J. Parasitol., 82(6), 1996 p. 936–940

UNUSUAL STRAIN OF *BORRELIA BURGDORFERI* ISOLATED FROM *IXODES DENTATUS* IN CENTRAL GEORGIA

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ABSTRACT: A new, unusual spirochete was cultured in Barbour–Stoenner–Kelly (BSK II) medium from the midgut and other tissues of the tick *Ixodes dentatus*. The tick was collected from leaf litter in an oak-pine wood lot in Bibb County ≈7.2 km from Macon in central Georgia during February 1993. Characterization by indirect immunofluorescence using 5 murine monoclonal antibodies, by sodium dodecyl sulfate–polyacrylamide gel electrophoresis of whole spirochetal lysates, and by polymerase chain reaction assay for several known DNA target sequences indicates that the spirochete is *Borrelia burgdorferi* sensu lato. It is genetically different from the B-31 reference strain of *B. burgdorferi* sensu stricto that is typical of strains causing Lyme borreliosis in North America. Range of infectivity and pathogenesis of the Bibb County isolate (BC-1) are unknown but being investigated. The BC-1 strain is the first *B. burgdorferi* isolate from *I. dentatus* in the southeastern United States (*I. dentatus* is not the common vector for Lyme borreliosis in humans). Additionally, the collection site was ≈322 km from the Atlantic coast, far distant from where most *B. burgdorferi* isolates have been obtained.

The only *Borrelia* species confirmed as an etiologic agent for Lyme borreliosis in North America is *Borrelia burgdorferi* Johnson, Schmid, Hyde, Steigerwalt and Brenner (Jaenson, 1991; Lane et al., 1991). Recently, however, 2 reports (Baranton et al., 1992; Welsh et al., 1992) indicated that there are 3 groups of spirochetes associated with Lyme borreliosis in humans in Europe, and that all of these groups can be generally categorized as *B. burgdorferi* sensu lato. These groups or "genospecies" included *Borrelia burgdorferi* sensu stricto; *Borrelia garinii* Baranton, Postic, Girons, Boerlin, Piffaretti, Assous and Grimont; and Group VS461 (Baranton et al., 1992). Genospecies VS461 is now classifed as *Borrelia afzelii* (Canica et al., 1993). Genospecies I corresponded to *B. burgdorferi* sensu stricto and contained more than 28 isolates from Europe and the United States, including the type strain (B-31) of this species. Genospecies II consisted of at least 13 isolates from Europe and Japan, including *B. garinii*. Genospecies III (*B. afzelii*) included isolates from Europe and Japan. More recently, a fourth genospecies (*Borrelia japonica*) has been described from Japanese *Ixodes ovatus* (Kawabata et al., 1993), and a fifth one has been isolated from *Ixodes dentatus* Marx and from cottontail rabbits (*Sylvilagus floridanus*) in New York (Anderson et al., 1989). Strains of *B. burgdorferi* reported from North America are generally less variable than those from Europe (Barbour et al., 1985), although there are several exceptions: (1) antigenically variable *Borrelia andersonii* isolated from cottontail rabbits and *Ixodes dentatus* from New York (Marconi et al., 1995); (2) an isolate from a veery songbird (*Catharus fuscescens* [Stephens]) (Barbour et al., 1985; Anderson et al., 1986), (3) a strain (25015) from *Ixodes scapularis* (formerly *Ixodes dammini*) from Milbrook, New York (Anderson et al., 1988; Fikrig et al., 1992), (4) a strain (DN 127) from *Ixodes pacificus* Cooley and Kohls from northern California (Bissett et al., 1988), (5) strains from *Ixodes neotomae* Cooley in California (Lane and Pascocello, 1989); and (6) from *Ixodes spinipalpis* Hadwen and Nuttall in California and Colorado (Craven and Dennis, 1993). Moreover, some *B. burgdorferi* isolates from cotton rats (*Sigmodon hispidus*) and cotton mice (*Peromyscus gossypinus*) in Georgia and Florida were more genetically heterogeneous than most isolates reported from the northeastern and north-central United States (Oliver et al., 1993, 1995). We now report the cultivation and characterization of an unusual *B. burgdorferi* strain isolated from *I. dentatus* from the transition zone of the coastal plain and piedmont of central Georgia, USA.

Received 7 February 1996; revised 11 June 1996; accepted 11 June 1996.

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MATERIALS AND METHODS

Tick collection and spirochete isolation

A questing unfed nymphal *I. dentatus* was collected from leaf litter in mid-February 1993, in an oak-pine wood lot in Bibb County ≈7.2 km southwest of Macon, Georgia. The area was damp and the temperature was ≈22 C. The nymph was surface-cleaned in 95% ethanol and rinsed in a 1:1 mixture of 10% Clorox solution and 95% ethanol. Midgut and other tissues were dissected in Barbour-Stoenner-Kelly (BSK II) medium (Barbour, 1984a) and then transferred to tubes of BSK II medium that contained 0.15% agarose (Seakem, FMC Bioproducts, Rockland, Maine), antibiotics, and other compounds (Sinsky and Piesman, 1989) formulated by a modification of previously reported techniques (Johnson et al., 1984). Cultures were incubated in 5% CO_2 atmosphere at 33–34 C and examined for spirochetes by darkfield microscopy twice weekly for 2 wk and, if spirochetes were not detected, weekly thereafter for 6 wk.

Immunofluorescent assays

The spirochetal isolate was examined by indirect immunofluorescence (Anderson et al., 1983; Magnarelli and Anderson, 1988) using 5 murine monoclonal antibodies. These included 2 *B. burgdorferi*-specific anti-outer surface protein A (OspA) monoclonals (H3TS and H5332), a *B. burgdorferi*-specific anti-outer surface protein B (OspB) monoclonal (H6831), a *Borrelia* (genus)-specific anti-flagellin monoclonal antibody (H9724), and a monoclonal antibody (9826) active against *Borrelia hermsii*.

SDS-PAGE

Each spirochetal culture was prepared for characterization by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) using whole spirochetal lysates (Laemmli, 1970; Persing et al., 1990). Five micrograms each of the B-31 spirochetal lysate and the BC-1 lysate were denatured in the presence of 5.0% 2-mercaptoethanol at 100 C for 10 min. Samples were loaded into a 4% stacking gel and resolved through a 10% separating gel. Both prestained and nonstained low molecular weight protein standards (Bio-Rad Laboratories, Richmond, California) were simultaneously electrophoresed for extrapolation of the molecular weights. The SDS-PAGE gel was then stained with Coomassie brilliant blue R-250, destained, and scanned with a Shimadzu

TABLE I. Primers and parameters for PCR amplification.

Primers	Locations	Sequences (5′ → 3′)	Parameters C (sec) 1	2	3	No. cycles
ospA	788	CTGCAGCTTGGAATTCAGGCACTT	95 (60)	50 (45)	72 (120)	45
	946	GTTTTGTAATTTCAACTGCTGACC				
ospA	149	TTATGAAAAAATATTTATTGGGAAT	95 (60)	50 (45)	72 (120)	45
	319	TGTCATCTGTTCGAACTCGAATTTC				
ospA	149	TTATGAAAAAATATTTATTGGGAAT	95 (60)	45 (45)	71 (120)	45
	459	TCTTCTACCGTTTTGTGATCA				
ospA	3′	ATTCCTCTTAAATACTCTAATAATTATCCTAGGGAG	95 (60)	50 (45)	72 (120)	45
	5′	CAGGAATTCAGTTATATTAATATAAAAGGAGAATATATTATG				
fla	245	AAGTAGAAAAAGTCTTAGTAAGAATGAAGGA	94 (30)	55 (60)	72 (120)	35
	855	AATTGCATACTCAGTACTATTCTTTATAGAT				
Chromosomal	147	CGAAGATACTAAATCTGT	94 (60)	37 (30)	60 (60)	30
	520	GATCAAATATTTCAGCTT				

scanning densitometer (model CS-9000U) at a wavelength of 570 nm interfaced with a CSTURBO analysis program (Shimadzu Corp., Tokyo, Japan). The total area of each protein peak was automatically determined, as well as the percentage of each peak relative to the number of visibly scanned bands, by using the CSTURBO analysis program. The molecular weights in Daltons (Da) of the predominant proteins were also determined by this program and extrapolated from known molecular weight protein standards.

Genetic analysis

The polymerase chain reaction (PCR) and specific primer sets were used to amplify several known DNA target sequences specifically found in *B. burgdorferi* reference strain B-31. Before performing PCR, the spirochetal lysates were centrifuged at 600 *g* for 15 min to sediment any cellular debris. A protein assay was then performed using a small aliquot of the supernatant protein. Before adding to the PCR reaction mixture, 0.6 μg of spirochetal protein from each isolate in 10 μl distilled water was heated at 100 C for 10 min to inhibit any proteolytic activity. The PCR reaction mixture contained 2.5 units of Taq DNA polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 200 μM each of the deoxynucleotide triphosphates, and 50 pmol of each of the appropriate oligonucleotide primers. Amplification of the target sequences was performed using 6 sets of primers and a Perkin-Elmer/Cetus (Norwalk, Connecticut) thermal cycler. Primers and parameters for PCR amplification are listed in Table I. One set of primers (245/855) amplified a 610-bp target sequence found in the *B. burgdorferi* B-31 strain flagellin (*fla*) gene (Johnson et al., 1992). A second set of primers (788/946) amplified a 158-bp target sequence found in the B-31 strain *ospA* gene (Persing et al., 1990), and 3 other sets of primers (149/319; 149/459; 3′/5′) also amplified sequences within the *ospA* gene. A sixth set of primers (147/520) amplified a 373-bp chromosomal target sequence present in 17 of 18 documented strains of *B. burgdorferi* worldwide (Rosa and Schwan, 1989). Pure genomic DNA from *B. burgdorferi* reference strain B-31 was used as a positive control, and sterile distilled water was a negative control for each PCR assay. All PCR-amplified products were electrophoresed in 2% agarose gels, stained with ethidium bromide, and documented for permanent record by Polaroid (Cambridge, Massachusetts) photography of the ultraviolet transilluminated gels.

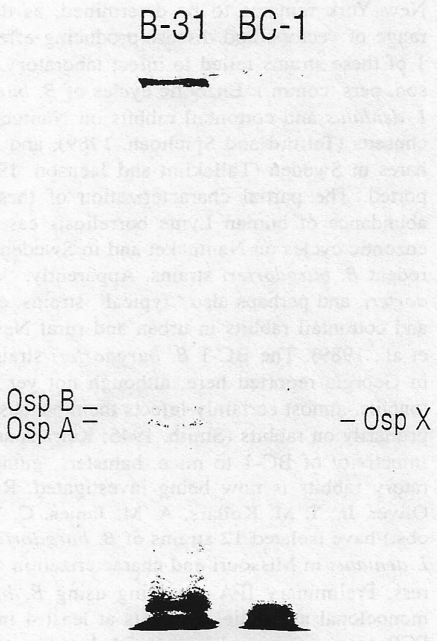

FIGURE 1. SDS-PAGE of whole spirochetal lysates. Note resolution of OspA and OspB bands in *B. burgdorferi* B-31 reference strain in left lane and of single Osp band (OspX) in BC-1 isolate in right lane.

RESULTS

The spirochetal isolate (BC-1) recovered from the unfed nymphal *I. dentatus* reacted positively to the *Borrelia* (genus-specific) H9724 monoclonal antibody but negatively to the *B. burgdorferi*-specific OspA (H3TS, H5332) and OspB (H6831) monoclonals and negatively to the *B. hermsii*-specific 9826 monoclonal antibody.

The major proteins identified by SDS-PAGE of the BC-1 isolate were compared to those of the B-31 reference strain. The major proteins, including the 31-kDa OspA and the 41-kDa flagellin, were identical to those previously reported for the B-31 strain (Barbour, 1984b). However, the BC-1 isolate differed in composition of the outer surface proteins compared to the B-31 reference strain (Fig. 1). There was a shift in the Osp proteins to a single band (OspX) at ≈32 kDa in the BC-1 isolate, compared to clearly resolved 31-kDa OspA and 34-kDa OspB bands in the B-31 strain (Fig. 1). With the exception of

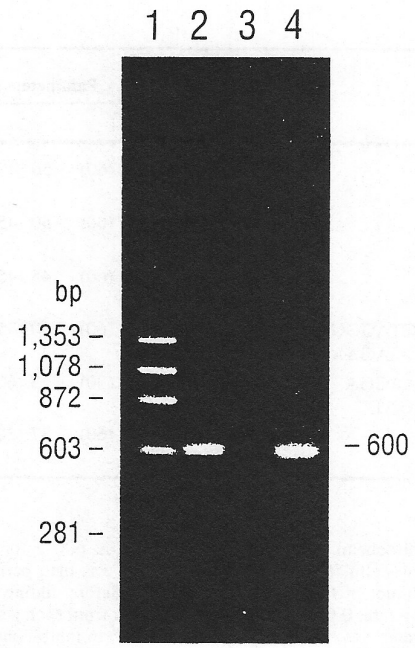

FIGURE 2. Agarose gel electrophoresis of amplified PCR products using primers for the *fla* gene of *B. burgdorferi*. Lane 1, DNA size standards; lane 2, *B. burgdorferi* B-31 (positive control); lane 3, sterile distilled water (negative control); lane 4, BC-1 isolate.

the single unique OspX band, other major proteins of the BC-1 isolate were similar to those of the B-31 reference strain.

Using PCR, the B-31 reference strain of *B. burgdorferi* consistently amplified DNA sequences specific for the *fla* primers (245/855), the chromosomal primers (147/520), and 4 sets of *ospA* primers (788/946; 149/319, 149/459, 3′/5′). The BC-1 *I. dentatus* isolate amplified DNA sequences specific for the 245/855 *fla* and the 149/319, 149/459, and 3′/5′ *ospA*, but not the 788/946 *ospA* nor the 147/520 chromosomal gene sequences. Agarose gel electrophoresis of amplified PCR products using primers for the *fla* gene of *B. burgdorferi* is illustrated in Figure 2.

DISCUSSION

Antigenically variable *B. burgdorferi* strains from 71 immature *I. dentatus* and from 6 cottontail rabbits in rural (Millbrook) and urban areas (New York City) of New York were reported (Anderson et al., 1989) and recently designated as the genospecies *B. andersonii* (Marconi et al., 1995). All of the New York rabbit and *I. dentatus* isolates reacted with *Borrelia* (genus-specific) monoclonal antibody H9724, but more than half failed to react with OspA H5332 monoclonal antibody and even fewer with the OspA H3TS monoclonal antibody. Results obtained from some of these New York isolates agree with those obtained from the BC-1, *I. dentatus*, Georgia isolate that also failed to react with H5332, H3TS, and OspB H6831 monoclonals. It is possible that several of the New York isolates may have been "typical" rodent strain *B. burgdorferi*, but others were clearly different, and some may have been composed of mixed strains. Interestingly, all *B. burgdorferi* isolates from *I. dentatus* on Nantucket Island, Massachusetts, reacted with monoclonal antibody H5332 (Telford and Spielman, 1989). Although spirochetes from the Nantucket Island *I. dentatus* were not thoroughly characterized, their reaction to the 1 monoclonal antibody reported combined with serologic results of rabbits and the fact that Nantucket is an intense focus of rodent strain *B. burgdorferi* sensu stricto suggest that the spirochetes from Nantucket *I. dentatus* were probably "typical" rodent strains and different from the BC-1 Georgia isolate; they also were different from some of the spirochetes reported from *I. dentatus* and rabbits from New York (Anderson et al., 1989) and now designated *B. andersonii* (Marconi et al., 1995).

SDS-PAGE protein profiles of 2 isolates from cottontail rabbits and 1 from *I. dentatus* from New York differed from one another and from all previously characterized *B. burgdorferi* strains from humans, ticks, and wildlife in North America (Anderson et al., 1989). Isolate 19857 from rabbit 6 had an OspA with a molecular weight of ≈31,250 Da but no clear OspB. Isolate 19865 from rabbit 8 had a heavy OspA band at 31,000 and an OspB band at 35,500 Da, whereas, the New York *I. dentatus* isolate (19941) had molecular weights of 30,500 and 34,250 Da, respectively. Thus, the *I. dentatus* isolate from Georgia differed from the New York isolates and the B-31 reference strain.

The failure of 1 of the 4 sets of *ospA* primers (788/946) to amplify DNA sequences known to be present in the B-31 index strain could result from a difference in base sequences at the primer binding sites caused by either mutations, deletions, or insertions. This also could explain the shift in the *osp* molecular weight of the BC-1 isolate, as delineated by SDS-PAGE.

The geographic distribution of the unusual *B. burgdorferi* strains (*B. andersonii*) isolated from *I. dentatus* and rabbits in New York remains to be determined, as do their infectivity, range of vectors, and disease-producing effects. Inoculation of 1 of these strains failed to infect laboratory mice (J. F. Anderson, pers. comm.). Enzootic cycles of *B. burgdorferi* involving *I. dentatus* and cottontail rabbits on Nantucket Island, Massachusetts (Telford and Spielman, 1989), and *Ixodes ricinus* and hares in Sweden (Tälleklint and Jaenson, 1993) have been reported. The partial characterization of these spirochetes and abundance of human Lyme borreliosis cases suggest that the enzootic cycles on Nantucket and in Sweden involve "typical" rodent *B. burgdorferi* strains. Apparently, "atypical" *B. burgdorferi*, and perhaps also "typical" strains, occur in *I. dentatus* and cottontail rabbits in urban and rural New York (Anderson et al., 1989). The BC-1 *B. burgdorferi* strain from *I. dentatus* in Georgia reported here, although not yet isolated from cottontails, almost certainly infects them because *I. dentatus* feeds primarily on rabbits (Smith, 1945; Keirans and Clifford, 1978). Infectivity of BC-1 to mice, hamsters, guinea pigs, and laboratory rabbits is now being investigated. Recently, we (J. H. Oliver, Jr., T. M. Kollars, A. M. James, C. W. Banks, unpubl. obs.) have isolated 12 strains of *B. burgdorferi* sensu lato from *I. dentatus* in Missouri and characterization of them is in progress. Preliminary IFA screening using *B. burgdorferi*-specific monoclonal antibodies suggests at least 4 immunologic types; PCR assay of several known DNA target sequences found in *B. burgdorferi* indicates that there are at least 3 molecular types.

It is unknown if antibodies to *B. burgdorferi* in jackrabbits from Texas (Rawlings, 1986) and California (Lane and Burg-

dorfer, 1988), and in other jackrabbits and cottontail rabbits from Texas (Burgess and Windberg, 1989), were due to "typical" rodent strains and/or "atypical" *I. dentatus*/rabbit strains of *B. burgdorferi*, or even possibly some other *Borrelia* spp. *Borrelia* spp. were isolated from 2 of 10 black-tailed jackrabbits from Texas, but cultures were contaminated and detailed characterization of the spirochetes was not done (Burgess and Windberg, 1989).

Published reports indicate more heterogeneity among European and California isolates of *B. burgdorferi* than among northeastern and north central American isolates (Barbour et al., 1985; Bissett et al., 1988; Wilske et al., 1988; Lane and Pascocello, 1989; Brown and Lane, 1992); the OspB protein showed more strain variability than OspA (Barbour, 1984b; Barbour et al., 1984, 1985; Barbour and Schrumpf, 1986). The OspA and OspB proteins, as revealed by SDS-PAGE, and differences in reactivity with monoclonal antibodies vary among some strains. Recently, a much greater degree of heterogeneity was found among *B. burgdorferi* isolates from the southern United States (Oliver et al., 1993, 1995).

Disease and its persistence in a susceptible host may be due, in part, to antigenic variation of the outer surface proteins of pathogenic microorganisms (Jonsson et al., 1992). The presence of clonal polymorphism of the OspB protein (Schwan and Burgdorfer, 1987; Bundoc and Barbour, 1989) and the finding that some Lyme borreliosis patients develop new antibodies directed against the OspB protein a year or more after initial infection suggest that antigenic variation of Osp proteins also might occur in *B. burgdorferi* (Craft et al., 1986).

Interestingly, stability of SDS-PAGE protein profiles and antigenicity of different *B. burgdorferi* strains were reported to change when the spirochetes were reintroduced into *I. ricinus* ticks (Hu et al., 1992). Initial isolates and reisolates of 8 Swiss strains and the B-31 North American strain of *B. burgdorferi* were compared by using protein profiles and reaction to western blot analysis of the strains to monoclonal antibody H5332 and a monospecific polyclonal antibody PoAb/anti-22 kDa. It appears that the changes reported were modifications due to effects of the internal environment of the tick; alternatively, perhaps differences were due to selection of a single strain from cultures that contained more than 1 strain. Strains NE56 and the North American B-31 did not show any changes after reintroduction with respect to the proteins with molecular masses of 31, 34, and 22–23 kDa.

ACKNOWLEDGMENTS

We thank M. S. Joiner for editorial and other assistance, C. W. Banks for tick and host colony maintenance, R. S. Lane for antibody screening, M. A. Wanchick for assistance with the laboratory cultures of spirochetes, and B. S. McGuire for performing SDS-PAGE. We are grateful to Barbara Johnson, Center for Infectious Diseases, Centers for Disease Control and Prevention, Ft. Collins, Colorado, David Persing, Mayo Clinic, Rochester, Minnesota, and Patricia Rosa, Rocky Mountain Laboratories, National Institutes of Health, Hamilton, Montana, for supplying primers for genetic analysis. Thanks to L. A. Durden for reviewing this manuscript. The research was supported in part by National Institutes of Health grants AI-09556, AI-24899, and AI-30026 to Georgia Southern University, Centers for Disease Control and Prevention Cooperative Agreement U50/CCU406614 to the Medical College of Georgia, and U50/CCU410281 to Georgia Southern University. Contents of this paper are solely the responsibility of the authors and do not necessarily represent the official views of CDC or NIH.

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