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Acquisition of Coinfection and Simultaneous Transmission of *Borrelia burgdorferi* and *Ehrlichia phagocytophila* by *Ixodes scapularis* Ticks

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The agents of Lyme disease (*Borrelia burgdorferi*) and human granulocytic ehrlichiosis (*Ehrlichia phagocytophila*) are both transmitted by the tick *Ixodes scapularis*. In nature, ticks are often infected with both agents simultaneously. We studied whether previous infection with either *Borrelia* or *Ehrlichia* in ticks would affect acquisition and transmission of a second pathogen. *Ehrlichia*-infected *I. scapularis* nymphs were fed upon *Borrelia*-infected mice, and *Borrelia*-infected *I. scapularis* nymphs were fed upon *Ehrlichia*-infected mice. The efficiency with which previously infected nymphal ticks acquired a second pathogen from infected hosts was compared to that of uninfected ticks. An average of 51% ± 15% of ticks acquired *Ehrlichia* from infected mice regardless of their prior infection status with *Borrelia*. An average of 85% ± 10% of ticks acquired *Borrelia* from infected mice regardless of their prior infection status with *Ehrlichia*. Also, we assessed the efficiency with which individual nymphs could transmit either agent alone, or both agents simultaneously, to individual susceptible hosts. An average of 76% ± 9% of *Borrelia*-infected ticks and 84% ± 10% of *Ehrlichia*-infected ticks transmitted these agents to mice regardless of the presence of the other pathogen. There was no evidence of interaction between the agents of Lyme disease and human granulocytic ehrlichiosis in *I. scapularis* ticks. The presence of either agent in the ticks did not affect acquisition of the other agent from an infected host. Transmission of the agents of Lyme disease and human granulocytic ehrlichiosis by individual ticks was equally efficient and independent. Dually infected ticks transmitted each pathogen to susceptible hosts as efficiently as ticks infected with only one pathogen.

The black-legged tick *Ixodes scapularis* is a vector of *Borrelia burgdorferi*, the etiologic agent of Lyme disease, as well as *Ehrlichia phagocytophila*, the etiologic agent of human granulocytic ehrlichiosis (HGE) (2, 4, 31, 32). In nature, nymphal and adult ticks are often infected with both agents simultaneously (3, 16, 29, 33). Nymphal or adult ticks can acquire both pathogens simultaneously from a single coinfected host during either larval or nymphal feeding (M. Levin, unpublished data). Alternatively, adult ticks may have acquired pathogens consecutively—one during larval feeding and a second during nymphal feeding. In nature, the prevalence of either pathogen in ticks increases significantly from the nymphal to the adult stage, and consequently, the prevalence of coinfection in questing adult ticks can be 7 to 10 times higher than in nymphs (16). This observation suggests that consecutive acquisition of different pathogens by individual ticks may happen more frequently than simultaneous acquisition.

Simultaneous infection with these two agents has also been documented in humans and rodents (16, 18–20). Infection with both agents may result from the bite of a single coinfected tick. However, there is no experimental evidence for simultaneous transmission of *Ehrlichia* and *Borrelia* by individual ticks. Moreover, the efficiency with which infected ticks can transmit either *E. phagocytophila* or *B. burgdorferi* to susceptible hosts has not been studied in detail. A recent study found that some laboratory mice fed upon by small numbers of *I. scapularis* ticks infected with *E. phagocytophila* failed to acquire infection, suggesting that *E. phagocytophila* may be transmitted less efficiently than *B. burgdorferi* (4). However, all transmission studies of *B. burgdorferi* published to date also involve groups of infected ticks (27, 28), and it is therefore not known if all individual ticks are capable of *Borrelia* transmission. Evidence from studies of tick-borne encephalitis suggests that the efficiency of transmission by a population of infected *Ixodes persulcatus* ticks is considerably less than 100% (10).

We questioned whether previous infection with either *B. burgdorferi* or *E. phagocytophila* in ticks would affect acquisition and/or transmission of a second pathogen. In order to determine this, we measured the efficiency with which previously infected nymphal ticks acquired a second pathogen from infected hosts and compared it to the efficiency of acquisition by uninfected ticks. We also measured the efficiency with which individual nymphs could transmit either agent alone or both agents simultaneously to individual susceptible hosts.

MATERIALS AND METHODS

The white-footed mouse (*Peromyscus leucopus*) is known to be a major reservoir for *B. burgdorferi*. It also has been shown to be susceptible to infection with *E. phagocytophila* (5, 16, 21, 32). Therefore, we used white-footed mice as hosts in our experiments. Two-month-old mice were derived from a specific-pathogen-free *P. leucopus* colony maintained in our laboratory. The maintenance and care of experimental animals complied with the National Institutes of Health guidelines for the humane use of laboratory animals. The mice were not exposed to ticks or pathogens prior to the experiments.

Infected *I. scapularis* nymphs were produced by allowing larval ticks to feed upon white-footed mice previously infected with either *B. burgdorferi* or *E. phagocytophila*. Both agents originated from nymphal ticks collected in Westchester County (N.Y.) and were maintained separately in a laboratory tick-mouse cycle. The identities of the agents had been previously confirmed by indirect immunofluorescence assay and by DNA sequencing of amplified PCR products (4, 14).

Infection with *B. burgdorferi* and *E. phagocytophila* in ticks and mice was determined by PCR. For PCR, individual nymphal or adult ticks or pools of engorged larvae were placed in sterile 1.5-cm^3 plastic vials, deep frozen in liquid nitrogen, ground with a sterile plastic pestle, and resuspended in 100 μl of

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Tris-borate buffer. DNA was extracted from the ticks with an IsoQuick nucleic acid extraction kit (ORCA Research Inc., Bothell, Wash.) to maximize sensitivity (30). Briefly, guanidine thiocyanate, a proprietary extraction matrix, and sodium dodecyl sulfate solution were added to a suspension, and the mixture was incubated at 65°C for 10 min. After separation of phases by centrifugation, the DNA was precipitated with sodium acetate and isopropanol and washed with 70% ethanol. The final DNA pellet was resuspended in 50 μl of RNase-free water, and 1 2.5-μl aliquot was used for each PCR test. Primers EHR521 (5′-TGT AGO CGG TTC GOT AAG TTA AAG-3′) and EHR747 (5′-GCA CTC ATC GTT TAC AGC GTG-3′) were used to amplify a 247-bp fragment of 16S ribosomal DNA from *E. phagocytophila* (24). Primers FLA297 (5′-CGG CAC ATA TTC AGA TGC AGA CAG-3′) and FLA652 (5′-CCT GTT GAA CAC CCT CTT GAA CC-3′), based on the published nucleotide sequence (6), were used to amplify a 378-bp fragment of the flagellin gene of *B. burgdorferi*. The amplification products were visualized in 2% agarose gels.

Acquisition experiment. Ten mice were each infected with *Borrelia* by allowing 10 *I. scapularis* nymphs from a *B. burgdorferi*-infected cohort to feed on them. Another 10 mice were each similarly infected with *Ehrlichia* by allowing 10 nymphs from an *E. phagocytophila*-infected cohort to feed on them. The infection in the nymphal cohorts prior to the investigation was assessed by testing representative samples of 25 ticks. The prevalence of infection in the *Borrelia*-infected cohort was 44.0% ± 10.1%, and the prevalence of infection in the *Ehrlichia*-infected cohort was 40.0% ± 10.0%.

Two weeks later, 25 nymphs from the *B. burgdorferi*-infected cohort were placed on each of five mice previously infected with *Ehrlichia*. The other five *Ehrlichia*-infected mice were each fed upon by 25 uninfected nymphs. Similarly, five *B. burgdorferi*-infected mice were infested with 25 nymphs from the *Ehrlichia*-infected cohort, and 25 uninfected nymphs fed upon the other five *B. burgdorferi*-infected mice. The engorged nymphs were collected and kept at 22°C and 98% relative humidity until they molted. Freshly moiled adult ticks were individually tested by PCR for infection.

Transmission experiment. Single-infected and coinfected nymphs were produced by allowing larval ticks to feed upon white-footed mice singly or simultaneously infected with *B. burgdorferi* and *E. phagocytophila* in the course of the previous experiment. These nymphs were placed individually on single naive mice and allowed to feed to repletion. The resulting engorged nymphs were collected and individually tested by PCR for infection.

Two weeks after the feeding by infected nymphs, the mice were infested with uninfected larval ticks for xenodiagnosis. The infection status of individual mice was assessed using 20 engorged xenodiagnostic larvae per mouse (four pools of five ticks). The tick pools were tested for both pathogens by PCR. Our previous study had shown that feeding density influences the acquisition of *B. burgdorferi* in larval *I. scapularis* (15). Therefore, the mice were infested with a large number of larvae (approximately 200) in order to maximize the sensitivity of xenodiagnosis. The xenodiagnostic larvae were derived from a colony of *I. scapularis* maintained in our laboratory by allowing them to feed on uninfected mice and rabbits for several generations. Representative samples of ticks from the colony are regularly tested to ensure that the colony is free of both tick-borne pathogens. Xenodiagnosis was performed only on mice from which individual replete nymphs that tested positive for either pathogen were collected.

Differences in prevalence of infection were analyzed using χ^2 and analysis of variance (ANOVA) statistics.

RESULTS AND DISCUSSION

Acquisition experiment. An average of 19 (12 to 23) nymphal ticks fed to repletion on each of the 20 infected mice and were tested for both agents as adults. When nymphs from the *Borrelia*-infected cohort fed upon five mice infected with *Ehrlichia*, 39 of the resulting adult ticks tested PCR positive and 47 tested PCR negative for *B. burgdorferi* (Table 1). The prevalence of *Borrelia* infection in adult ticks (45.3% ± 10.6%) did not differ from that in the same cohort of nymphs tested prior to feeding (44.0% ± 19.9%). When nymphs from the *Ehrlichia*-infected cohort fed upon five mice infected with *B. burgdorferi*, 48 of the resulting adult ticks tested PCR positive and 47 tested PCR negative for *Ehrlichia* (Table 2). Again, the difference in *Ehrlichia* infection between nymphal ticks prior to feeding (40.0% ± 19.6%) and the resulting adult ticks (50.5% ± 10.1%) was not statistically significant.

Nymphs may be able to acquire pathogens not only from an infectious host but also from infected ticks during cofeeding (7, 22, 23, 25). However, transmission by cofeeding did not increase the prevalence of either *B. burgdorferi* or *Ehrlichia* in our experiment. Therefore, we assume that the ticks which tested positive for *B. burgdorferi* after feeding on mice (Table 1) were infected with *B. burgdorferi* prior to feeding. The same assumption applies to the adult ticks that tested positive for *Ehrlichia* (Table 2).

TABLE 1. Acquisition of *E. phagocytophila* by ticks infected with *B. burgdorferi* and by uninfected ticks

Mouse	No. of ticks infected with *B. burgdorferi*		No. of uninfected ticks		$P_{\chi}{}^{2a}$
	Tested	Acquiring *E. phagocytophila* (%)	Tested	Acquiring *E. phagocytophila* (%)	
PI-387	11	6 (54.5)	9	4 (44.4)	0.65
PI-388	6	3 (50.0)	6	3 (50.0)	1.00
PI-389	8	5 (62.5)	12	8 (75.0)	0.85
PI-390	7	4 (57.1)	7	3 (42.9)	0.59
PI-391	7	2 (28.6)	13	5 (38.5)	0.67
Total	39	20 (51.3 ± 15.9)[b]	47	24 (51.1 ± 14.4)[b]	0.98

[a] P_{χ^2}, probability for a chi-square test distribution.
[b] ±95% confidence interval.

A total of 44 ticks from the *Borrelia*-infected cohort acquired *Ehrlichia* during feeding upon five infected mice (Table 1). The efficiency of *Ehrlichia* acquisition by nymphal ticks from the same cohort varied among individual mice but did not differ between ticks that were or were not previously infected with *B. burgdorferi* ($P_{\chi^2} = 0.98$). On the average, approximately 50% of nymphs from the *Borrelia*-infected cohort acquired *Ehrlichia* from infected mice regardless of their prior infection status with *B. burgdorferi* (Table 1).

When a cohort of exclusively uninfected nymphs fed upon the second group of five mice infected with *Ehrlichia*, a total of 43 of 96 resulting adult ticks (44.8% ± 10.0%) acquired the infection. Individual mice transmitted *Ehrlichia* to 30.0 to 60.1% of feeding ticks. The difference in acquisition of *Ehrlichia* by a cohort of *B. burgdorferi*-infected nymphs and a cohort of uninfected nymphs was not statistically significant ($P_{\mathrm{ANOVA}} = 0.22$).

A total of 81 ticks from the *Ehrlichia*-infected cohort acquired *Borrelia* during feeding upon five infected mice (Table 2). The efficiency of *B. burgdorferi* acquisition by nymphal ticks from the same cohort varied among individual mice but did not differ between ticks that were or were not previously infected with *Ehrlichia* ($P_{\chi^2} = 0.97$). An average of 85.3% ± 7.2% of nymphs from the *Ehrlichia*-infected cohort acquired *B. burgdorferi* from infected mice regardless of their prior infection status with *Ehrlichia* (Table 2).

When a cohort of exclusively uninfected nymphs fed upon an

TABLE 2. Acquisition of *B. burgdorferi* by ticks infected with *E. phagocytophila* and by uninfected ticks

Mouse	No. of ticks infected with *E. phagocytophila*		No. of uninfected ticks		$P_{\chi}{}^{2a}$
	Tested	Acquiring *B. burgdorferi* (%)	Tested	Acquiring *B. burgdorferi* (%)	
PI-397	6	4 (66.7)	14	10 (71.4)	0.83
PI-398	8	7 (87.5)	9	7 (77.8)	0.60
PI-399	11	11 (100.0)	7	7 (100.0)	1.00
PI-400	12	11 (91.7)	8	8 (100.0)	0.40
PI-401	11	8 (72.7)	9	8 (88.9)	0.39
Total	48	41 (85.4 ± 10.0)[b]	47	40 (85.1 ± 10.3)[b]	0.97

[a] P_{χ^2}, probability for a chi-square test distribution.
[b] ±95% confidence interval.

TABLE 3. Transmission of *B. burgdorferi* and *E. phagocytophila* to mice by individual *I. scapularis* nymphs

Infection in nymphs	No. of ticks fed	Transmitting *B. burgdorferi*[a]	Transmitting *E. phagocytophila*[a]
		No. (%)	No. (%)
B. burgdorferi only	38	31 (81.6 ± 12.5)	
B. burgdorferi and *E. phagocytophila*	30	21 (70.0 ± 16.7)	25 (83.3 ± 13.6)
E. phagocytophila only	21		18 (85.7 ± 15.3)

[a] ± 95% confidence interval.

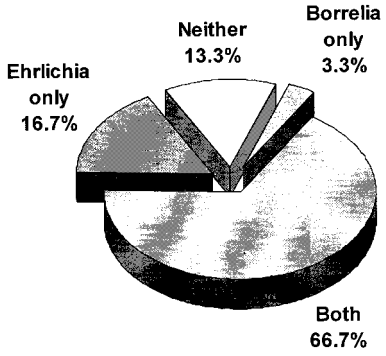

FIG. 1. Transmission of *B. burgdorferi* and *E. phagocytophila* by individual *I. scapularis* nymphs simultaneously infected with both pathogens.

additional five mice infected with *B. burgdorferi*, a total of 88 of 105 resulting adult ticks (83.8% ± 7.1%) acquired the infection. Individual mice transmitted *B. burgdorferi* to 73.9 to 90.5% of feeding ticks. The difference in acquisition of *B. burgdorferi* by a cohort of *Ehrlichia*-infected nymphs and a cohort of uninfected nymphs was not statistically significant ($P_{ANOVA} = 0.37$).

Thus, previous infection with *B. burgdorferi* or *E. phagocytophila* in nymphal *I. scapularis* did not affect the ability of the ticks to acquire a second pathogen from infected hosts.

Transmission experiment. A total of 98 mice were successfully fed upon by individual nymphal ticks. Of those 98 nymphs, 89 were infected with either *B. burgdorferi* or *E. phagocytophila* or both as detected by PCR performed on the engorged ticks (Table 3). Xenodiagnostic results showed that 31 of 38 (81.6%) ticks infected with only *B. burgdorferi* transmitted the spirochete to mice compared to 70% (21 of 30) transmission success when ticks were simultaneously infected with both *Borrelia* and *Ehrlichia* (Table 3). This difference between the two groups of ticks was not statistically significant ($P_{\chi^2} = 0.27$). When ticks were infected with *Ehrlichia* only, 18 of 21 (85.7%) transmitted it to susceptible mice, as determined by xenodiagnosis (Table 3). Of 30 dually infected ticks, 18 (83.3%) transmitted *Ehrlichia*. Thus, there also was no difference in the efficiency of transmission of *E. phagocytophila* between ticks infected with one or both pathogens ($P_{\chi^2} = 0.82$). The efficiency of transmission did not differ significantly between *B. burgdorferi* and *Ehrlichia* either in ticks infected with one pathogen ($P_{\chi^2} = 0.69$) or in dually infected ticks ($P_{\chi^2} = 0.22$).

In another study of pathogen transmission by individual *I. scapularis* ticks, six of seven nymphs that fed to repletion transmitted *B. burgdorferi* to hamsters (26). However, the ticks themselves were not examined, and it was not known whether the nontransmitting ticks were infected. Our data show that only 70 to 81% of infected *I. scapularis* nymphs transmit *B. burgdorferi* to susceptible hosts even when fed to repletion. The efficiency of transmission of *E. phagocytophila* by infected ticks is 83 to 86% and is not significantly different from that of *B. burgdorferi*. The differential infectivity of ticks is likely to be related to the variability of pathogen concentration among infected ticks (1, 11–13, 17). This has been shown to occur in ticks transmitting spring-summer tick-borne encephalitis virus (8, 9).

Of 30 ticks infected with *Borrelia* and *Ehrlichia* simultaneously, 20 successfully transmitted both pathogens while 4 failed to transmit either (Fig. 1). Our results suggest that transmission of the agents of Lyme disease and HGE by individual ticks is equally efficient and independent. Simultaneous infection with the agents of Lyme disease and HGE has been observed both in human patients and in wild animals (16, 18–20). Mixed infections in hosts may originate either from the bite of a single tick infected with two pathogens or from multiple bites of singly infected ticks. Simultaneous transmission of *B. burgdorferi* and *Babesia microti* by individual *I. scapularis* nymphs has been previously reported (26). The present study provides evidence that dually infected ticks are capable of simultaneous transmission of *B. burgdorferi* and *E. phagocytophila* and that infection of ticks with one of these pathogens does not interfere with transmission of the other.

There was no evidence of interaction between the agents of Lyme disease and HGE in *I. scapularis*. The presence of either agent in ticks did not interfere with acquisition of the other agent from an infected host. Transmission of the agents of Lyme disease and HGE by individual ticks was equally efficient and independent. Dually infected ticks transmitted each pathogen to susceptible hosts as efficiently as ticks infected with only one pathogen, and most dually infected ticks were able to transmit both pathogens to a susceptible host.

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