Published in Vector Borne Zoonotic Diseases 2, issue 1, 3-9, 2002
which should be used for any reference to this work

Investigations on the Mode and Dynamics of Transmission and Infectivity of *Borrelia burgdorferi* Sensu Stricto and *Borrelia afzelii* in *Ixodes ricinus* Ticks

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ABSTRACT

***Borrelia burgdorferi* sensu lato (sl), the agent of Lyme disease, is transmitted to the host during the blood meal of *Ixodes* ticks. In most unfed ticks, spirochetes are present in the midgut and migrate during blood feeding to the salivary glands, from which they are transmitted to the host via saliva. In the present study, the efficiency of *Ixodes ricinus* ticks to transmit *B. afzelii* and *B. burgdorferi* sensu stricto (ss) and their infectivity for mice were examined in relation to the duration of the blood meal. In addition, we investigated whether these two *Borrelia* species can penetrate intact skin. Three modes of infection of mice were studied: tick-bite infection, inoculation of tick homogenates, and transcutaneous infection by topical application of tick homogenates on mouse skin. Transmission of *B. burgdorferi* sl from *I. ricinus* nymphs to mouse increased with duration of tick attachment. *B. afzelii*-infected ticks start to transmit infection earlier (≤48 h) than *B. burgdorferi* ss-infected ticks. As previously shown for *B. burgdorferi* ss in *Ixodes scapularis*, *B. burgdorferi* ss and *B. afzelii* in unfed *I. ricinus* were noninfectious for mice when tick homogenates were inoculated. However, the inoculation of homogenates of ticks fed for 24 h readily produced infection in mice. Therefore, *B. burgdorferi* ss and *B. afzelii* spirochetes are potentially infectious in the tick before natural transmission can occur. None of the mice (n = 33) became infected by transcutaneous transmission when tick homogenates were applied on mouse skin, showing that *B. burgdorferi* ss and *B. afzelii* are unable to penetrate intact skin, in contrast to relapsing fever spirochetes. This study also shows that *B. afzelii* is transmitted by *I. ricinus* to the host earlier than *B. burgdorferi* ss and that *I. ricinus* seems to be a more efficient vector of *B. afzelii* than *B. burgdorferi* ss.**

Key Words: Transmission—*Ixodes ricinus*—*Borrelia burgdorferi* sensu lato—Lyme borreliosis.

INTRODUCTION

BORRELIA BURGDORFERI sensu lato (sl), the agent of Lyme disease, is transmitted to the host via infected saliva during the blood meal of *Ixodes* ticks. In most unfed ticks spirochetes are present in the midgut and migrate during blood feeding to the salivary glands from which they are transmitted to the host via saliva (Ribeiro et al. 1987, Zung et al. 1989, Gern et al. 1990, 1996, de Silva and Fikrig 1995, Schwan and Piesman 2000).

Borrelia transmission does not occur at the beginning of the blood uptake but later. In fact, the transmission efficiency increases with the duration of the blood meal, as described for the North American vector *Ixodes scapularis* infected with *B. burgdorferi* sensu stricto (ss). Nymphal ticks must be attached to the host for at least 24 h before transmission of *B. burgdor-*

feri ss starts, and a high level of transmission is reached after ≥48 h of attachment (Piesman et al. 1987, Piesman 1993, des Vignes et al. 2001, Ohnishi et al. 2001). Similarly, the transmission efficiency increases in relation to the duration of the blood meal in the European vector *Ixodes ricinus* (Kahl et al. 1998). However, an early transmission with high efficiency was described for *I. ricinus*: 50% of animals were infected by *B. burgdorferi* sl after 16.7 h of tick attachment (Kahl et al. 1998). The observations of high infection rates in salivary glands of unfed *I. ricinus* collected in Switzerland suggested that systemically infected ticks can transmit *Borrelia* early after attachment to hosts (Lebet and Gern 1994, Leuba-Garcia et al. 1994). In contrast, systemic infections are rare in *I. scapularis* (Burgdorfer and Hayes 1989). All these observations seem to indicate that the transmission dynamics of the various *Borrelia* species might be different in *I. ricinus* compared with *B. burgdorferi* ss in *I. scapularis*.

Not only the efficiency of *Borrelia* transmission but also the *Borrelia* infectivity are influenced by the tick blood meal (Piesman 1993, Ohnishi et al. 2001). Piesman (1993) reported that *Borrelia* spirochetes in the midgut of unfed ticks are noninfectious. Host infection could not occur when unfed *I. scapularis* nymphs infected with *B. burgdorferi* ss (JD1 strain) were homogenized and injected intradermally into mice. Host infection was detected only if the inoculated ground nymphs had previously been attached to a host for at least 24 h. It is not known if other pathogenic *Borrelia* species infecting *I. ricinus* are also noninfectious for the vertebrate host before the beginning of blood feeding.

Among the genus *Borrelia*, additional modes of transmission than the salivary route have been described. *Borrelia duttoni*, the agent of African relapsing fever, is transmitted via saliva but also via coxal fluid by the soft tick *Ornithodoros moubata*, and *Borrelia recurrentis*, the agent of louse-borne relapsing fever, is transmitted by contamination of the bite wound with infectious hemolymph of *Pediculus humanus humanus* lice (Felsenfeld 1971). Moreover, in addition to these modes of transmission, *B. duttoni* and *B. recurrentis* are able to establish an infection in a host by active passage through intact skin (Burgdorfer 1976). Today, it remains unknown if *B. burgdorferi* sl is able to penetrate intact skin. If so, the removal of ticks by naked hands could represent a risk of *Borrelia* transmission to humans.

In the present study, the transmission efficiency and the infectivity of *Borrelia afzelii* and *B. burgdorferi* ss were examined in *I. ricinus* in relation to the duration of the blood meal. In addition, we investigated whether these two *Borrelia* species can penetrate intact skin.

MATERIALS AND METHODS

Mice, Borrelia, *and ticks*

AKR/N mice from a breeding colony maintained at the Institute of Zoology in Neuchâtel (Switzerland) were used in this study.

Four *Borrelia* isolates were used: *B. burgdorferi* ss ZS7 isolated from a female tick collected in the Freiburg (Germany) area (Schaible et al. 1989), *B. burgdorferi* ss NE1849 isolated from a female tick collected in the Neuchâtel (Switzerland) area, *B. afzelii* NE496 obtained from the midgut of a free-living *I. ricinus* adult collected in Aarberg (Switzerland) (Leuba-Garcia et al. 1998), and *B. afzelii* NE2963 isolated from a biopsy of a BALB/c mouse infected by *I. ricinus* nymphs collected in Neuchâtel. Isolates were typed with a restriction fragment length polymorphism on the polymerase chain reaction (PCR) product according to Postic et al. (1994). Briefly, 4 mL of culture medium was centrifuged and washed two times, and then the pellet was suspended in 50 μL of ultrafiltered water. After incubation at 100°C for 10 min the thermolysates were stored at −20°C until use for PCR. Primers used to amplify the variable spacer region between two repeated genes encoding for ribosomal 23S and 5S were as follows: primer 1 (5′-CTGCGAGTTCGCGGGAGA-3′) and primer 2 (5′-TCCTAGGCATTCACCATA-3′). Ten microliters of each sample was added to 40 μL of PCR mix containing 23.7 μL of H_2O (nanopure), 5 μL of 10 × Taq buffer, 5 μL of primer 1 (5 pmol), 5 μL of primer 2 (5 pmol), 1 μL of deoxynucleotide triphosphates (200 μM), and 0.3 μL of *Taq* polymerase (1.5 U). Each amplification reaction was carried out for 35 cycles. Denaturation was per-

formed for 1 min at 95°C, annealing at 50°C for 1 min, and extension at 72°C for 1 min. PCR products were electrophoresed in a 1% agarose gel and stained with ethidium bromide. PCR products were first digested with *Mse*I restriction endonuclease for 2 h at 37°C and then electrophoresed in a 16% acrylamide gel for 90 min at 120 V. Digested DNA was stained with ethidium bromide.

To obtain infected nymphs, larvae from a laboratory colony free of *B. burgdorferi* sl and maintained at the Institute of Zoology of Neuchâtel were allowed to feed on mice infected with either isolates (NE1849 and ZS7) of *B. burgdorferi* ss or isolates (NE2963 and NE496) of *B. afzelii*. Replete larvae were maintained at room temperature and 95% relative humidity until they molted. The infection rates in nymphs were evaluated using immunofluorescence, as previously described (Gern et al. 1997b). Tick infection rates reached 70% for *B. burgdorferi* ss ZS7, 60% for *B. burgdorferi* ss NE1849, 80% for *B. afzelii* NE496, and 60% for *B. afzelii* NE2963.

Infection of mice

Three modes of infection of AKR/N mice were considered in this study:

Tick-bite infection. The back of an AKR/N mice was sheared, and a hollow plastic cap (15 mm in diameter) was glued with wax (Mbow et al. 1994). Six infected *I. ricinus* nymphs were placed on each mouse inside the plastic cap and allowed to feed for determined intervals: 24, 48, 72, or 96 h. Ticks collected from each mouse were ground as described below and used to infect additional mice.

Needle inoculation of tick homogenates. Unfed ticks and ticks fed for 24, 48, 72, or 96 h (maximum of five nymphs per mouse) were separately ground in 250 μL of BSK-H medium. These homogenates were used to infect AKR/N mice either by needle inoculation or by transcutaneous infection after topical application on intact skin.

For needle infection, each AKR/N mouse was inoculated intradermally with 60 μL of tick homogenate.

Transcutaneous infection. To test transcutaneous infection, the back of a mouse was sheared, and 60 μL of the infected tick homogenate was applied on the intact skin for ~15 min. Mice were exposed to homogenates of ticks infected by *B. burgdorferi* ss ZS7, *B. burgdorferi* ss NE1849, *B. afzelii* NE496, and *B. afzelii* NE2963; only unfed ticks and ticks fed for 48 and 96 h were used.

Before any infection, mice were anesthetized by intramuscular injection of 0.03 mL of a mixture of 0.5:0.8 of Narketan® (Chassot AG, Bern, Switzerland) and Xylasol® (Gräub AG, Bern), respectively.

To examine *Borrelia* infection in mice, ear biopsies were taken at days 30 and 60 after infection. Ear biopsies were obtained from anesthetized mice using little scissors after cleaning the skin with 70% ethanol. Skin samples were placed into 4-mL tubes containing BSK-H-supplemented medium, according to Sinsky and Piesman (1989), and incubated at 34°C to allow isolation of *B. burgdorferi* ss and *B. afzelii*. Cultures were screened for the presence of spirochetes by dark-field microscopy after 10 days and 4 weeks of incubation.

BSK medium containing ear biopsies was screened using the PCR protocol described by Postic et al. (1994) to detect *Borrelia* DNA as described above. Mice were considered as infected when spirochetes were isolated from ear biopsies or when *Borrelia* DNA was detected by PCR in culture fluid containing ear biopsies.

Statistics were calculated using the Fisher's exact test with the R program (version 0.90.0) on a Linux machine.

RESULTS

Infection of mice through tick bite

All mice exposed to the bite of *B. burgdorferi* ss-infected ticks for 24 and 48 h ($n = 18$) remained uninfected (Table 1). In contrast, one of seven (14%) and four of eight (50%) mice exposed for the same time periods to *B. afzelii*-infected ticks became infected. The difference of transmission between the two species is statistically significant for ticks attached for ≤48 h [one of 18 and five of 15 (33%), respectively, Fisher's test $p = 0.01$]. The transmission risk in-

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Table 1. Infection Rates of Mice Infected by Tick Bite and by Inoculation of Tick Homogenates

	Infection rate at given feeding interval by genospecies									
	0 h		*24 h*		*48 h*		*72 h*		*96 h*	
	Bb	*Ba*	*Bb*	*Ba*	*Bb*	*Ba*	*Bb*	*Ba*	*Bb*	*Ba*
Naturally infected mice	ND	ND	0/10	1/7	0/8	4/8	2/4	5/5	2/5	2/4
Injected mice	0/7	0/8	6/9	5/9	4/11	10/11	4/4	5/5	7/8	5/7

AKR/N mice were infected by different modes: tick bite or inoculation of tick homogenates. Ticks infected with *B. burgdorferi* or *B. afzelii* were permitted to feed on mice for 24, 48, 72, or 96 h. These ticks as well as unfed infected ticks were subsequently homogenized and injected into other mice. Infection in mice was monitored by cultivation of ear biopsy and PCR amplification.

creased with the duration of tick attachment (Table 1). When mice were exposed to ticks for ≥72 h, four of nine (44%) and seven of nine (78%) mice exposed to *B. burgdorferi* ss- and *B. afzelii*-infected ticks, respectively, acquired infection (Table 1). The difference of transmission between the two species is no more significant for ticks attached for ≥72 h (Fisher's test $p = 0.33$).

Infection of mice through inoculation of tick homogenates

All mice ($n = 15$) inoculated with homogenates of unfed nymphs infected with *B. burgdorferi* ss or *B. afzelii* remained uninfected (Table 1). Among mice inoculated with homogenates of *B. burgdorferi* ss- and *B. afzelii*-infected ticks fed for ≤48 h, 10 of 20 (50%) and 15 of 20 (75%), respectively, became infected. This is not statistically different ($p = 0.19$). Inoculation of homogenates of *B. burgdorferi* ss- and *B. afzelii*-infected ticks fed for 24 h already induced infection in mice: six of nine (67%) and five of nine (56%), respectively. When *B. burgdorferi* ss- and *B. afzelii*-infected ticks fed for ≥72h were homogenized and injected into mice, 11 of 12 (92%) and 10 of 12 (83%) mice, respectively, became infected (Table 1).

Comparison of infection success by tick bite and tick homogenate inoculation during the second part of the blood meal (72 and 96 h), when tick-bite transmission is known to be the highest (Piesman 1993, Kahl et al. 1998, des Vignes et al. 2001, Ohnishi et al. 2001), showed that there is a difference between *B. burgdorferi* ss and *B. afzelii*. Success of transmission of *B. burgdorferi* ss by *I. ricinus* was significantly lower via natural tick bite transmission ($n = 4/9$) than via homogenate inoculation ($n = 11/12$) (Table 1) (Fisher's test $p = 0.046$), and this is not the case for *B. afzelii*. In fact, success of transmission via tick bite ($n = 7/9$) and via homogenate inoculation ($n = 10/12$) was not statistically different for *B. afzelii* (Table 1) (Fisher's test $p = 1$). The same phenomenon was observed for each *B. burgdorferi* ss isolate (NE1849 and ZS7) and for each *B. afzelii* isolate (NE496 and NE2963) (data not shown).

Infection of mice via topical application of tick homogenate

The topical application of homogenates of tick infected with *B. burgdorferi* ss (ZS7, nine mice; NE1849, nine mice) and *B. afzelii* (NE496, nine mice; NE2963, six mice) on the intact skin of mice did not produce any infection in mice, whether ticks were fed or not.

DISCUSSION

It is now well documented that *B. burgdorferi* sl is transmitted via saliva during the blood meal and that dissemination from the midgut to the salivary glands is necessary for efficient transmission to the host (Ribeiro et al. 1987, Zung et al. 1989, Gern et al. 1990, 1996, de Silva et al. 1995, Schwan and Piesman 2000). We investigated the mode and dynamics of transmission and infectivity of two species of *Borrelia* in *I. ricinus*. *B. burgdorferi* ss- and *B. afzelii*-infected ticks were used because ticks infected by these two species are easy to obtain from laboratory infected mice, which is not the

case for *Borrelia garinii* (Fikrig et al. 1995, Gern et al. 1997a, Dolan et al. 1998, Hu et al. 2001). The experiments were carried out with two different isolates of *B. burgdorferi* ss and two of *B. afzelii*.

We observed that the success of transmission of *B. burgdorferi* sl from *I. ricinus* to mice increased with duration of attachment. This confirms previous results with *I. scapularis* (Piesman 1993, des Vignes et al. 2001, Ohnishi et al. 2001) and *I. ricinus* (Kahl et al. 1998). However, we observed an earlier transmission by *I. ricinus* ticks infected by *B. afzelii* than by *B. burgdorferi* ss. In fact, during the first 48 h of attachment, *B. burgdorferi* ss-infected ticks did not infect the 18 exposed mice, whereas *B. afzelii*-infected ticks transmitted infection to 33% of mice (five of 15). So the observations of *B. burgdorferi* ss in *I. ricinus* ticks are similar to what observed for *B. burgdorferi* ss in *I. scapularis* (Piesman 1993, des Vignes et al. 2001, Ohnishi et al. 2001), while *B. afzelii*-infected ticks showed a different dynamic of transmission to the host. Different hypothesis could explain this earlier transmission of B. afzelii. First, we can hypothesize that *B. afzelii*-infected ticks had a systemic infection. In *I. ricinus*, high salivary gland infection rates by *B. burgdorferi* sl have been observed in unfed ticks (Lebet and Gern 1994, Leuba-Garcia et al. 1994), whereas in *I. scapularis*, systemic infections by *B. burgdorferi* ss are rare (Burgdorfer and Hayes 1989). Although *Borrelia* species responsible for systemic infection in *I. ricinus* have never been identified, our results suggest that very invasive *B. afzelii* may be responsible for a more frequent systemic infection in unfed ticks. A second explanation to the different dynamics of transmission observed at the beginning of tick attachment between *B. burgdorferi* ss- and *B. afzelii*-infected ticks might be related to different strategies used by spirochetes once in the host skin. Shih et al. (1992, 1993) and Gern and Rais (1996) reported that *B. burgdorferi* ss remains at the tick bite site and disseminate later. Moreover, Ohnishi et al. (2001) described the presence of *B. burgdorferi* ss in the skin attached to the rostrum of ticks that had been removed from the host. We suggest that when ticks are removed at the beginning of the blood meal, when the number of *B. burgdorferi* ss transmitted by the tick is still low in the host skin (Ohnishi et al. 2001), the few transmitted spirochetes are removed with the skin attached to the tick mouthparts. This removal of spirochetes would prevent the establishment of *B. burgdorferi* ss infection in mice. In contrast, *B. afzelii*, which is often associated with dermatological manifestations, might disseminate in the host skin more rapidly from the tick bite site. When *B. afzelii*-infected ticks are removed during the first 48 h of attachment, a part of the spirochetes could have already been able to migrate in the skin and establish the infection in mice.

Looking at detailed results obtained with the four different isolates (data not shown), we observed that the success of tick transmission to the host appears to be more isolate-dependent than species-dependent. In fact, *B. afzelii* (NE496)-infected ticks transmitted *Borrelia* more efficiently, infecting 100% of mice when attached for $\geq$72h, than ticks infected by the two *B. burgdorferi* ss (ZS7 and NE1849) and the other *B. afzelii* (NE2963) isolates, which transmitted *Borrelia* to only 40–50% of mice. Fingerle et al. (2002) demonstrated that in *B. afzelii* and *B. garinii* capillary-infected *I. ricinus*, the velocity of dissemination of spirochetes in the tick organs varies among isolates. This fact can explain the difference in success of tick transmission observed in our study between the two *B. afzelii* isolates.

Looking at results of mice infection via inoculation of homogenates of ticks, we observed that *B. burgdorferi* ss and *B. afzelii* in unfed *I. ricinus* are noninfectious. Host infection was detected only if the inoculated ground *I. ricinus* had previously been attached to a host for 24 h; this confirms previous observations obtained with *B. burgdorferi* ss and *I. scapularis* (Piesman 1993), although infectivity of inoculated spirochetes was higher in our study, reaching 67% for *B. burgdorferi* ss. This means that *B. burgdorferi* ss and *B. afzelii* spirochetes are infectious in the tick before natural transmission occurs. Interestingly, Piesman (1995) showed that inoculation of homogenized salivary glands produced infection in mice only if *B. burgdorferi* ss ticks had previously engorged for 60 h. This suggests that spirochetes become infectious in the tick before reaching the sali-

vary glands. In our work, the localization of the spirochetes in the ticks is not known since the entire ticks were injected to mice.

At the end of the blood meal, when tick transmission is known to be the highest (Piesman 1993, Kahl et al. 1998, des Vignes et al. 2001, Ohnishi et al. 2001), *B. burgdorferi* ss-infected ticks showed a lower infection success in mice than the same ticks injected into mice. That means that *B. burgdorferi* ss spirochetes are very infective in the tick, but that they are not transmitted with the same efficacy via *I. ricinus* bite. This is not the case for *B. afzelii*. In addition, *B. burgdorferi* ss is transmitted only after 48 h of tick attachment, whereas *B. afzelii* is transmitted earlier. All this together suggests that *I. ricinus* is a more efficient vector for *B. afzelii* than for *B. burgdorferi* ss.

An additional aim of this study was to investigate if *B. burgdorferi* sl can penetrate intact skin. Usually, various methods are used to remove *I. ricinus* ticks (Kahl et al. 1998). Although tweezers are frequently used, some individual removes attached ticks with naked hands. Up to now it was unknown whether this was a risk for acquiring Lyme disease spirochetes. Although it is well known that *B. burgdorferi* sl is transmitted via saliva (Piesman et al. 1987), other *Borrelia* species, like *B. duttoni* and *B. recurrentis*, the agents of African relapsing fever and louse-borne relapsing fever, respectively, are able to infect vertebrates by active penetration through intact skin (Burgdorfer 1976). In this study, we observed that homogenates of ticks infected by *B. afzelii* (NE2963, NE496) or *B. burgdorferi* ss (ZS7, NE1849) applied on mouse skin never infected mice. *B. burgdorferi* ss and *B. afzelii* appear not to be able to penetrate intact skin. However, whether the other *Borrelia* species belonging to the *B. burgdorferi* sl complex are able to cross intact skin remains to be tested. We conclude that tick bite is a prerequisite for transmission of *B. burgdorferi* ss and *B. afzelii*.

ACKNOWLEDGEMENTS

We warmly thank Pierre-François Humair for critical reading of a previous version of the manuscript, Emmanuelle Medjitna and Yves Cheminade for technical assistance, and Jean-Luc Perret for statistics. This paper is part of the Ph.D. thesis of Mara Crippa.

ABBREVIATIONS

PCR, polymerase chain reaction; sl, sensu lato; ss, sensu stricto.

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