</i>	Mosquito	C7-10	Fallon and Stollar (1987)
<i>Mus musculus</i>	Mouse	L929	ATTC, CCL-1
<i>Cercopithecus aethiops</i>	Monkey	Vero	ATCC, CCL-81
	(African green)		

Cell lines were challenged using cell-free rickettsiae (see text) and tested for ability to support *R. peacockii* replication.

Table 2Influence of initial infection level on yield of *R. peacockii* infected cells in DAE100-A or ISE6 cells^a

Initial infection level		Yield at time of harvest ^b		
No. cells per culture (×10 ⁶)	% cells infected	No. cells per culture (×10 ⁶)		% cells infected
		Total	Infected	
DAE100A				
0.4	10	7.8 ±0.3	5.7 ± 0.04	73.5±2.8
1.3	33	6.4 ±0.6	5.3 ±0.4	82.7±1.6
2.0	50	5.1 ±0.6	4.6 ± 0.5	91.4±1.4
ISE6				
0.3	7	11.2 ±0.5	5.0 ± 0.4	44.6±1.4
0.4	10	9.8 ±1.1	4.6 ± 0.3	47.5±3.9
0.8	20	6.8 ±0.8	3.7 ± 0.1	53.6±7.5
1.3	33	5.7 ±1.0	3.5 ± 0.2	60.8±1.9

^a Replicated cultures (three 12.5 cm² flasks) were each seeded with a total of 4 ×10⁶ cells.^b DAE100A harvested 14 days post-inoculation; ISE6 10 days post-inoculation. Values are means ± SD of triplicated cultures.