Immunity Reduces Reservoir Host Competence of Peromyscus leucopus for Ehrlichia phagocytophila

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Infection with Ehrlichia phagocytophila in white-footed mice is transient and followed by a strong immune response. We investigated whether the presence of acquired immunity against E. phagocytophila precludes white-footed mice from further maintenance of this agent in nature. Mice were infected with E. phagocytophila via tick bite and challenged either 12 or 16 weeks later by Ixodes scapularis nymphs infected with the same agent. Xenodiagnostic larvae fed upon each mouse simultaneously with challenged nymphs and 1 week thereafter. Ticks were tested for the agent by PCR, and the prevalence of infection was compared to that in ticks that fed upon nonimmune control mice. Only 30% of immunized mice sustained cofeeding transmission of E. phagocytophila between simultaneously feeding infected and uninfected ticks, compared to 100% of control mice. An average of 6.3% of xenodiagnostic ticks acquired Ehrlichia from previously immunized mice when fed 1 week after the challenge, compared to 82.5% infection in the control group. Although an immune response to a single infection with E. phagocytophila in white-footed mice provided only partial protection against reinfection with the same agent, the majority of mice were rendered reservoir incompetent for at least 12 to 16 weeks. Immunity acquired by mice during I. scapularis nymphal activity in early summer may exclude a large proportion of the mouse population from maintaining E. phagocytophila during the period of larval activity later in the season.

Human granulocytic ehrlichiosis (HGE) is a recently recognized tick-borne disease caused by an obligate intracellular bacterium believed to be identical to Ehrlichia phagocytophila (9, 17, 32, 38, 41). Infection with E. phagocytophila has been reported in a variety of mammalian species including rodents, dogs, horses, deer, and humans (3, 8, 22, 47). In the eastern United States, E. phagocytophila is maintained in a natural cycle between the black-legged tick Ixodes scapularis and its vertebrate hosts (14, 43), as is Borrelia burgdorferi, the etiologic agent of Lyme disease. Small rodents, especially the white-footed mouse (Peromyscus leucopus), are important hosts for immature I. scapularis and major reservoirs for B. burgdorferi. Larval ticks acquire infection while parasitizing infected mice and then transmit it to new susceptible hosts during nymphal feeding. Mice, once infected with B. burgdorferi, remain infective for ticks for many months (16, 31). Their prolonged infectivity is essential for continuous transmission of B. burgdorferi between infected nymphs active from May to June and uninfected larvae that parasitize the same mice in August and September (19). Granulocytic Ehrlichia and antibodies to E. phagocytophila have also been found in a variety of wild rodent species, including white-footed mice (7, 11, 33, 35, 43, 45, 47). Together with laboratory experiments (15, 30, 35, 43), these findings show that the white-footed mouse is susceptible to E. phagocytophila and often exposed to infection in nature. However, its role in the natural cycle of E. phagocytophila remains unclear (30). In contrast to that with B. burgdorferi, infection with E. phagocytophila in susceptible rodent hosts appears to be transient. Untreated C3H mice resolve an infection within 60 days (42). Laboratory mice (Mus musculus) inoculated with E. phagocytophila mount an immune response, which partially protects animals against challenge (23, 42). Such protection, if it occurs in nature, may preclude previously infected hosts from further maintenance of the agent.

Here, we show that previous exposure of white-footed mice to E. phagocytophila provides partial protection against homologous challenge, thus reducing their susceptibility to additional infection and subsequent infectivity to ticks, consequently diminishing their role in the natural maintenance of E. phagocytophila.

MATERIALS AND METHODS

Two-month-old mice, derived from a colony that has been maintained in our laboratory for several generations, were infected with E. phagocytophila and consequently challenged with the same agent either 12 or 16 weeks thereafter. The susceptibility of immune mice to reinfection and their subsequent infectivity for xenodiagnostic ticks were compared to the same parameters for Ehrlichia-naive control mice. The strain of E. phagocytophila used in our experiments originated from I. scapularis nymphs collected at a site in Westchester County (N.Y.) where HGE is endemic (39). The agent is maintained in our laboratory in a tick-mouse cycle, where infected I. scapularis nymphs are produced by allowing uninfected larval ticks to feed upon mice previously exposed to E. phagocytophila-infected nymphs. Uninfected xenodiagnostic larvae were derived from a separate I. scapularis colony maintained for several generations in our laboratory by feeding on uninfected mice and rabbits. Host sera are routinely screened for Ehrlichia antibodies, and representative samples of ticks from the colony are regularly tested by PCR to ensure that the colony is free of tick-borne pathogens.

Primary infections. Two groups of mice were initially infected with E. phagocytophila via exposure to infected ticks. Six mice were each infected with 10 infected I. scapularis nymphs 12 weeks prior to the challenge (experimental group I). Another 10 mice were each infected with 10 infected nymphs 16 weeks prior to the challenge (experimental group II). Infection in both groups of mice was confirmed by xenodiagnostic infestation with approximately 200 uninfected larvae 1 week after the original nymphal infestation. Additionally, serum samples were collected from the retro-orbital sinus of each experimental mouse on the day of nymphal infestation and weekly for 4 weeks thereafter. Sera were tested for the presence of specific antibodies against E. phagocytophila by indirect immunofluorescence assay (IFA).

All mice from experimental groups I and II were subjected to yet another xenodiagnostic infestation 11 and 15 weeks, respectively, after infection (7 days before the challenge) to ensure that mice had resolved the infection. Simultaneously, four tick-naive mice (control group) were also infected with xenodiagnostic larvae to verify that they were indeed free of Ehrlichia. High feeding density enhances pathogen acquisition in larval I. scapularis (29); therefore, mice were infested with approximately 300 larvae to increase the sensitivity of xeno-
diagnosis. Thirty engorged larvae per mouse were tested by PCR in pools of 5 for *E. phagocytophila*.

**Challenge.** All 16 previously infected mice and 4 naïve control mice were each challenged by 10 nymphs infected with *E. phagocytophila*. The homology of challenge was ensured by using infected nymphs that had fed as larvae upon the same mouse. Challenge of all mice was performed on the same day to guarantee uniformity among the challenging ticks. The prevalence of infection in the challenging nymphs was estimated to be 40%, as assessed by testing a representative sample of 20 ticks by PCR. Approximately 100 xenodiagnostic larvae fed upon each mouse simultaneously with challenging nymphs to assess the possibility and efficiency of transmission of *E. phagocytophila* between cofeeding ticks. Another group of approximately 100 larvae fed upon each of the 20 mice 1 week after the challenge.

Engorged nymphs and larvae were collected daily, as they detached after replation, and kept at 22°C and 95% relative humidity. Ticks ingesting an agent with a blood meal may not be able to transmit it transtadially (15). Therefore, at this stage of the experiment, both challenging and xenodiagnostic ticks were tested after they molted to the next stage. All adult ticks that had fed as challenging nymphs, and a sample of 30 nymphs derived from xenodiagnostic ticks were tested by PCR. Additionally, blood and serum samples were collected from the retro-orbital sinus of each mouse 1 week before the challenge, on the day of challenge, and 7 days later. Blood was tested for *E. phagocytophila* by PCR, and serum was tested for the presence of specific immunoglobulin G (IgG) antibodies by IFA. Positive mouse blood samples and ticks were tested for the presence of *E. phagocytophila* DNA as described before (30). Briefly, individual adult and nymphal ticks and pools of replete larvae were placed in sterile 1.5-cm³ plastic vials, deep-frozen in liquid nitrogen, ground with a sterile plastic pestle, and resuspended in 50 µl of Tris-EDTA buffer. DNA from ground ticks and blood samples were extracted using the IsoQuick nucleic acid extraction kit (ORCA Research Inc., Bothell, Wash.). Primers EHR-521 (5'-TGT AGG CCG TTC GGT GAG TAA AAC-3') and EHR-747 (5'-GCA CTC GTC GTT TAC AGC GTG-3') were used to amplify a 247-bp fragment of 16S ribosomal DNA from *E. phagocytophila* (37). The amplification products were visualized in 2% agarose gels stained with ethidium bromide.

**Transmissibility test.** It is possible that some positive PCR results may be due to remnants of ehrlichial DNA retained in a tick gut after feeding and not due to actual infection. To assess the viability of the pathogen acquired by ticks from immune mice, an additional sample of 25 ticks that fed as larvae upon each of the 16 immune and 4 control mice 1 week after the challenge were placed on an additional 20 naïve mice. Nymphs were allowed to feed to replation. At 7 days after the infestation, mice were bled and infected with ~100 xenodiagnostic *I. scapularis* larvae to confirm the infection. Blood samples and two pools of 10 replete larvae from each mouse were tested for *E. phagocytophila* by PCR.

**IFDi.** Mouse sera were tested for IgG antibodies reactive with the cultured isolate of *E. phagocytophila* in an IFA developed by Aquila Biopharmaceuticals (Worcester, Mass.). The antigen provided by Aquila Biopharmaceuticals was derived from a human promyelocyte cell culture (HL-60) infected with *E. phagocytophila*. Sera were initially screened at a dilution of 1:40 (30, 46). Twofold serial dilutions of reactive samples in phosphate-buffered saline (pH 7.4) up to a dilution of 1:5,120 were examined. Positive control serum was obtained in our laboratory from a *I. persulcatus* mouse infected with the same isolate of *E. phagocytophila*, and infection was confirmed by PCR with blood and xenodiagnosis.

**RESULTS**

**Primary infection.** Xenodiagnostic *I. scapularis* larvae acquired infection from all 16 mice in experimental groups I and II when fed 1 week after the initial infestation with infected nymphs (11 and 15 weeks, respectively, prior to the challenge). Also, all 16 experimental mice seroconverted within 7 to 14 days after nymphal infestation. Sera collected from all mice on day 14 postinfestation were reactive to *E. phagocytophila* at dilutions ≥1:640 (mode, 1:2,560). Thus, all mice in both experimental groups became infected with *E. phagocytophila* and developed a strong immune response.

On the day of challenge (12 weeks after the original infection), sera from all six mice in group I were reactive against *E. phagocytophila* at dilutions ≥1:320 (mode, 1:1,280). All 10 mice in group II also were seropositive on the day of challenge (16 weeks after the original infection) with antibody titers that were ≥1:160 (mode, 1:640). However, none of these mice had *E. phagocytophila* DNA in the blood that was detectable by PCR, and *E. phagocytophila* was not found in any of 30 xenodiagnostic ticks that fed upon each mouse 1 week before the challenge. Thus, all mice successfully resolved the infection and were not infectious for xenodiagnostic ticks prior to the challenge.

**Challenge.** First, we assessed the prevalence of infection among adult ticks used as challenging nymphs (Table 1) and compared it to that for the same cohort prior to the challenge (40% ± 10%). Six to ten ticks from each mouse in all three groups molted to the adult stage and were available for PCR. The average prevalence of *Ehrlichia* infection in ticks that fed upon four control mice (41.7% ± 5.5%) did not differ from the original prevalence of infection in challenging nymphs. The average prevalence of infection in ticks that fed upon six mice from group I at 12 weeks postinfestation was only half that in challenging nymphs (21.1% ± 4.5%). This difference was statistically significant (P = 0.0329). The average prevalence of infection in ticks that fed upon 10 mice from group II at 16 weeks postinfection (34.9% ± 5.3%) was not significantly different from that for the cohort of challenging nymphs prior to the feeding (P = 0.4850). Thus, the prevalence of infection in challenging nymphs was reduced after the nymphs fed with recently acquired immunity but not after they fed upon naïve mice.

Next, we measured prevalence of infection in xenodiagnostic ticks that fed simultaneously with challenging nymphs. Every mouse in the control group sustained transmission of the agent between simultaneously feeding infected nymphs and uninfected larvae, whereas only one-third of immune mice in groups I and II sustained that type of transmission (Table 2). Overall, 10.8% ± 1.6% of larval ticks acquired *Ehrlichia* during feeding. However, only 1.0 to 1.1% of larval ticks acquired *Ehrlichia* while cofeeding with infected nymphs on previously exposed mice (Table 2). The efficiency of *E. phagocytophila* transmission during cofeeding on immune mice differed significantly from that during cofeeding on control mice (P by analysis of variance [PANOVA] = 0.0035 and 0.0001 for groups I and II, respectively) and did not differ between the two experimental groups (PANOVA = 0.7487).

One week after the challenge, ehrlichial DNA was detected in 10.8% ± 1.6% of the control group ticks and in 1.1 ± 0.7% of the infected groups II ticks. No infection was detected in any of the xenodiagnostic ticks that cofed with ticks from group I on day 14 postinfection (Table 2).

**TABLE 2. E. phagocytophila infection in xenodiagnostic ticks that fed upon control and immune mice simultaneously with challenging nymphs**

<table>
<thead>
<tr>
<th>Mouse groupa</th>
<th>No. of mice with cofeeding transmission/ no. of mice in group</th>
<th>No. of ticks tested</th>
<th>% Infected ticks ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4/4</td>
<td>120</td>
<td>10.8 ± 1.6</td>
</tr>
<tr>
<td>I</td>
<td>2/6</td>
<td>180</td>
<td>1.1 ± 0.7</td>
</tr>
<tr>
<td>II</td>
<td>3/10</td>
<td>300</td>
<td>1.0 ± 0.05</td>
</tr>
</tbody>
</table>

a Mouse groups are as defined in the footnote to Table 1.
by PCR in the blood of all 4 control mice but only in 2 of 6 mice challenged 12 weeks postinfection and in 3 of 10 mice challenged 16 weeks postinfection. Xenodiagnostic ticks fed 1 week after the challenge acquired *E. phagocytophila* from each of 4 mice in the control group, from 5 of 6 mice in group I, and from 7 of 10 mice in group II (Table 3). The average prevalences of infection in xenodiagnostic ticks that fed upon immune mice (6.0 and 6.7% in groups I and II, respectively) were significantly lower than that in ticks that fed at the same time upon control mice (82.5%) (\(P_{\text{ANOVA}} < 0.001\)) and did not differ between the two experimental groups (\(P_{\text{ANOVA}} = 0.7258\)) (Table 3).

**Transmissibility test.** When nymphs that fed as larvae upon each of 20 challenged mice were placed on 20 naïve mice, 14 of the naïve mice became infected with *E. phagocytophila*, as confirmed by both PCR with blood and xenodiagnosis. Nymphs from each of four control mice successfully transmitted *E. phagocytophila* to new animals, as did ticks from 4 of 6 mice challenged at 12 weeks postinfection and from 6 of 10 mice challenged at 16 weeks postinfection. This provided direct evidence that a positive PCR result for molted xenodiagnostic ticks was indicative of a viable agent and not just remnants of DNA.

**Discussion**

Infection with *E. phagocytophila* causes a disease affecting both humans and domestic animals. The ability of susceptible hosts to mount protective immunity in response to ehrlichial infection and the scope and duration of protection are important for understanding of epidemiology and epizootiology of the disease as well as for the development of protective strategies. Results of our experiment indicate that an infection with *E. phagocytophila* in white-footed mice renders only partial protection against reinfection. Five of 6 mice were reinfected 12 weeks after the initial infection, and 7 of 10 mice were reinfected when challenged at 16 weeks. Although reactivation of a persistently infected mouse is theoretically possible, it is unlikely, as the agent was not detected in mice by either massive xenodiagnostic infestation or PCR with blood prior to the challenge. Also, chronic or latent infection has not been described and does not seem to occur in mice, horses, sheep, or cattle experimentally infected with *E. phagocytophila* (6).

Humans, cattle, sheep, horses, and mice mount humoral immune responses to infection with *E. phagocytophila* within 2 weeks, and antibodies continue to be detected for many months, but their significance in protective immunity is not clearly established (6, 20, 24, 28, 46, 48). Partial protection from homologous challenge after natural or experimental infection with *E. phagocytophila* has been reported for sheep (40, 49), goats (40), hares (2, 36), cattle (44), and laboratory mice (42). However, this protection seems to be incomplete. The duration of protective immunity also appears to be variable. Some animals may be reinfected with *Ehrlichia* in a few months, while others may resist reinfecion for over a year (49).

Stamp and Watt (40) reported that goats were protected from reinfection with *E. phagocytophila* for a year, while Hudson (26) found that the immunity to this pathogen is short-lived in cattle. Reinfection with *E. phagocytophila* has been reported in a human case 2 years after successful treatment of HGE (25).

Thus, a single infection with *E. phagocytophila* does not necessarily provide long-term protection against reinfection. Our results indicate that white-footed mice that have been infected in early summer, during the peak of nymphal activity, may again become partially susceptible to the same agent by the time of larval activity at the end of summer and early in the fall. If infected, a few nymphs that are active in August and September can transmit the infection to previously immune mice. Acquired immunity against *E. phagocytophila* does not affect tick feeding and molting success. However, this acquired immunity appears to disrupt further maintenance of *E. phagocytophila*.

A proportion of infected nymphs seem to lose *E. phagocytophila* after feeding on immune mice. A decrease in the prevalence of infection between flat nymphal and adult ticks occurred only for ticks that fed upon immune mice, and it was more apparent in ticks that fed upon mice at 12 weeks postinfection than in ticks that fed at 16 weeks. The mechanisms and locations of the interaction between host Igs and an intracellular parasite such as *E. phagocytophila* inside a tick are unclear. Unlike *B. burgdorferi*, which resides primarily in the midgut of an unfed tick and thus is easily accessible to host-derived antibodies ingested by a tick during blood feeding (13, 18), *E. phagocytophila* is not restricted to the midgut. It develops a generalized infection in a variety of internal organs in the vector, including the salivary glands (12).

Host Igs, including IgG antibodies, ingested by the tick during blood feeding are able to cross the gut wall and retain their immunological properties in tick hemolymph. This phenomenon has been demonstrated for several species of ixodid and argasid ticks (1, 4, 5, 10, 21, 34). However, vaccine-derived antibodies ingested by *Dermacentor andersoni* with a blood meal did not appear to affect the development of *Anaplasma marginale* in previously infected ticks in two trials, and there was no significant effect of tick exposure to host antibodies on the development of salivary gland infection or transmission of *A. marginale* by ticks (27). Our data indicate that *E. phagocytophila* is affected to some degree by host immune mechanisms and that feeding on immune animals decreases the efficiency of transfadial transmission of *E. phagocytophila* in ticks (Table 1).

Also, acquired immunity against *E. phagocytophila* significantly diminishes the efficiency of transmission of the agent from infected nymphs to cofeeding larvae. Our experiment provides direct evidence for successful transmission of *E. phagocytophila* between simultaneously feeding infected and uninfected ticks on nonimmune hosts (Table 2). This particular route of transmission may play an important part in a persistent circulation of the agent in nature, especially considering the relatively short duration of infectiousness in mice compared to that of *B. burgdorferi*. Therefore, a 90% decrease in the efficiency of cofeeding transmission in immune mice would significantly reduce their reservoir competence for *E. phagocytophila*. Third, mice that had become reinfected with *E. phagocytophila* in spite of acquired immunity remained much less infectious for larval ticks (Table 3).

In our experiment, 12 of 16 immune mice produced infected xenodiagnostic ticks when infested 7 days after the challenge; however only 5 of them tested positive by PCR performed on blood samples collected at the same time. Apparently, PCR did not detect ehrlichial DNA in the blood samples from several immune mice that were still infectious for xenodiagnostic

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**Table 3. *E. phagocytophila* infection in xenodiagnostic ticks that fed upon control and immunized mice 1 week after the challenge**

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of mice with infection/SE</th>
<th>No. of ticks tested</th>
<th>% Infected ticks ± SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4/2: 120</td>
<td>82.5 ± 5.5</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>5/5: 180</td>
<td>6.7 ± 2.1</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>7/10: 300</td>
<td>6.0 ± 2.2</td>
<td></td>
</tr>
</tbody>
</table>

* Mouse groups are as defined in the footnote to Table 1.
ACKNOWLEDGMENTS

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REFERENCES


Whether the decreased infectivity of immune mice is due to low bacteriaemia or to inefficient transstadial transmission in ticks, mice infected with E. phagocytophila a second time are at least 90% less infectious for ticks than are mice infected for the first time. Thus, white-footed mice once exposed to E. phagocytophila, though susceptible to reinfection, have greatly diminished ability to sustain natural transmission of the agent for at least 3 to 4 months. Our field observations in Connecticut have shown that up to 50 to 60% of a population of P. leucopus may carry antibodies against E. phagocytophila during summer (30). Together with the results of the present study, these findings imply that a large proportion of a population of white-footed mice are exposed to E. phagocytophila early in summer during the activity period of nymphal I. scapularis but become reservoir incompetent for this agent by the time of larval activity 2 to 3 months later in the season. Therefore, other host species of I. scapularis are likely to be involved in the maintenance of E. phagocytophila in nature (30).

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