JOURNAL OF CLINICAL MICROBIOLOGY, Dec. 2002, p. 4735–4737 Vol. 40, No. 12
0095-1137/02/$04.00+0 DOI: 10.1128/JCM.40.12.4735–4737.2002

Apodemus Species Mice Are Reservoir Hosts of *Borrelia garinii* OspA Serotype 4 in Switzerland

D. Huegli,[1] C. M. Hu,[1] P.-F. Humair,[1] B. Wilske,[2] and L. Gern[1]*

Institut de Zoologie, University of Neuchâtel, Neuchâtel, Switzerland,[1] and Max von Pettenkofer Institut, Munich, Germany[2]

Received 25 February 2002/Returned for modification 19 June 2002/Accepted 4 September 2002

Among *Borrelia burgdorferi* sensu lato isolates, seven outer surface protein A (OspA) serotypes have been described: serotypes 1 and 2 correspond to *B. burgdorferi* sensu stricto and *Borrelia afzelii*, respectively, and serotypes 3 to 7 correspond to *Borrelia garinii*. In Europe, serotype 4 has never been isolated from *Ixodes ricinus* ticks until recently, although this serotype has been frequently isolated from cerebrospinal fluid from patients. In Europe, *B. afzelii* and *B. burgdorferi* sensu stricto were found associated with rodents and *B. garinii* was found associated with birds. In this study, the reservoir role of *Apodemus* mice for *B. garinii* OspA serotype 4 was demonstrated by xenodiagnosis. *Apodemus* mice are the first identified reservoir hosts for *B. garinii* OspA serotype 4.

Borrelia burgdorferi sensu lato, the agent of Lyme borreliosis, is a complex including at least 10 genospecies (1, 2, 4, 13, 14, 16, 17, 20, 23). Among them, three species are recognized as pathogenic for humans: *B. burgdorferi* sensu stricto, *Borrelia afzelii*, and *Borrelia garinii*. In Europe, the tick *Ixodes ricinus* is the main vector of these pathogens.

Wilske et al. (24) defined seven outer surface protein A (OspA) serotypes among these three *Borrelia* species: serotype 1 corresponds to *B. burgdorferi* sensu stricto, serotype 2 corresponds to *B. afzelii*, and serotypes 3 to 7 correspond to *B. garinii*. Strikingly, *B. garinii* serotype 4 was never cultivated from *I. ricinus* until recently (10), although this serotype has frequently been cultivated from cerebrospinal fluid from patients from Germany, The Netherlands, Denmark, and Slovenia (18, 24, 25). In some areas of Europe where these species are indigenous, *B. afzelii* and *B. burgdorferi* sensu stricto are maintained by rodents and *Borrelia valaisiana* and *B. garinii* are maintained by birds (for a review, see reference 12). In the laboratory, it is difficult to obtain ticks infected by *B. garinii* from infected mice (3, 5). However, Hu et al. (10) obtained *B. garinii* serotype 4-infected ticks from laboratory mice. This finding, and the presence of *B. garinii* DNA in rodents and in xenodiagnostic ticks that fed on rodents in the eastern part of Europe (21; G. Khanakah et al., Abstr. VI Int. Conf. Lyme Borreliosis, abstr. PO77W, 1994), urged us to investigate whether rodents in Switzerland transmit serotype 4 to *I. ricinus* ticks.

From May to September 2000, small rodents were captured in three areas in Switzerland (Staatswald, Kernenried, and Langendorf) where *Borrelia* spp. are endemic. *B. burgdorferi* infection in rodents was monitored by tick xenodiagnosis. For xenodiagnosis, infection-free *I. ricinus* larvae from our laboratory colony (7) were placed on the head of each rodent, allowed to feed until repletion, and examined as nymphs for *Borrelia* spp. Each nymph was cleaned with 70% ethanol and then cut into two pieces. One half of the nymph was examined by immunofluorescence (IF) analysis using fluorescein isothiocyanate-conjugated polyclonal antibodies prepared from a pool of Lyme borreliosis patient sera and which detect all *Borrelia* species (6). The other half was used for *Borrelia* isolation as described by Gern et al. (6).

PCR and restriction fragment length polymorphism (RFLP) analysis of the *rrf-rrl* intergenic spacer were used for the identification of *Borrelia* species as described by Postic et al. (19). The pellet from 1 ml of spirochete growth culture (initial tube containing the tick) was washed, and DNA suspension was used as the template for amplification. The PCR products were analyzed by RFLP using *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 as previously described (8), using monoclonal antibody (MAb) L32 1G3, which specifically reacts with the OspA of *B. garinii* serotype 4 (24).

The N-terminal end of the OspA shows a variable region of amino acids which allows the determination of serotypes (24). The sequences of *ospA* genes of various *Borrelia* serotypes (24, 26) were aligned, and the following primers in the 5′ end of the gene were designed: primer OspA-1F (5′-CAAAATGTTAG CAGCCTTG-3′) and primer OspA-3R (5′-GTGTATTCAAG TCTGGTTCC-3′). These primers allow the amplification of a DNA fragment of 389 bp, including the variable region described by Wilske et al. (24). Reaction volumes were 50 μl and contained 1.5 mM $MgCl_2$, 0.2 mM deoxynucleoside triphosphates, a 0.2 μM concentration of each primer, 1× *Taq* buffer, 1.25 U of *Taq* DNA polymerase (QIAGEN, Basel, Switzerland), and ultrafiltered H_2O. The PCR amplification was carried out for 35 cycles (denaturation at 94°C for 30 s, primer annealing at 55°C for 30 s, and extension at 72°C for 1 min) with an initial denaturation step at 94°C for 2 min and a final extension step at 72°C for 10 min. The presence of PCR products was checked by using a 1% agarose gel and visualized by UV transillumination after ethidium bromide staining. PCR

* Corresponding author: Institut de Zoologie, Emile Argand 11, 2007 Neuchâtel 7, Switzerland. Phone: 41 32 718 3000. Fax: 41 32 718 3001. E-mail: lise.gern@unine.ch.

TABLE 1. *Borrelia* infection rate of xenodiagnostic ticks that fed on *Apodemus mice* and characterization of *Borrelia* isolates from these ticks

Animal no.	Rodent species	Swiss capture site	Infection rate[a]	Characterization of isolates (*n*)[b]
1	*A. sylvaticus*	Staatswald	9/10	*B. garinii*, OspA type 4 (1)**
2	*A. sylvaticus*	Staatswald	9/10	*B. garinii*, OspA type 4 (6)
3	*A. sylvaticus*	Staatswald	6/10	*B. garinii*, OspA type 4 (2)
4	*A. flavicollis*	Staatswald	2/10	*B. afzelii* (1), *B. garinii*, OspA type 4 (1)
5	*A. sylvaticus*	Staatswald	9/10	*B. afzelii* (2)
6	*A. flavicollis*	Kernenried	9/10	*B. afzelii* (5)
7	*A. sylvaticus*	Kernenried	8/10	*B. afzelii* (2)
8	*A. sylvaticus*	Kernenried	6/10	*B. afzelii* (4)
9	*A. sylvaticus*	Langendorf	2/10	*B. afzelii* (2)
10	*A. sylvaticus*	Langendorf	8/10	*B. afzelii* (1)

[a] Number of ticks infected relative to total number of ticks examined, determined by IF analysis.
[b] Determined by PCR-RFLP, *ospA* sequencing, and Western blotting. *n*, number of isolates.

products were purified with a QIAquick PCR purification kit (QIAGEN). Cycle sequencing was performed by the dideoxy chain termination method using an ABI PRISM BigDye Terminator cycle sequencing kit (Applied Biosystems, Rotkreuz, Switzerland). Cycle sequencing parameters were as follows: denaturation at 96°C for 15 s, primer annealing at 53°C for 15 s, and extension at 60°C for 4 min. The cycle sequencing products were cleaned by ethanol precipitation and applied to an ABI PRISM 310 genetic analyzer (Applied Biosystems). DNA sequences were checked with Sequence Navigator software (Applied Biosystems). The identities of sequences were assigned by a search of the GenBank database.

A total of 29 rodents, captured in three areas where *Borrelia* is endemic and belonging to *Apodemus sylvaticus* ($n = 24$) and *Apodemus flavicollis* ($n = 5$), were subjected to xenodiagnosis. Seventeen mice (59%) transmitted spirochetes to xenodiagnostic ticks as determined by IF analysis and/or *Borrelia* isolation. *Borrelia* isolates ($n = 27$) obtained from xenodiagnostic ticks that fed on 10 individuals were characterized by PCR-RFLP analysis of the *rrf*(5S)-*rrl*(23S) intergenic spacer. *B. afzelii* was the dominant species among isolates from ticks that fed on mice (17 of 27, 63%), followed by *B. garinii* (10 of 27, 37%) (Table 1). Six out of 10 *Apodemus* mice transmitted only *B. afzelii* to xenodiagnostic ticks, whereas 3 individuals transmitted only *B. garinii*. One additional individual transmitted both species to xenodiagnostic ticks. The rates of *B. garinii* infection in xenodiagnostic ticks, determined by IF analysis, were as high as rates of *B. afzelii* infection (Table 1). Immunoblotting with MAb L32 1G3 revealed that all 10 *B. garinii* isolates belonged to OspA serotype 4. Sequencing of the amplified *ospA* gene confirmed the identification of species and serotypes obtained by PCR-RFLP and immunoblotting. *ospA* sequences of all *B. garinii* isolates matched the *ospA* sequence of serotype 4 type strain PBi. All mice which transmitted *B. garinii* serotype 4 to ticks came from one capture site only (Staatswald, Switzerland) (Table 1).

Serotyping of European isolates allowed us to distinguish seven OspA serotypes: serotypes 1 and 2 correspond to *B. burgdorferi* sensu stricto and *B. afzelii*, respectively, whereas serotypes 3 to 7 are included in *B. garinii* (24). Interestingly, the role of ticks as vectors of *B. garinii* serotype 4 has only recently been demonstrated (10). It was shown that *I. ricinus* nymphs collected in the field can transmit serotype 4 to laboratory mice and that these mice transmitted serotype 4 to xenodiagnostic ticks (10). This was surprising because *B. garinii* is usually associated with birds in nature (see reference 12) and because ticks infected by *B. garinii* are difficult to obtain from mice infected in the laboratory (3, 5). Since the association of *B. garinii* with rodents has been reported occasionally in Europe (21; Khanakah et al., Abstr. VI Int. Conf. Lyme Borreliosis, 1994.), we investigated the role of rodents as reservoirs for *B. garinii* serotype 4. *B. afzelii* was the *Borrelia* species most frequently transmitted from *Apodemus* mice to xenodiagnostic ticks. This confirms previous observations describing rodents as reservoir hosts for *B. afzelii* in Switzerland (9, 11). In the present study, rodents from one of the three studied areas transmitted *B. garinii* serotype 4 to xenodiagnostic ticks, whereas rodents from the two other areas transmitted only *B. afzelii* to ticks. This demonstrates that *Apodemus* mice can act as reservoirs for *B. garinii* serotype 4. Interestingly, the association of *B. afzelii* with *B. garinii* serotype 4 observed here in xenodiagnostic ticks corroborates previous observations (10). A mixture of *B. garinii* type 4 and *B. afzelii* was also isolated from the cerebrospinal fluid of a patient (24). This indicates that *B. garinii* serotype 4 may be present in mixed infections in rodents, humans, and ticks in nature, and that serotype 4 may be overgrown in culture by other types, possibly *B. afzelii*, which is more easily isolated from ticks (6). This could explain the previous difficulty in obtaining OspA serotype 4 isolates from ticks collected in the field (25). The fact that mice serve as a reservoir for *B. garinii* serotype 4 and not other *B. garinii* serotypes might be explained by serum resistance (10, 15). Interestingly, serotype 4 was resistant to human serum whereas the other *B. garinii* subtypes were sensitive (22).

In conclusion, the present study identified *Apodemus* mice as reservoirs of *B. garinii* serotype 4 in Switzerland.

We thank Olivier Rais and Yves Cheminade for their technical assistance, the Laboratoire de Botanique évolutive (University of Neuchâtel) for the use of the sequencer, and Yong-Min Yuan for his help.

We acknowledge the Swiss National Science Foundation (no. 32-57098.99) and the Roche Research Foundation for supporting P.-F.H.

REFERENCES

1. **Baranton, G., D. Postic, I. Saint Girons, P. Boerlin, J. C. Piffaretti, M. Assous, and P. A. D. Grimont.** 1992. Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS 461 associated with Lyme borreliosis. Int. J. Syst. Bacteriol. **42:**378–383.
2. **Canica, M. M., F. Nato, L. Du Merle, J. C. Mazie, G. Baranton, and D. Postic.** 1993. Monoclonal antibodies for identification of *Borrelia afzelii* sp. nov. associated with late cutaneous manifestations of Lyme borreliosis. Scand. J. Infect. Dis. **25:**441–448.
3. **Dolan, M. C., J. Piesman, M. L. Mbow, G. O. Maupin, O. Péter, M. Brossard, and W. T. Golde.** 1998. Vector competence of *Ixodes scapularis* and *Ixodes ricinus* (Acari: *Ixodidae*) for three genospecies of *Borrelia burgdorferi*. J. Med. Entomol. **35:**465–470.
4. **Fukunaga, M., A. Hamase, K. Okada, and M. Nakao.** 1996. *Borrelia tanukii* sp. nov. and *Borrelia turdae* sp. nov. found from Ixodid ticks in Japan: rapid species identification by 16S rRNA gene-targeted PCR analysis. Microbiol. Immunol. **40:**877–881.
5. **Gern, L., C. M. Hu, P. Voet, P. Hauser, and Y. Lobet.** 1997. Immunization with a polyvalent OspA vaccine protects mice against *Ixodes ricinus* tick bites

infected by *Borrelia burgdorferi* ss, *Borrelia garinii* and *Borrelia afzelii*. Vaccine **15:**1551–1557.
6. **Gern, L., C. M. Hu, E. Kocianova, V. Vyrostekova, and J. Rehacek.** 1999. Genetic diversity of *Borrelia burgdorferi* sensu lato isolates obtained from *Ixodes ricinus* ticks collected in Slovakia. Eur. J. Epidemiol. **15:**665–669.
7. **Graf, G. F.** 1978. Copulation, nutrition et ponte chez *Ixodes ricinus* L. (Ixodoidea: Ixodidae), 1ère partie. Bull. Soc. Entomol. Suisse **51:**89–97.
8. **Hu, C. M., S. Leuba-Garcia, M. D. Kramer, A. Aeschlimann, and L. Gern.** 1994. Comparison in the immunological properties of *Borrelia burgdorferi* isolates from *Ixodes ricinus* derived from three endemic areas in Switzerland. Epidemiol. Infect. **112:**533–542.
9. **Hu, C. M., P. F. Humair, R. Wallich, and L. Gern.** 1997. *Apodemus* sp. rodents, reservoir hosts for *Borrelia afzelii* in an endemic area in Switzerland. Zentbl. Bakteriol. **285:**558–564.
10. **Hu, C. M., B. Wilske, V. Fingerle, Y. Lobet, and L. Gern.** 2001. Transmission of *Borrelia garinii* OspA serotype 4 to BALB/c mice by *Ixodes ricinus* ticks collected in the field. J. Clin. Microbiol. **39:**1169–1171.
11. **Humair, P. F., O. Rais, and L. Gern.** 1999. Transmission of *Borrelia afzelii* from *Apodemus* mice and *Clethrionomys* voles to *Ixodes ricinus* ticks: differential transmission pattern and overwintering maintenance. Parasitology **118:**33–42.
12. **Humair, P. F., and L. Gern.** 2000. The wild hidden face of Lyme borreliosis in Europe. Microbes Infect. **2:**915–922.
13. **Johnson, R. C., G. P. Schmid, F. W. Hyde, A. G. Steigerwalt, and D. J. Brenner.** 1984. *Borrelia burgdorferi* sp. nov.: etiologic agent of Lyme disease. Int. J. Syst. Bacteriol. **34:**496–497.
14. **Kawabata, H., T. Masuzawa, and Y. Yanagihara.** 1993. Genomic analysis of *Borrelia japonica* sp. nov. isolated from *Ixodes ovatus* in Japan. Microbiol. Immunol. **37:**1–6.
15. **Kurtenbach, K., H. S. Sewell, N. H. Ogden, S. E. Randolph, and P. A. Nuttall.** 1998. Serum complement sensitivity as a key factor in Lyme disease ecology. Infect. Immun. **66:**1248–1251.
16. **Le Fleche, A., D. Postic, K. Girardet, O. Péter, and G. Baranton.** 1997. Characterization of *Borrelia lusitaniae* sp. nov. by 16S ribosomal DNA sequence analysis. Int. J. Syst. Bacteriol. **47:**921–925.
17. **Marconi, R. T., D. Liveris, and I. Schwartz.** 1995. Identification of novel insertion elements, restriction fragment length polymorphism patterns, and discontinuous 23S rRNA in Lyme disease spirochetes: phylogenetic analyses of rRNA genes and their intergenic spacers in *Borrelia japonica* sp. nov. and genomic group 21038 (*Borrelia andersonii* sp. nov.) isolates. J. Clin. Microbiol. **33:**2427–2434.
18. **Marconi, R. T., S. Hohenberger, S. Jauris-Heipke, U. Schulte-Spechtel, C. P. LaVoie, D. Röβler, and B. Wilske.** 1999. Genetic analysis of *Borrelia garinii* OspA serotype 4 strains associated with neuroborreliosis: evidence for extensive genetic homogeneity. J. Clin. Microbiol. **37:**3965–3970.
19. **Postic, D., M. V. Assous, P. A. D. Grimont, and G. Baranton.** 1994. Diversity of *Borrelia burgdorferi* sensu lato evidenced by restriction fragment length polymorphism of *rrf* (5S)-*rrl* (23S) intergenic spacer amplicons. Int. J. Syst. Bacteriol. **44:**743–752.
20. **Postic, D., N. Marti Ras, R. S. Lane, M. Hendson, and G. Baranton.** 1998. Expanded diversity among californian *Borrelia* isolates and description of *Borrelia bissettii* sp. nov. (formerly *Borrelia* group DN127). J. Clin. Microbiol. **36:**3497–3504.
21. **Richter, D., S. Endepols, A. Ohlenbusch, H. Eiffert, A. Spielman, and F. R. Matuschka.** 1999. Genospecies diversity of Lyme disease spirochetes in rodent reservoirs. Emerg. Infect. Dis. **5:**291–296.
22. **van Dam, A. P., A. Oei, R. Jaspars, C. Fijen, B. Wilske, L. Spanjaard, and J. Dankert.** 1997. Complement-mediated serum sensitivity among spirochetes that cause Lyme disease. Infect. Immun. **65:**1228–1236.
23. **Wang, G., A. P. van Dam, A. Le Fleche, D. Postic, O. Péter, G. Baranton, R. de Boer, L. Spanjaard, and J. Dankert.** 1997. Genetic and phenotypic analysis of *Borrelia valaisiana* sp. nov. (*Borrelia* genomic groups VS116 and M19). Int. J. Syst. Bacteriol. **47:**926–932.
24. **Wilske, B., V. Preac-Mursic, U. B. Göbel, B. Graf, S. Jauris, E. Soutschek, E. Schwab, and G. Zumstein.** 1993. An OspA serotyping system for *Borrelia burgdorferi* based on reactivity with monoclonal antibodies and OspA sequence analysis. J. Clin. Microbiol. **31:**340–350.
25. **Wilske, B., U. Busch, H. Eiffert, V. Fingerle, H. W. Pfister, D. Rössler, and V. Preac-Mursic.** 1996. Diversity of OspA and OspC among cerebrospinal fluid isolates of *Borrelia burgdorferi* sensu lato from patients with neuroborreliosis in Germany. Med. Microbiol. Immunol. **184:**195–201.
26. **Zumstein, G., R. Fuchs, A. Hofmann, V. Preac-Mursic, E. Soutschek, and B. Wilske.** 1992. Genetic polymorphism of the gene encoding the outer surface protein A (OspA) of *Borrelia burgdorferi*. Med. Microbiol. Immunol. **181:**57–70.