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Transmission of *Borrelia garinii* OspA Serotype 4 to BALB/c Mice by *Ixodes ricinus* Ticks Collected in the Field

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In Europe, *Borrelia garinii* OspA serotype 4 has been isolated from the cerebrospinal fluid of patients but, up to now, has never been identified among culture isolates from *Ixodes ricinus* ticks. This information raises the question of whether OspA serotype 4 is transmitted by *I. ricinus* in nature. In the present study, *I. ricinus* nymphs collected in an area of endemicity in southern Germany were allowed to feed on mice. Cultivation of ear biopsy specimens showed that six of seven *B. garinii*-infected mice were infected by OspA serotype 4. In contrast, very few *B. garinii* OspA serotype 4 organisms were isolated directly from the ticks which infected the mice; most isolates were *B. afzelii*. The infected mice transmitted mainly OspA serotype 4 to xenodiagnostic ticks, preferentially in combination with *B. afzelii*.

Borrelia burgdorferi sensu lato is the agent that causes Lyme borreliosis, a multisystemic disorder involving the skin, heart, joints, and nervous system in humans (23). Among *B. burgdorferi* isolates from different biological sources, 10 genospecies have been described (1, 2, 4, 13–16, 19, 25). Among them, three species—*B. burgdorferi* sensu stricto, *B. afzelii,* and *B. garinii*—are recognized as being pathogenic for humans. In Europe, the tick *Ixodes ricinus* is the main vector of these pathogens to animals and humans (11).

Wilske et al. (27) defined seven outer surface protein A (OspA) serotypes of *B. burgdorferi* sensu lato. These serotypes correlated well with the three delineated most frequent genospecies: serotype 1 corresponds to *B. burgdorferi* sensu stricto, serotype 2 corresponds to *B. afzelii,* and serotypes 3 to 7 correspond to *B. garinii*. This considerable heterogeneity among *B. garinii* isolates was confirmed on a genetic basis (26). Strikingly, *B. garinii* serotype 4 isolates have been cultivated from cerebrospinal fluid (CSF) from patients in Germany, The Netherlands, Denmark, and Slovenia and even have been cultivated from CSF more frequently than other serotypes but have never been isolated from ticks (24, 27, 28). Therefore, we determined whether *I. ricinus* can transmit *B. garinii* OspA serotype 4 to mice and whether mice can infect *I. ricinus* with this serotype.

I. ricinus nymphs were collected by flagging vegetation in the Munich area (Germany). One portion of these ticks was used to evaluate the *B. burgdorferi* infection rate. Each nymph was cut into two pieces. One half was examined by immunofluorescence (IF) using a fluorescein isothiocyanate-conjugated polyclonal antibody which was prepared from a pool of Lyme borreliosis patient sera and which detects all *Borrelia* species (6); the other half was used for *Borrelia* isolation (6). The other portion of the field-collected nymphs was used to challenge 8-week-old female BALB/c mice. Challenge nymphs (14 nymphs/mouse) were placed in a capsule on the back of the mice and collected 5 to 6 days later, after natural detachment. Each derived adult tick was placed into a tube containing BSK II medium (22), incubated at 34°C, and examined by dark-field microscopy for 2 months. *B. burgdorferi* infection in mice was monitored by spirochete isolation from ear biopsy specimens 1 month after the infectious tick bite and by xenodiagnosis (5). For xenodiagnosis, infection-free *I. ricinus* larvae from our laboratory colony (8) were placed on the head of each mouse. Derived unfed nymphs were prepared for *B. burgdorferi* isolation and IF (6).

PCR and restriction fragment length polymorphism (RFLP) analyses were used for the identification of *Borrelia* species (18). The pellet from 1 ml of initial culture containing tick or ear biopsy specimen was used for PCR. The variable intergenic spacer between tandemly repeated 23S (*rrl*)-5S (*rrf*) ribosomal genes of *B. burgdorferi* sensu lato was used as a template for amplification. The PCR products were analyzed by the RFLP technique using the *Mse*I restriction endonuclease to identify the genospecies of *B. burgdorferi* sensu lato. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot analysis were performed (9). Monoclonal antibodies were used for *B. garinii* serotyping (27). In addition, restriction analysis of *ospA* amplicons was applied to isolates which were not serotype 4 (K. Trebesius, C. Teufel, V. Fingerle, and B. Wilske, Abstr. Microbiology 2000, poster 15.P.14.15, p. 162, 2000).

Twenty-one field-collected nymphs were examined: Two nymphs were found infected by both IF and cultivation, and two additional ticks were found infected either by IF or by cultivation (infection rate: 4 of 21, or 19%). Three isolates were obtained from these four infected ticks. All three *Borrelia* isolates were identified as *B. afzelii*.

B. burgdorferi isolates were obtained from 19 out of 22 mice challenged by field-collected nymphs. These mice were found to be infected by *B. burgdorferi* sensu stricto ($n = 2$), *B. afzelii* ($n = 9$), and *B. garinii* ($n = 7$). One isolate could not be identified by RFLP analysis. Characterization of isolates from *B. garinii*-infected mice showed that mice 1 through 6 were infected by serotype 4, whereas mouse 7 was infected by a mixture of serotypes 5 and 6. A total of 22 isolates (including 1

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TABLE 1. Determination of *Borrelia* isolates from ticks which fed on mice infected by *B. garinii*

Mouse	Infecting serotype	Challenge ticks					Xenodiagnostic ticks					
		No. of:		No. of isolates of[b]:			No. of IF-positive ticks[c]	No. of:		No. of isolates of:		
		Isolates	Tubes[a]	Bg	Ba	Bg + Ba		Isolates	Tubes[d]	Bg[e]	Ba	Bg + Ba
1	4	4	14	0	4	0	8	2	3	**1**	1	0
2	4	1	11	0	1	0	5	5	5	**3**	0	2
3	4	3	12	1[f]	2	0	8	1	1	0	1	0
4	4	5	9	0	5	0	9	2	2	**1**	1	0
5	4	8	13	0	8	0	6	4	4	**4**	0	0
6	4	1	13	0	0	1	3	5	6	**5**	0	0
7	5 + 6	6	9	6	0	0	0	0	1			

[a] Each culture tube was inoculated with one challenge tick.
[b] Bg, *B. garinii*; Ba, *B. afzelii*.
[c] Ten ticks were tested.
[d] Each culture tube was inoculated with five xenodiagnostic ticks.
[e] Results shown in bold indicate that the isolates reacted with L32 1G3, a monoclonal antibody against *B. garinii* OspA serotype 4.
[f] Identified by RFLP analysis in one culture tube with nonmotile spirochetes.

isolate with nonmotile spirochetes) were recovered from challenge ticks which fed on the six *B. garinii* serotype 4-infected mice. *B. afzelii* clearly dominated among these isolates, whereas *B. garinii* was rare (Table 1). In contrast, six *B. garinii* isolates were obtained from challenge ticks which fed on mouse 7: three were serotype 5, two were serotype 6, and one was not analyzed. Some of the challenge ticks did not molt.

All mice infected with *B. garinii* serotype 4 transmitted spirochetes to xenodiagnostic ticks, as observed by IF (Table 1). However, no isolate was recovered from 60 xenodiagnostic ticks which fed on *B. garinii* serotype 4-infected mice when the ticks were incubated individually in tubes. This result was surprising, since between 30 and 90% of these ticks were found by IF to be infected (Table 1). Therefore, we repeated the isolation using the rest of the xenodiagnostic ticks, but we inoculated each tube with five ticks instead of one. Here, isolates were obtained from ticks which fed on all *B. garinii* serotype 4-infected mice. *B. garinii*, *B. afzelii,* and a mixture of both species were observed (Table 1). All *B. garinii* isolates were serotype 4. Mouse 7 did not transmit spirochetes to xenodiagnostic ticks, as observed by IF, and no isolate could be obtained from these ticks when they were incubated individually in BSK II medium or when five ticks were incubated in a tube.

In Europe, *B. burgdorferi* sensu stricto (13), *B. garinii* (1), and *B. afzelii* (2) have been frequently isolated from ticks and reservoir hosts and are associated with Lyme borreliosis. In the present study, these three genospecies were transmitted to mice by nymphs. Characterization of the isolates from *B. garinii*-infected mice demonstrated that six out of seven mice were infected by serotype 4, meaning that serotype 4 was the main *B. garinii*-associated serotype transmitted to mice by field-collected nymphs. This result is interesting, since serotype 4 had never been cultured directly from *I. ricinus* ticks before (27, 28), although sequences of OspA serotype 4 had been identified in field-collected ticks (3). One explanation for the fact that this serotype had never been isolated from ticks before is that spirochetes of *B. garinii* serotype 4 may be present in low numbers in ticks. In fact, the success of isolation may depend on the number of spirochetes present in ticks (6). This notion may explain why we did not isolate *B. garinii* serotype 4 when we incubated challenge and xenodiagnostic ticks individually in BSK II medium, whereas successful isolation occurred when more than one tick was incubated in a tube. The other explanation is that serotype 4 may be present in mixed infections in ticks in nature and is overgrown in cultures by other serotypes, possibly those of *B. afzelii,* which can be isolated easily from ticks (6).

Interestingly, in the present study, in xenodiagnostic ticks which fed on serotype 4-infected mice, *B. garinii* serotype 4 was frequently associated with *B. afzelii*. In contrast, *B. garinii* serotype 4 was never associated with *B. afzelii* in specimens obtained from mouse tissue. In order to see if this phenomenon could be reproduced, we used xenodiagnostic ticks which fed on mice 1 to 5 and placed them after molting on 10 mice (two mice for each group). Ear biopsy cultivation allowed *Borrelia* isolation from 5 out of 10 mice. Only *B. garinii* serotype 4 was isolated from the mice, although *B. afzelii* was present in some of the xenodiagnostic ticks used to challenge these mice. This result may indicate that serotype 4 is very invasive in vertebrates, as has been shown recently for some *B. burgdorferi* sensu stricto clones (21), and/or that the greater serum resistance of serotype 4 may facilitate dissemination of this *Borrelia* serotype into mice (24). OspA serotype 4 strains are nearly identical in their OspC phenotype and *ospC* sequences as well as their plasmid profiles. Also, the otherwise very heterogeneous upstream homology box RFLP pattern is highly similar (12, 17, 28). This information is an indication that OspA serotype 4 is a recently emerged clone with potentially higher virulence than that of other serotypes. Interestingly, we have found a double infection of the salivary glands of a tick removed from a child who developed multiple erythema migrans (V. Fingerle and B. Wilske, unpublished results). A CSF isolate from a patient with neuroborreliosis contained both *B. afzelii* and *B. garinii* OspA serotype 4 (28). These are indications that *B. garinii* OspA serotype 4 may be preferentially cotransmitted with *B. afzelii* not only to mice but also to humans.

In nature, specific maintenance cycles have been described for *B. afzelii* and small mammals (10). However, nonspecific maintenance cycles involving small mammals and *B. garinii* have been described in Russia (7) and eastern parts of Europe (20; G. Khanakah et al., Abstr. VI Int. Conf. Lyme Borreliosis, abstr. PO77W., 1994). In view of our results showing that *B. garinii* serotype 4 easily infects mice and xenodiagnostic

ticks, it is possible that serotype 4 is the serotype of *B. garinii* circulating among small mammals and ticks in Russia and eastern parts of Europe.

Two groups of *B. garinii*-infected mice could be distinguished: one group (mice 1 through 6), infected by serotype 4, easily infected xenodiagnostic ticks (tick infection rate: 30 to 90%), and one group (mouse 7), infected by serotypes 5 and 6, did not transmit these serotypes to ticks. Interestingly, although *B. garinii*-infected ticks are difficult to obtain from mice infected in the laboratory (5), this does not appear to be the case for *B. garinii* serotype 4.

In conclusion, we demonstrated that *B. garinii* serotype 4 can be transmitted to mice through the bites of field-collected *I. ricinus* and that it is often associated with *B. afzelii* in ticks.

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