ENZOOTIC TRANSMISSION OF THE AGENT OF HUMAN GRANULOCYTIC EHRlichiosis AMONG COTTONTAIL RABBITS

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Abstract. To determine whether the agent of human granulocytic ehrlichiosis (HGE) (Anaplasma phagocytophilum) may be maintained in a parallel enzootic cycle between cottontail rabbits and their ticks, we sampled these hosts from a zoonotic site during five transmission seasons. Evidence of infection was sought by microscopy and polymerase chain reaction from rabbit blood or splenic tissue, and from ticks collected from rabbits or from vegetation. Approximately 27% of all rabbits sampled contained evidence of active infection, and 66% were seropositive. The vectorial capacity of Ixodes dentatus was demonstrated by xenodiagnosis studies; in addition, 2% of host-seeking nymphs were infected. Haemaphysalis leporispalustris was not a competent vector. Because of their propensity to densely inhabit peridomestic sites, and because I. dentatus may be transported by birds, a parallel cycle of transmission in cottontail rabbits would facilitate introduction and perpetuation of the agent of HGE.

INTRODUCTION

Zoonotic agents are commonly perpetuated in parallel transmission cycles, with primary and secondary reservoir hosts and vectors that may dynamically interact. For example, yellow fever virus in South America is primarily maintained in a sylvatic cycle comprising monkeys and Hemagogus mosquitoes in the jungle, becoming a public health threat when transmitted between humans by Aedes aegypti in urban settings. The sylvatic enzootic cycle serves as a reservoir for the virus, which becomes greatly amplified within the urban anthropoconotic cycle, and which may, in turn, reestablish a sylvatic cycle.1 The zoonotic guild of microbes maintained by northern deer ticks (Ixodes dammini) and white-footed mice (Peromyscus leucopus) in the northeastern United States appears to follow a different pattern of perpetuation, with I. dammini serving as both maintenance and zoonotic vector. Lyme disease spirochetes (Borrelia burgdorferi s.l.) may, however, perpetuate in parallel between cottontail rabbits (Sylvilagus floridanus) and the relatively host-specific, non-human biting I. dentatus.2 In this manner, should mice become scarce for several transmission seasons, as part of a typical demographic pattern,3 spirochetes may be efficiently maintained within rabbits and their ticks. Rabbits serving as hosts to larval I. dammini would thereby help to reestablish the deer tick-mouse cycle by providing infectious deer tick nymphs to feed on mice as they become more abundant.

The agent of human granulocytic ehrlichiosis (HGE), Anaplasma phagocytophilum, appears to be maintained in the same enzootic cycle as that for the agents of Lyme disease and human babesiosis (Babesia microti). Mice are frequently infected, are competent reservoirs, and serve as important hosts for subadult deer ticks.4 However, A. phagocytophilum seems to have as great a vertebrate host range as does B. burgdorferi, infecting mice, deer, sheep, raccoons, skunks, and cattle.5–10 Accordingly, it may also be perpetuated in parallel cycles. Similar to Lyme disease spirochetes, such additional cycles would buffer the agent of HGE against extinction during the years of low mouse density, and may help explain the wide distribution, including Europe and the southern and western United States, of enzootic HGE.11–15

In contrast, B. microti appears to mainly infect small rodents and remains limited as a zoonosis to certain coastal New England and upper Midwestern sites and has not been found in I. scapularis from the south or I. pacificus from California.13,16

It may be that cottontail rabbits help maintain the agent of HGE in a parallel cycle as they do that of Lyme disease. Rabbits are among the most common animals on Nantucket Island, along with mice and deer. Introduced to coastal New England in the early 1900s, the eastern cottontail, Sylvilagus floridanus, has proliferated and displaced the native New England cottontail S. transitionalis.17 Rabbits have great reservoir capacity inasmuch as they are abundant in peridomestic settings, long-lived, and are infected by ticks with great host-specificity. All three stages of I. dentatus, as well as those for Haemaphysalis leporispalustris, feed mainly on rabbits. In addition, infections may be easily bridged to humans or other animals because subadult I. dammini may also feed on rabbits. We described the reservoir capacity of cottontail rabbits for the agent of HGE on Nantucket Island by determining the prevalence of infection in these hosts and the ticks that infest them. In addition, we evaluated the competence of rabbits and rabbit-feeding ticks as hosts for the agent of HGE.

MATERIALS AND METHODS

Sample collection. Cottontail rabbits were shot or live trapped (Tomahawk Live Traps, Tomahawk, WI) on Nantucket Island during April–October from 1998 to 2002; one sampling was undertaken during February 2002. This population of rabbits has previously been identified as S. floridanus.2 All work was performed under a scientific collecting permit issued by the Massachusetts Division of Fisheries and Wildlife. Whole blood was collected from live animals anesthetized with ketamine/xylazine; spleens and whole blood, when possible, were removed from those that were shot. Rabbits were visually inspected for ticks and those that were apparent were removed by gentle traction with forceps. Live rabbits were then caged over water overnight to collect ticks as they
RESULTS

Identification and isolation of HGE in rabbits. We amplified *A. phagocytophilum* DNA from 27% of the rabbits. We sequenced 900 basepairs of the 16S rDNA target from a random sample of our amplicons (GenBank #AY144728), and this sequence was 100% similar to that from HGE patients on the island. Of five HL60 cultures that were inoculated with PCR-positive rabbit blood, all developed inclusions that were indistinguishable from the NCH-1 strain within 10 days of initiating the cultures. One such isolate was subpassaged twice and preserved as a cryostable at −70°C.

Incidence and seroprevalence in rabbits. Transmission of the agent of HGE is intense in cottontail rabbits on Nantucket Island. Each year, except for 1998, at least 20% of our rabbits were demonstrated to have active infections as suggested by the presence of amplifiable DNA (Table 1). In 1998, rabbits were sampled only in May and September, but the wide CI for our estimated prevalence would imply that 1998 did not differ from other years with respect to transmission. The rate of HGE infection, as assessed by PCR, for the one February collection was not significantly different than what we detected the rest of the year (12.5%, 95% CI = 0.3−52). The majority of the rabbits tested each year were seropositive by the IFAT, indicating that they had been exposed to infection. To determine whether the force of HGE transmission differed on a seasonal basis, incidence (as measured by PCR) was stratified by month (Figure 1). Rabbits generally appeared to be actively infected regardless of month.

Infection rates in ticks removed from rabbits. To identify the vector of the rabbit HGE, ticks removed from rabbits were tested in pools of six for evidence of infection by PCR. Three species of ticks were identified from these rabbits: *I. dammini*, *I. dentatus*, and *I. leporispalustris*. To eliminate possible false-positive results due to blood meal contamination, ticks collected from PCR-positive rabbits were not included in this analysis. All three species of ticks showed evidence of infection with HGE by the PCR. (Table 2). The estimated minimum infection rates (MIRs) for all species range from 2% to 6% with overlapping CIs. These estimates are well within the range that we expect to see in host-seeking *I. dammini* in this site field. Interestingly, the known vector of this agent, *I. dammini*, was less frequently infected than were the other two ticks.

Complementary to our molecular data, we also determined the prevalence of infection in female ticks by microscopy. Salivary glands from partially fed ticks were dissected and stained by the Feulgen reaction as described previously. *Ehrlichia* were readily identifiable in the salivary glands of *I.

### Table 1

<table>
<thead>
<tr>
<th>Year</th>
<th>DNA (95% CI)</th>
<th>Serology (95% CI)</th>
<th>No. tested</th>
</tr>
</thead>
<tbody>
<tr>
<td>1998</td>
<td>0 (0–20)</td>
<td>56% (23–83)</td>
<td>17</td>
</tr>
<tr>
<td>1999</td>
<td>46% (26–67)</td>
<td>91% (59–99)</td>
<td>24</td>
</tr>
<tr>
<td>2000</td>
<td>21% (10–37)</td>
<td>45% (27–64)</td>
<td>38</td>
</tr>
<tr>
<td>2001</td>
<td>31% (19–46)</td>
<td>ND</td>
<td>51</td>
</tr>
<tr>
<td>2002</td>
<td>26% (16–38)</td>
<td>ND</td>
<td>73</td>
</tr>
<tr>
<td>Overall</td>
<td>27% (21–33)</td>
<td>66% (56–76)</td>
<td>203</td>
</tr>
</tbody>
</table>

* CI = confidence interval; ND = not determined.
**FIGURE 1.** Incidence of human granulocytic ehrlichiosis in rabbits by month as measured by a polymerase chain reaction. Bars show the mean ± SD.

dentatus and had the same morphology as previously described within *I. dammini*. Salivary glands from *Haemaphysalis*, however, were difficult to score because their acinar cytoplasm does not adequately increase in area during feeding and therefore obscures the microscopic detection of salivarian pathogens. Because *Ehrlichia* do not seem to invade the salivary glands of ticks until after the molt (Telford III SR, unpublished data), we could be certain that any HGE seen in the glands were due to infection from the previous stage and not from the rabbit from which it was removed. Therefore, ticks were analyzed regardless of the infection status of the rabbit. We compared the infection rates of female *I. dentatus* by microscopy to those by the PCR (Table 3). Although infection rates appear to differ between the two methods, these differences are not statistically significant. We conclude that HGE is capable of infecting the salivary glands of *I. dentatus* and that our MIRs of ticks tested by the PCR are not grossly inflated due to contaminating blood meal.

**Infection rates in molted ticks.** We tested the capacity of rabbit-derived ticks to retain infection through the molt. Engorged larvae collected from rabbits were allowed to molt to nymphs, and were then tested for infection in pools of five by the PCR. Approximately 5% of *I. dentatus* and *I. dammini* nymphs collected from PCR-positive rabbits remained infected after the molt (Table 4). In contrast, none of the *H. leporispalustris* remained infected after the molt. A similar proportion of *I. dentatus* (5%) become infected regardless of the PCR status of the rabbit that provided the blood meal. However, none of the *I. dammini* tested from PCR-negative rabbits became infected, suggesting that *I. dentatus* may be a more competent vector.

**Effect of immunity on reservoir competence of rabbits.** To determine whether antibody precluded a circulating rickettsemia, we stratified the rabbits by IFAT titer and determined the mean number of rabbits that were positive by PCR for each titer (Figure 2). Most rabbits that were PCR positive were also seropositive. Furthermore, a robust humoral response, as defined by a high serum antibody titer, did not appear to diminish the probability that infection would be detected by the PCR.

**Prevalence of infection in host-seeking ticks.** Questing nymphal ticks were collected by dragging a cloth over the vegetation and grass in sites where rabbits were observed, and were pooled in groups of five and tested by the PCR. Both *I. dentatus* and *I. dammini* yielded PCR-positive ticks, although at widely different rates. Human granulocytic ehrlichiosis agent was more than five times more prevalent in *I. dammini* than in *I. dentatus* (Table 5). None of the *H. leporispalustris* was positive. We conclude that both *I. dammini* and *I. dentatus* may maintain HGE, whereas *H. leporispalustris* may not.

**DISCUSSION**

Our comprehensive field observations demonstrate that HGE is maintained in a robust enzootic cycle among cotton-tail rabbits on Nantucket Island. The majority of the rabbits sampled over a five-year period showed some evidence of infection. Of 203 tested, 27% had active infection as assessed by PCR, and 66% showed evidence of past infection as assessed by seroreactivity to HGE antigen (Table 1). Transmission over the summer months is consistently high (Figure 1), suggesting that either the rabbits maintain infection for a long period of time or they are constantly reinfected throughout the summer months.

Reservoir competence may be affected by host immunity. Seroconversion of a host often signals the onset of immunity, clearance of organisms from the blood, and the loss of the ability to infect ticks. White-footed mice have been characterized as poorly competent reservoirs of HGE because they seroconvert and seem to lose their infection within a few weeks. We examined this issue with rabbits, specifically asking the question whether rabbits that were seropositive could be actively rickettsemic, i.e., whether rabbits positive by the IFAT were also positive by PCR. Every PCR-positive rabbit (except one) was seropositive, and increasing IFAT titer did not influence the proportion of PCR-positive animals (Figure 2). We conclude that a robust antibody response does not necessarily reduce rabbit reservoir competence, assuming that the presence of amplifiable DNA within peripheral blood

**TABLE 2**

Minimum infection rate of ticks collected from rabbits*

<table>
<thead>
<tr>
<th>Species</th>
<th>Nymphs</th>
<th>Males</th>
<th>Females</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>I. dentatus</em></td>
<td>6% (0–27)</td>
<td>4% (1–9)</td>
<td>5% (2–10)</td>
</tr>
<tr>
<td><em>I. dammini</em></td>
<td>2% (0–8)</td>
<td>–</td>
<td>0 (0–10)</td>
</tr>
<tr>
<td><em>H. leporispalustris</em></td>
<td>2% (0–8)</td>
<td>0 (0–46)</td>
<td>6% (0–27)</td>
</tr>
</tbody>
</table>

* Tick pools from polymerase chain reaction–positive rabbits are excluded from the analysis. CI = confidence interval; *I. = Ixodes; *H. = Haemaphysalis.*
I. dammini becomes competent under laboratory conditions, the sum of our observations allow us to conclude that competence is classically demonstrated under laboratory conditions, as well as transmitting infection to people. Although vector competence is classically demonstrated under laboratory conditions, the sum of our observations allow us to conclude that I. dammini is a competent vector for HGE, but that H. leporispalustris is not. We were able to identify HGE in the salivary glands of female I. dentatus, implying the capacity to transmit during feeding. The agent of HGE was efficiently maintained through the molt of larvae to nymphs, demonstrating transstadial transmission. Approximately 5% of I. dentatus and I. dammini nymphs collected from PCR-positive rabbits remained infected after the molt (Table 4), but none of the H. leporispalustris did so. This suggests that although Haemaphysalis were exposed to infectious organisms, they seem to be incompetent vectors for HGE.

Because ehrlichiae do not seem to be maintained by transovarial transmission, analysis of nymphs derived from larvae engorging on field-collected rabbits provides a xenodiagnosis, which is the definitive assay for reservoir competence. A similar proportion of I. dentatus (5%) became infected regardless of the PCR status of the rabbit that provided the blood meal, suggesting that xenodiagnosis is more sensitive than the PCR. Xenodiagnosis, of course, is well documented as a sensitive method for detecting Lyme disease spirochetes. It may be that ehrlichiae concentrate within neutrophils infiltrating the site of tick feeding, and that peripheral blood neutrophils are less likely to be infected. Interestingly, xenodiagnosis on rabbits demonstrated that I. dammini is less competent than is I. dentatus. Perhaps rabbits serve as relatively poor hosts for deer ticks, thereby providing diminished blood meal quality that may affect competence. Alternatively, rabbit-infecting ehrlichiae may be specifically adapted to rabbits and tick rabbit. Recently, unique 16S rDNA sequence variants from an agent of HGE that do not appear to be associated with human disease have been described from Rhode Island. The limited number of amplification products that we sequenced, however, demonstrates that the rabbit-infecting agent does not differ from that of NCH-1, the index case of HGE in the northeastern United States. Furthermore, blood from field-collected rabbits efficiently infects laboratory mice (a sensitive model for HGE infection) with the agent of HGE, producing infections that do not appear to differ morphologically or behaviorally from those initiated from white-footed mouse or infected human blood.

**TABLE 4**

<table>
<thead>
<tr>
<th>Species</th>
<th>Rabbit PCR</th>
<th>MIR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. dentatus</td>
<td>–</td>
<td>5.1% (3.1–8.0)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5.6% (1.8–12.5)</td>
</tr>
<tr>
<td>H. leporispalustris</td>
<td>–</td>
<td>0 (0–9.2)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0 (0–24.7)</td>
</tr>
<tr>
<td>I. dammini</td>
<td>–</td>
<td>0 (0–16.8)</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>5% (0.1–25)</td>
</tr>
</tbody>
</table>

* MIR = minimum infection rate; CI = confidence interval; I = Ixodes; H = Haemaphysalis.

**TABLE 5**

<table>
<thead>
<tr>
<th>Species</th>
<th>MIR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I. dentatus</td>
<td>1.7% (0.5–4.2)</td>
</tr>
<tr>
<td>H. leporispalustris</td>
<td>0 (0–8.8)</td>
</tr>
<tr>
<td>I. dammini</td>
<td>10.9% (4–22)</td>
</tr>
</tbody>
</table>

* MIR = minimum infection rate; CI = confidence interval; I = Ixodes; H = Haemaphysalis.

Because ehrlichiae do not seem to be maintained by transovarial transmission, analysis of nymphs derived from larvae engorging on field-collected rabbits provides a xenodiagnosis, which is the definitive assay for reservoir competence. A similar proportion of I. dentatus (5%) became infected regardless of the PCR status of the rabbit that provided the blood meal, suggesting that xenodiagnosis is more sensitive than the PCR. Xenodiagnosis, of course, is well documented as a sensitive method for detecting Lyme disease spirochetes. It may be that ehrlichiae concentrate within neutrophils infiltrating the site of tick feeding, and that peripheral blood neutrophils are less likely to be infected. Interestingly, xenodiagnosis on rabbits demonstrated that I. dammini is less competent than is I. dentatus. Perhaps rabbits serve as relatively poor hosts for deer ticks, thereby providing diminished blood meal quality that may affect competence. Alternatively, rabbit-infecting ehrlichiae may be specifically adapted to rabbits and tick rabbit. Recently, unique 16S rDNA sequence variants from an agent of HGE that do not appear to be associated with human disease have been described from Rhode Island. The limited number of amplification products that we sequenced, however, demonstrates that the rabbit-infecting agent does not differ from that of NCH-1, the index case of HGE in the northeastern United States. Furthermore, blood from field-collected rabbits efficiently infects laboratory mice (a sensitive model for HGE infection) with the agent of HGE, producing infections that do not appear to differ morphologically or behaviorally from those initiated from white-footed mouse or infected human blood.

Whether this rabbit-maintained enzootic cycle of HGE contributes to human risk is not known. Often, multiple cryptic cycles exist in nature that help buffer the organism from local extinction, but may not directly contribute to zoonotic risk. Subadult I. dammini may serve as the bridge between the rabbit cycle and the mouse cycle. Indeed, more I. dammini are found on rabbits in years in which the local mouse population is at a cyclic nadir (Goethert HK, unpublished data). Accordingly, during such times interventions focused solely on mice would be relatively ineffective.

The rabbit-feeding I. dentatus is not thought to frequently bite humans, although a recent study of Lyme disease risk in a Maryland community found that one in four Ixodes that were submitted for identification by tick-bitten residents were I. dentatus. Perhaps I. dentatus attack people more often than is assumed. To quantify the risk associated with I. dentatus, we determined entomologic inoculation rates (EIR) for I. dammini as well as I. dentatus within our study site. Infected I. dammini were five times more prevalent than I. dentatus (Table 5). This was unexpected because our data suggested that I. dentatus seem to become infected more readily than I. dammini (Table 4). Perhaps HGE infection is less stable within I. dentatus than it is in I. dammini. Alternatively, our estimates may be a result of the sampling method. Because I. dentatus are much more patchily distributed than I. dammini, it may be that our low EIR estimate for I. dentatus derives from the collection of patches of negative ticks averaged with patches of positive ticks. Regardless, the presence of I. dentatus may have implications for risk because their hosts tend to
to accumulate around peoples’ lawns, and the ticks themselves are readily found on lawns, unlike I. dammini.

Finally, the competence of I. dentatus for the agent of HGE has implications for the potential distribution of this pathogen. Immature I. dentatus are known to feed on birds. As with Lyme disease spirochetes, the agent of HGE could easily be transported to start new foci of disease. Cottontail rabbits are common throughout much of the eastern and central United States, and therefore the probability that a founder infected tick will find a suitable host seems great. In addition, HGE risk may be apparent in areas where I. dentatus is present but I. dammini is not.

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