Coinfection with *Anaplasma phagocytophilum* Alters *Borrelia burgdorferi* Population Distribution in C3H/HeN Mice

Kevin Holden,1 Emir Hodzic,1 Sunlian Feng,1 Kimberly J. Freet,1 Rance B. Lefebvre,2 and Stephen W. Barthold1*

Center for Comparative Medicine, Schools of Medicine and Veterinary Medicine, University of California at Davis, Davis, California 95616,1 and Department of Pathology, Microbiology, and Immunology, School of Veterinary Medicine, University of California at Davis, Davis, California 956162

Received 17 December 2004/Accepted 14 January 2005

*Borrelia burgdorferi*, the agent of Lyme disease, and *Anaplasma phagocytophilum*, the agent of human anaplasmosis, are both transmitted by *Ixodes* sp. ticks and may occasionally coinfect a host. The population distributions of tick-transmitted *B. burgdorferi* infection were assessed using quantitative PCR targeting the flaB gene of *B. burgdorferi* in the ear, heart base, quadriceps muscle, skin, and tibiotarsal joint tissue of C3H mice previously infected with *A. phagocytophilum*. Population distributions of *Anaplasma* infection were assessed by targeting the p44 gene. *A. phagocytophilum* in blood and serologic response to both agents were evaluated. Spirochete numbers were increased in the ears, heart base, and skin of coinfected mice, but *Anaplasma* numbers remained constant. Antibody response to *A. phagocytophilum*, but not *B. burgdorferi*, was decreased in coinfected mice. These results suggest that coinfection with *A. phagocytophilum* and *B. burgdorferi* modulates pathogen burden and host antibody responses. This may be explained by the ability of *A. phagocytophilum* to functionally impair neutrophils, important cells in the early defense against *B. burgdorferi* infection.

*Borrelia burgdorferi*, the causative agent of Lyme disease, is the most common tick-borne pathogen in the United States and is a significant cause of morbidity throughout the world (30). Lyme disease is a multisystemic disease that may be characterized by dermatological, musculoskeletal, and neurological manifestations. The spirochete bacterium is transmitted to humans via the bite of infected ticks belonging to the genus *Ixodes*. In recent years, another zoonotic pathogen, *Anaplasma phagocytophilum*, the agent of human anaplasmosis (formerly designated as human granulocytic ehrlichiosis, or HGE) has been detected in *Ixodes* ticks in both the United States and Europe (5, 26). *Anaplasma phagocytophilum* is an obligate, intracellular bacterium that infects the granulocytes, primarily neutrophils, of mammals. Clinical manifestations of human anaplasmosis may include a wide array of symptoms involving the hematopoietic, immune, and nervous systems; involvement can range from a mild, self-limiting disease to a severe, life-threatening condition (2). Since *A. phagocytophilum* shares a common vector with *B. burgdorferi*, cases of anaplasmosis often occur in areas where Lyme disease is endemic (2, 23).

Inevitably, questing ticks coinfect with *B. burgdorferi* and *A. phagocytophilum* have been identified in these regions (17, 29). Furthermore, the simultaneous acquisition, coinfection, and transmission of both of these agents in the tick vector to the laboratory mouse have recently been established (15, 20).

Dual infections with *B. burgdorferi* and *A. phagocytophilum* have been documented in both human patients, wild rodents, and laboratory mice (20, 21, 33, 34). In humans, several distinctive clinical presentations aid in the differential diagnosis of Lyme disease from anaplasmosis. However, in coinfection scenarios, patients may present with a confusing mixture of manifestations, making diagnosis problematic (21, 24, 25). Undoubtedly, anaplasmosis may complicate the disease severity and prognosis of Lyme disease (7, 31). The frequency of coinfection and the resulting clinical outcome in humans is largely unknown and has been the focus of several studies (2, 31). Ultimately, the immunosuppressive nature of anaplasmosis may invariably affect the outcome and duration of *B. burgdorferi* infection.

The pathogenesis of Lyme disease and anaplasmosis has been well documented in murine model systems (10, 13, 14, 16, 28, 35). However, only two studies to date have focused on investigating the coinfection phenomenon (33, 34). Zeidner et al. reported that when cotransmitted by ticks, *B. burgdorferi* and *A. phagocytophilum* act synergistically to modulate host immune responses, possibly providing a greater opportunity for either pathogen to escape initial immune surveillance (34). Moreover, Thomas et al. showed that in addition to modulation of host immune responses, coinfected mice suffered from higher pathogen burdens and more severe arthritis when *B. burgdorferi* and *A. phagocytophilum* were cotransmitted via syringe inoculation (33). Thus, simultaneous coinfection with *B. burgdorferi* and *A. phagocytophilum* appears to enhance the pathogenesis of Lyme disease in laboratory mice. However, a number of alternative coinfection scenarios may exist in nature. Perhaps a more frequent occurrence is that hosts may acquire one infection before the other. Given the evidence that *Anaplasma* infection can be immunosuppressive, it is important to consider this effect on subsequent tick-borne infection with *B. burgdorferi*, particularly in light of the fact that *B. burgdorferi* has been shown to significantly influence the immune status of the host (9, 18, 22, 28, 33).
The purpose of this study was to determine the effect that an established *A. phagocytophilum* infection has upon a subsequent infection with *B. burgdorferi*. Previously, our laboratory had developed accurate, sensitive, molecular techniques for assessing population dynamics and distribution of *B. burgdorferi* and *A. phagocytophilum* in mice by the use of real-time quantitative PCR (qPCR) (12, 13, 14). Herein we have utilized these tools to demonstrate that prior tick-borne infection with *A. phagocytophilum* alters the population distribution and antibody response in mice subsequently infected with tick-borne *B. burgdorferi*.

**MATERIALS AND METHODS**

**Mice.** Specific-pathogen-free, 3- to 5-week-old C3H/HeN (Frederick Cancer Research Center, Frederick, MD) and C3H/Snn.Ccr-csid (SCID) mice (Harlan, Indianapolis, IN) were used in this study based upon their susceptibility to infection and disease with both *B. burgdorferi* and *A. phagocytophilum* (16, 28, 33). Mice were maintained in isolated isolator cages within an infectious disease containment room and fed commerical mouse diet and water ad libitum. Mice were euthanized by carbon dioxide asphyxiation.

**Bacteria.** A low-passage, clonal strain of *B. burgdorferi* N40 sensu stricto was maintained in modified BSK II medium supplemented with 6% rabbit serum (1). Cells were enumerated in a bacterial counting chamber as described previously (14). For the development of *B. burgdorferi*-infected ticks (see below), five C3H mice were each inoculated intradermally at the thoracic dorsal midline with 10^7 mid-log-phase spirochetes. The NCH-1 isolate of *A. phagocytophilum* was maintained via serial passage from infected SCID mice to naive SCID mice every 3 weeks by intraperitoneal inoculation of 0.1 mL EDTA-anticoagulated blood. For the development of *A. phagocytophilum*-infected ticks (see below), five C3H mice were inoculated intraperitoneally with blood from infected SCID mice.

**Ticks.** Mated adult female Ixodes scapularis ticks were kindly provided by Durland Fish of Yale University, New Haven, Connecticut. The egg mass from a single tick produced the uninfected larvae for experimental use. Three groups of five C3H mice each were infected with *B. burgdorferi* or *A. phagocytophilum* or sham inoculated with sterile BSK II medium (negative control). After 2 weeks, infection was confirmed by PCR (see below) of collected ear notches (for *B. burgdorferi*) or determination of morulae on peripheral blood smears and PCR (for *A. phagocytophilum*) using blood collected by tail bleeding. Larval ticks were allowed to attach and engorge upon three groups of five C3H mice anesthetized with a ketamine-xylazine cocktail and maintained in individual cages in order to generate *B. burgdorferi*-infected, *A. phagocytophilum*-infected, or uninfected nymphs. Engorged larvae were collected and allowed to molt and harden into nymphs. Tick rearing was conducted in an incubator at 21°C with 95% relative humidity and fed commerical mouse diet and water ad libitum. Tissues chosen for analysis were individually stored, snap-frozen, and kept at -20°C until they could be used in an enzyme-linked immunosorbent assay (ELISA).

**MATERIALS AND METHODS**

**Mice.** Specific-pathogen-free, 3- to 5-week-old C3H/HeN (Frederick Cancer Research Center, Frederick, MD) and C3H/Snn.Ccr-csid (SCID) mice (Harlan, Indianapolis, IN) were used in this study based upon their susceptibility to infection and disease with both *B. burgdorferi* and *A. phagocytophilum* (16, 28, 33). Mice were maintained in isolated isolator cages within an infectious disease containment room and fed commerical mouse diet and water ad libitum. Mice were euthanized by carbon dioxide asphyxiation.

**Bacteria.** A low-passage, clonal strain of *B. burgdorferi* N40 sensu stricto was maintained in modified BSK II medium supplemented with 6% rabbit serum (1). Cells were enumerated in a bacterial counting chamber as described previously (14). For the development of *B. burgdorferi*-infected ticks (see below), five C3H mice were each inoculated intradermally at the thoracic dorsal midline with 10^7 mid-log-phase spirochetes. The NCH-1 isolate of *A. phagocytophilum* was maintained via serial passage from infected SCID mice to naive SCID mice every 3 weeks by intraperitoneal inoculation of 0.1 mL EDTA-anticoagulated blood. For the development of *A. phagocytophilum*-infected ticks (see below), five C3H mice were inoculated intraperitoneally with blood from infected SCID mice.

**Ticks.** Mated adult female Ixodes scapularis ticks were kindly provided by Durland Fish of Yale University, New Haven, Connecticut. The egg mass from a single tick produced the uninfected larvae for experimental use. Three groups of five C3H mice each were infected with *B. burgdorferi* or *A. phagocytophilum* or sham inoculated with sterile BSK II medium (negative control). After 2 weeks, infection was confirmed by PCR (see below) of collected ear notches (for *B. burgdorferi*) or determination of morulae on peripheral blood smears and PCR (for *A. phagocytophilum*) using blood collected by tail bleeding. Larval ticks were allowed to attach and engorge upon three groups of five C3H mice anesthetized with a ketamine-xylazine cocktail and maintained in individual cages in order to generate *B. burgdorferi*-infected, *A. phagocytophilum*-infected, or uninfected nymphs. Engorged larvae were collected and allowed to molt and harden into nymphs. Tick rearing was conducted in an incubator at 21°C with 95% relative humidity. Ten percent of molted nymphs from each infection group were indi"
groups of five C3H mice each were established. Group 1 mice were infected with *A. phagocytophilum*-positive ticks on day 0 and then infected with *B. burgdorferi*-positive ticks on day 8. Group 2 mice were infected with *A. phagocytophilum*-positive ticks and then infected with uninfected ticks on day 8. Group 3 mice were infected with uninfected ticks and then infected with *B. burgdorferi*-positive ticks on day 8. Group 4 mice were infected with uninfected ticks on day 0 and day 8. At each feeding, 4 ticks were allowed to feed to engorgement, and all feedings were performed on the same time schedule, with mice maintained in individual cages. Previous studies have shown that *B. burgdorferi* infection and disease reach their peak in C3H mice at 14 days after syringe inoculation (13, 14). Thus, on day 24 (16 days after *B. burgdorferi*-positive or -negative tick exposure), mice were necropsied. Blood and tissues were collected for PCR, culture, and histology.

All mice in groups 1 and 2 (infected with *A. phagocytophilum*-infected ticks) had confirmed infections with *A. phagocytophilum*, based upon positive PCR results and the presence of morulae in peripheral blood smears on day 7. Mice in groups 3 and 4 (infected with uninfected ticks) were negative. On day 24, all mice in groups 1 and 3 were culture and PCR positive for *B. burgdorferi* and all mice in groups 2 and 4 were negative. Therefore, all experimental mice were determined to be suitable for analysis.

Quantitative values for *A. phagocytophilum* were calculated based on the number of *p44* gene copy numbers present in 50 μL of blood collected at necropsy (day 24). Amplification of the *A. phagocytophilum p44* gene by qPCR was successful in blood samples from all mice in groups 1 and 2 on day 24 that were previously shown to be infected with the agent on day 7 (Fig. 1). However, there was no significant difference (P = 0.063) in copy numbers, and therefore bacterial populations, between mice that were singly infected with *A. phagocytophilum* (group 2) and those that were coinfected with *B. burgdorferi* (group 1).

The populations of *B. burgdorferi* in various tissues were quantified by calculating the number of *flaB* copies present in DNA per milligram of tissue. Evaluation of two different skin sites showed differences in spirochete population distribution. Mice coinfected with *A. phagocytophilum* and *B. burgdorferi* (group 1) were found to have significantly higher levels of spirochetes present in both the ear and skin than mice singly infected with *B. burgdorferi* (group 3) (Fig. 2) (P = 0.026 and 0.0034, respectively). No *flaB* copy numbers were found in the skin of any mice that were only infected with *B. burgdorferi* (group 3). Analysis of heart base tissues also showed a difference in spirochete populations (Fig. 2). Coinfected mice (group 1) were consistently found to have significantly greater *B. burgdorferi* populations than mice singly infected with *B. burgdorferi* (group 3) (P = 0.017). No significant differences in spirochete population distribution were identified between coinfected (group 1) mice and *B. burgdorferi*-infected mice (group 3) in tibiotarsal joints and quadriceps muscle (Fig. 2) (P = 0.379 and 0.585, respectively).

Antibody titers against *B. burgdorferi* or *A. phagocytophilum* lysates were determined for each treatment group. The antibody response against *A. phagocytophilum* at day 24 after tick-borne infection differed between mice singly infected with the agent (group 2) and those coinfected with *B. burgdorferi* (group 1). With sera from five mice in each infection group, average titers for the *A. phagocytophilum*-infected group were 2,700, whereas average titers from the coinfected group were 900, indicating a statistically lower (P = 0.374) overall antibody response. The antibody response against *B. burgdorferi* at day 17 after tick-borne infection was similar for mice singly infected with *B. burgdorferi* (group 3) and those coinfected (group 1). With sera from five mice in each infection group, the average titer for *B. burgdorferi*-infected mice was 2,700 and the average titer for the coinfected mice was also 2,700.

Histopathologic examination of mouse hearts failed to elucidate significant differences in carditis prevalence or severity.
between coinfected (group 1) and singly infected B. burgdorferi (group 3) mice. Analysis of joints revealed that coinfected mice showed a low prevalence of mild arthritis (data not shown), whereas singly infected mice showed no arthritis in any of the joints examined.

**DISCUSSION**

Previously, B. burgdorferi and A. phagocytophilum coinfection studies in mice have focused on the cotransmission of these agents simultaneously and the resulting coevolution of disease (33, 34). However, a wide range of coinfection scenarios exist in nature, and the frequency of their occurrences is unknown. Although ticks may harbor both pathogens, prevalence studies in endemic areas throughout the United States have shown that coinfection rates may be as little as 1 to 6% (6, 17) or as high as 26% (3), whereas the rate of ticks singly infected with B. burgdorferi can be up to 40% (6) and the rate of ticks singly infected with A. phagocytophilum can be up to 20% (19). In addition, transmission time, from tick to host, may differ for each pathogen (20). When one considers that natural hosts, such as mice and deer, and incidental hosts such as humans, may be infected with more than one tick at a given time, it makes it difficult to assign the order or timing in which coinfections might take place. Simultaneous infection is the simplest situation to create in a controlled experimental setting. Yet perhaps a more frequent occurrence is that hosts acquire one infection prior to the other. Due to the documented immunosuppressive nature of *Anaplasma* infection (2, 4) and its nature of causing either symptomatic or asymptomatic infections (7), an important avenue of exploration is the effect that this infection may have upon subsequently acquired tick-borne B. burgdorferi. It is well established that infection population dynamics and disease susceptibility in mice infected with B. burgdorferi are significantly affected by immune competence of the host (13). Thus, preinfection with an agent such as *A. phagocytophilum*, which may cause immunosuppression, may therefore have a significant effect upon subsequent B. burgdorferi infection. Thus, in this study, we elected to initially infect mice with tick-borne *A. phagocytophilum* and then at the approximate peak of infection (14) we challenged them with tick-borne B. burgdorferi. This design offered the opportunity to test the hypothesis that prior infection with *A. phagocytophilum*, at an interval at which effects on the host are likely to be maximal, has an effect upon subsequent infection with B. burgdorferi.

The current study found increases in B. burgdorferi populations in the subcutis, skin, ear, and heart base of mice coinfected with *A. phagocytophilum*. An interesting observation was that none of the mice that were singly infected with B. burgdorferi were PCR positive for skin, yet they were culture positive at the inoculation site. In contrast, skin and subcutis from all of the mice that were coinfected were PCR positive and culture positive, respectively. Although this may appear contrary, it is important to note that subcutaneous fascia directly beneath the skin (subcutis) was cultured, and PCR was performed on the overlying skin. Thus, spirochetes were present in both the skin and subcutis in coinfected mice and in significantly greater amounts in the ear samples of coinfected mice (which consist of skin, subcutis, and cartilage). This phenomenon of persistence in the skin may lead support to observations made by Thomas et al., who noted that acquisition of both B. burgdorferi and A. phagocytophilum by feeding ticks is enhanced in coinfection scenarios (33).

The immunosuppressive effects of *Anaplasma* infection appear to be varied. Human patients enduring this disease can readily become susceptible to opportunistic infections (4, 7). Featured defects in host immunity during this infection include T- or B-cell suppression, with notable decreases in CD4 and CD8 cell counts, and impaired lymphoproliferation of isolated lymphocytes (7). Also of importance, recent studies have shown that neutrophil function, specifically phagocytic ability, can be significantly impaired in anaplasmosis (4). This is of particular interest because studies have shown that B. burgdorferi spirochetes are susceptible to phagocytosis and a novel oxidative burst mechanism exhibited by these cells (32). Although B. burgdorferi employs a variety of immune evasion mechanisms to survive and disseminate in the host, neutrophils are thought to play a key role in the early defense against infection. Impairment of this innate ability, in concert with the overall picture of immunosuppression, may explain the increase in B. burgdorferi population distribution in coinfected mice.

Despite the increased numbers of spirochetes in several tissues of coinfected mice, including heart and joint, histopathologic observations did not reveal more severe disease. Spirochetes were increased in heart tissue of coinfected mice, yet there were no appreciable difference in carditis severity, and were found in approximately equal numbers in quadriiceps muscle and tibiotarsal joint of singly and coinfected mice, and histopathology did not suggest a significant increase in arthritis severity in the coinfected mice. This may have been due to immune suppression from *A. phagocytophilum* and lack of an appropriate inflammatory response. Alternatively, it may be a reflection of the mouse model, as disease is relatively mild or absent in older mice, and by necessity of the experimental design, disease could not be evaluated until the mice were 8 to 9 weeks of age.

Coinfection did not significantly impair antibody response to either pathogen. In spite of the fact that coinfected mice harbored larger populations of spirochetes in several tissues, ELISA results indicated that the antibody responses to B. burgdorferi were not significantly different between coinfected and singly infected mice. This is similar to findings by Thomas et al. (33) in which mice were simultaneously coinfected. Although our results show that coinfection with B. burgdorferi does not alter levels of *A. phagocytophilum* in the blood, coinfection does appear to alter the antibody response to *A. phagocytophilum*. The observation of a diminished antibody response in coinfected mice in this study is consistent with previous reports by Zeidner et al., who further noted that differences in cytokine profiles indicated that potential immune modulation mechanisms were active in a coinfection scenario (33, 34).

We have shown that coinfection with *A. phagocytophilum* can influence the population distribution of spirochetes during infection of a murine host. By some means of immunomodulation, this allows B. burgdorferi to persist longer in particular tissues and to exist in overall higher numbers within the host. Although *A. phagocytophilum* has its own immune evasion capabilities, it is likely that such a coinfection scenario aids this...
bacterium in continued persistence. Additional studies are needed in order to prove that anaplasmosis invariably affects the clinical presentation and duration of Lyme disease.

ACKNOWLEDGMENTS

We thank Edward Lorenzana for technical support.

This work was supported by Public Health Service grant AI26815 from the National Institute of Allergy and Infectious Diseases, National Institutes of Health.

REFERENCES


