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Reactivity of Human Lyme Borreliosis Sera with a 39-Kilodalton Antigen Specific to *Borrelia burgdorferi*

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***Borrelia burgdorferi* is the causative agent of Lyme borreliosis, a spirochetal illness with a variety of acute clinical manifestations that may lead to debilitating neurological and arthritic complications. Diagnosis is difficult because symptoms mimic a variety of unrelated clinical conditions, spirochetes cannot always be isolated from infected patients, and current serological tests are frequently inconclusive because of the presence of cross-reacting non-*B. burgdorferi* antibodies. To identify antigens specific to *B. burgdorferi* that could be used in the serodiagnosis of Lyme borreliosis, we screened a *Borrelia* DNA expression library in *Escherichia coli* for antigens reactive with human Lyme borreliosis sera. One clone carried a 6.3-kilobase *Eco*RI chromosomal fragment (pSPR33), which encoded two species-specific antigens with molecular masses of 28 (P28) and 39 (P39) kilodaltons (kDa). These two antigens were immunologically distinct from OspA, OspB, and the 41-kDa flagellin. Ninety-four serum specimens from patients having Lyme borreliosis were tested for reactivity with P39. All of 33 the serum specimens with immunofluorescence assay titers of ≥1:256, 13 of 17 serum specimens with titers of 1:128, and 14 of 44 serum specimens with titers of ≤1:64 reacted with P39. Notably, many sera reactive to P39 did not appear to react with the 41-kDa flagellin. Therefore, antibody to P39 could be mistaken for antibody to the 41-kDa flagellin in tests of human sera by Western blot (immunoblot). Twenty-five control serum specimens, which included sera from syphilitic, relapsing fever, and amyotrophic lateral sclerosis patients as well as from 10 normal individuals, did not react to P39. Our data suggest that P39 may be a useful antigen for the serological confirmation of Lyme borreliosis.**

Lyme borreliosis in humans is a multisystemic disorder caused by infection with the tick-borne spirochete, *Borrelia burgdorferi* (11, 25, 40). Since the first epidemiological investigations of this disease in south-central Connecticut (40–42), cases of Lyme borreliosis in humans have now been reported in 43 states of the United States (12), five provinces of Canada (13), numerous countries throughout Europe and Asia (1, 18, 32), and possible restricted foci in Australia (43) and Africa (20, 39). Between 1982 and 1988, reports of 13,825 cases of Lyme borreliosis were received by the Centers for Disease Control, Atlanta, Ga., from all 50 states of the United States (12), making this disease the most prevalent arthropod-borne infection in the country.

With the dramatic increase in awareness, prevalence, and geographical distribution of Lyme borreliosis, a tremendous new demand has been placed on clinical laboratories to serologically confirm cases (36; L. A. Magnarelli, Editorial, J. Am. Med. Assoc. **262**:3464–3465, 1989) or to rule out this disease in differential diagnoses. However, many potential problems exist with the currently available serological tests for Lyme borreliosis, which may result in either false-positive or false-negative results (L. A. Magnarelli, Editorial, J. Am. Med. Assoc. **262**:3464–3465, 1989). Some studies have focused on using the flagellar protein of *B. burgdorferi* to increase the sensitivity of serological tests (21, 22) because earlier studies demonstrated that it appeared to be the 41-kilodalton (kDa) flagellar subunit (flagellin) of the spirochete that generated the earliest antibody response in infected humans (5, 14, 19). One of two potential problems with using flagellar protein, however, is that flagella of other *Borrelia* species (6) and possibly *Treponema pallidum* (10, 15) share epitopes common to the flagella of *B. burgdorferi*. Secondly, in most studies that have screened human sera by immunoblot analysis (5, 14, 16, 29), antibodies bound the protein that has an apparent migration of 41-kDa and has been assumed, but not proven, to be flagellin.

In our efforts to develop a better serological test for Lyme borreliosis, we have cloned a 39-kDa nonflagellar antigen (P39) of *B. burgdorferi* in *Escherichia coli*. This antigen is immunoreactive with most sera from human patients with Lyme borreliosis and may represent an antigenic component of the spirochete that at times has been mistaken for the 41-kDa flagellin.

MATERIALS AND METHODS

Bacterial strains. *B. burgdorferi* strains used in this study (Table 1) have been previously described or were kindly provided by John Anderson (Connecticut Agriculture Experiment Station, New Haven), Alan MacDonald (Southampton Hospital, Southampton, N.Y.), and Glenna Teltow and Julie Rawlings (Medical Entomology Section, Bureau of Laboratories, Texas Department of Health, Austin). The five strains representing *Borrelia hermsii* (HS1), *Borrelia coriaceae* (Co53), *Borrelia parkeri*, *Borrelia turicatae*, and *Borrelia anserina* have been described previously (35). Borrelia organisms were cultured at 32°C in BSK-II medium as previously described (2).

DNA purifications. Total DNA was purified from 500-ml stationary-phase borrelial cultures by a modification of a previously described method (4). Cells were recovered by centrifugation, washed in 20 ml of phosphate-buffered saline plus 5 mM $MgCl_2$, and suspended in 2.4 ml of TES (50 mM Tris, pH 8.0; 50 mM EDTA; 15% [wt/vol] sucrose). Lysozyme was added to a final concentration of 1 mg/ml, and then the cell suspension was left on ice for 10 min. Cells were lysed by addition of 3 ml of TES plus 1% (vol/vol) sodium

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TABLE 1. Summary of *B. burgdorferi* strains used in this study, all of which expressed P28 and P39

Strain	Biological source[a]	Geographical source (yr isolated)	Source of strain (reference)	In vitro passage[b]
Sh-2-82 (P6)	Id	New York (1982)	Schwan et al. (33)	L
Sh-2-82 (P246)	Id	New York (1982)	Schwan et al. (33)	H
B31	Id	New York (1982)	Schwan et al. (33)	H
CA-2-87	Ip	California (1987)	Schwan et al. (33)	L
CA-3-87	Ip	California (1987)	Schwan et al. (33)	L
NY-1-86	H	New York (1986)	Schwan et al. (33)	L
ECM-NY-86	H	New York (1986)	Schwan et al. (33)	L
NY-6-86	H	New York (1986)	A. MacDonald	L
NY-13-86	H	New York (1986)	A. MacDonald	L
CT20004	Ir	France (1985)	J. Anderson	L
CT22921	Rp	New York (1986)	J. Anderson	L
CT26816	Rm	Rhode Island (1985)	J. Anderson	L
CT19678	Rp	New York (1986)	J. Anderson	L
CT21343	Rp	Wisconsin (1986)	J. Anderson	L
CT21305	Rp	Connecticut (1986)	J. Anderson	L
CT21721	Id	Wisconsin (1986)	J. Anderson	L
CT27985	Id	Connecticut (1988)	J. Anderson	L
TX1352	Aa	Texas (1989)	J. Rawlings	H
PE92	D	Texas (1989)	J. Rawlings	H
BR4-3028	H	Texas (1989)	J. Rawlings	H

[a] Id, Tick (*Ixodes dammini*); Ip, tick (*Ixodes pacificus*); Ir, tick (*Ixodes ricinus*); Aa, tick (*Amblyomma americanum*); H, human; Rp, rodent (*P. leucopus*); Rm, rodent (*Microtus* sp.); D, dog.

[b] L, Low (strains passed for ≤10 passages); H, high (strains passed for ≥20 passages).

deoxycholate and gently mixed for 10 min at room temperature. Proteinase K (1 mg) was then added, and the sample was incubated at 37°C for 1 h. The DNA suspension was then extracted twice with 1 volume of phenol-chloroform (1:1 [vol/vol]) and once with chloroform-isoamyl alcohol (24:1 [vol/vol]). The DNA was ethanol precipitated, washed twice with 70% ethanol, and suspended to a final concentration of 1 mg/ml in TE (10 mM Tris, pH 7.6; 1 mM EDTA).

Recombinant plasmid pSPR33 was isolated from *E. coli* for mapping studies from 500-ml cultures and purified as previously described (37), except that two consecutive dye-buoyant density gradients were performed (30) in a VTi80 rotor (Beckman Instruments, Inc., Fullerton, Calif.) at 70,000 rpm for 4 h at 18°C. The supercoiled circular plasmid portion was diluted with 2 volumes of water after removal of the ethidium bromide and then ethanol precipitated. The plasmid DNA was then suspended in a minimal volume of TE.

Agarose gel electrophoresis and Southern blot analysis. Undigested total DNA was electrophoresed in 0.4% agarose gels (12 V for 16 h). Southern blot procedures, including the transfer of DNA from agarose gels to nitrocellose, high-stringency hybridization (which permitted 10% base pair mismatch), and autoradiography, were as previously described (38), except that the prehybridization and hybridization buffers and temperatures were as described by Schwan et al. (35). The DNA probe was recovered from agarose gels by using GeneClean (BIO 101, Inc., La Jolla, Calif.) and labeled with [α-^{32}P]dCTP (3,000 Ci/mmol) by nick translation according to the directions of the manufacturer (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). The probe was boiled for 4 min and quenched on ice immediately before addition to the hybridization buffer.

Restriction endonucleases were purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and digestions were performed as recommended by the manufacturer.

Construction and screening of DNA library. Total DNA (1 μg) from *B. burgdorferi* Sh-2-82, which had been passaged no more than six times, was digested with *Eco*RI, ligated to the dephosphorylated arms of the expression vector λZAPII (Stratagene, La Jolla, Calif.) and packaged and plated according to the directions of the manufacturer.

Recombinant plaques were screened by immunoblot with a human Lyme borreliosis serum (1:100) from Long Island, N.Y., following adsorption of plaque antigens to nitrocellulose filters (28). After blocking for 1 h at 25°C in TSE-Tween (50 mM Tris, pH 7.4; 150 mM NaCl; 5 mM EDTA; 0.05% Tween 20), filters were incubated with serum diluted in TSE-Tween with gentle rocking at 25°C for 1 h. They were then washed for 1 h with four changes of TSE-Tween, and the bound antibody was detected by incubating the filters with ^{125}I-labeled protein A (500,000 cpm/ml) for 1 h with rocking. Each filter was then washed four times for 15 min each time with TSE-Tween, dried, and autoradiographed with X-AR5 film (Eastman Kodak Co., Rochester, N.Y.). A recombinant plaque that reacted with human serum was plaque purified, and the phagemid carrying the *Borrelia* DNA was excised from the λ sequences with the aid of the helper phage R407 according to the directions of the supplier (Stratagene). Miniplasmid preparations (28) of positive clones were examined by agarose gel electrophoresis after their digestion with *Eco*RI to determine the insert size.

Serological reagents and Western blot (immunoblot) analysis. Rabbit sera prepared against whole-cell lysates of *E. coli* carrying either pSPR33 (anti-pSPR33) or the vector pBluescript SK (anti-*E. coli*) were prepared as follows. Bacterial cells were recovered from 16-h cultures, washed once, and suspended in phosphate-buffered saline to a final concentration of 10^8 cells per ml. The cells were killed by incubation for 30 min at 56°C and disrupted by sonication on ice (2 min at an output of 4; sonifier-cell disrupter model 185, Branson Sonic Power Co., Danbury, Conn.). New Zealand White rabbits were immunized (without adjuvant) intramuscularly with 1.5 ml of the cell sonic extract and boosted with the same immunogen at 21 and 42 days after the primary immunization. Sera were collected every 2 weeks thereafter for 4 months and pooled, and 5-ml samples were adsorbed

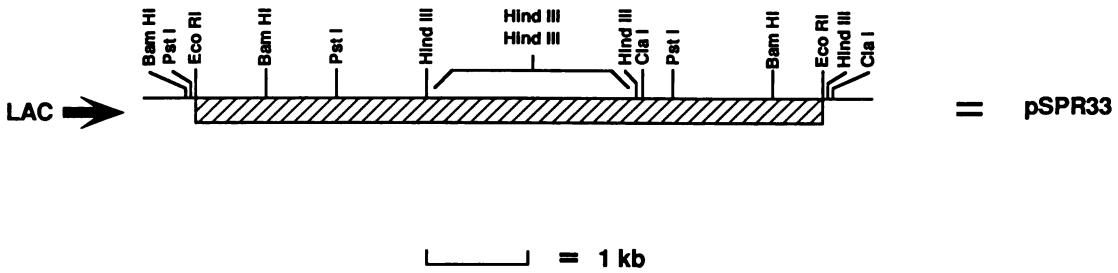

FIG. 1. Genetic map of pSPR33. Spirochete DNA is denoted (▨), and the arrow indicates the direction that the *Lac* promoter was transcribed. There were no restriction sites within the spirochete *Eco*RI fragment for *Acc*I, *Kpn*I, *Xba*I, *Xho*I, or *Sma*I. The *Hin*dIII fragment indicated by the bracket had two internal *Hin*dIII sites that were not mapped.

with *E. coli* XL1-blue cells (Stratagene) collected from 500-ml cultures and incubated with rotation at 37°C for 4 h. The bacteria were removed by centrifugation in a VTi80 rotor at 40,000 rpm for 30 min. This process was repeated twice, and adsorbed sera were then filtered through a sterile, 0.22-μm-pore-size filter (Millipore Corp., Bedford, Mass.) and stored at −20°C. Anti-pSPR33 and anti-*E. coli* sera were used at dilutions of 1:500 and 1:50, respectively. Tissue culture supernatants were the source of monoclonal antibodies H5332 (9), H5TS (8), and H9724 (6) and were used at a dilution of 1:100.

Human syphilitic sera were kindly provided by Wayne Hogrefe and Jane Markley (Hillcrest Biologicals, Cypress, Calif.), amyotrophic lateral sclerosis sera were provided by Jeffrey Smith (ALS Clinic, Mount Sinai Medical Center, New York, N.Y.) and Alan MacDonald, and relapsing fever sera were collected from patients from Oregon and Washington. Normal sera were obtained from staff and laboratory personnel at Rocky Mountain Laboratories. Human Lyme borreliosis sera were provided by Alan MacDonald and were collected from patients clinically diagnosed with Lyme borreliosis from Long Island, N.Y.

Immunofluorescence assay (IFA) titers of Lyme borreliosis and relapsing fever sera were determined as previously described (11). *B. burgdorferi* B31 and *B. hermsii* HS1, respectively, were used as the antigens in the IFAs.

Immunoblot analyses of whole-cell lysates were performed essentially as previously described elsewhere (34) except that cells were prepared as follows. Cells were recovered from liquid cultures by centrifugation (8,000 × *g* for 5 min) and suspended in phosphate-buffered saline to give an optical density of 0.2 at 600 nm. Cells from 2 ml of this suspension were recovered by centrifugation and suspended in 100 μl of distilled water and 50 μl of sample buffer (0.2 M Tris [pH 6.8], 30% [vol/vol] glycerol, 3% [wt/vol] sodium dodecyl sulfate [SDS], 0.002% [wt/vol] bromophenol blue). Samples were then boiled for 4 min, and 20 μl was loaded onto a 12.5% SDS-polyacrylamide gel. Gel electrophoresis, immunoblotting, and detection of bound antibody with ^{125}I protein A have been described previously (34).

Immune electron microscopy. Intact flagella were isolated from cultures of *B. burgdorferi* Sh-2-82 as previously described (6). Strain Sh-2-82 had been passaged in vitro six times. Flagella were adsorbed onto grids, and bound antibody was detected as described elsewhere (8). Tissue culture supernatants were the source of the flagellum-specific monoclonal antibody H9724 and were diluted 1:100. Anti-pSPR33 antiserum was used at a dilution of 1:50.

RESULTS

Cloning and genetic analysis of *Borrelia* DNA. To identify *B. burgdorferi* antigens that induce an antibody response during the course of infection, a DNA library of *B. burgdorferi* containing *Eco*RI fragments was constructed in *E. coli* with the λ expression vector λZAPII. The library was screened for *Borrelia* antigens with a convalescent-phase serum from a human Lyme borreliosis patient (IFA titer, 1:1,024). Positive clones were detected at a frequency of 5%, and one was plaque purified to yield a homogeneous bacteriophage preparation. Excision of the cloned fragment from the purified phage produced the phagemid portion containing a 6.3-kilobase-pair (kb) *Eco*RI fragment, designated plasmid pSPR33 (Fig. 1). The fragment was isolated from an agarose gel, radiolabeled, and shown to hybridize with a similar-size fragment in *Eco*RI-digested total DNA from six North American *B. burgdorferi* isolates and one European isolate (Fig. 2). Southern blot analysis of undigested DNA from seven

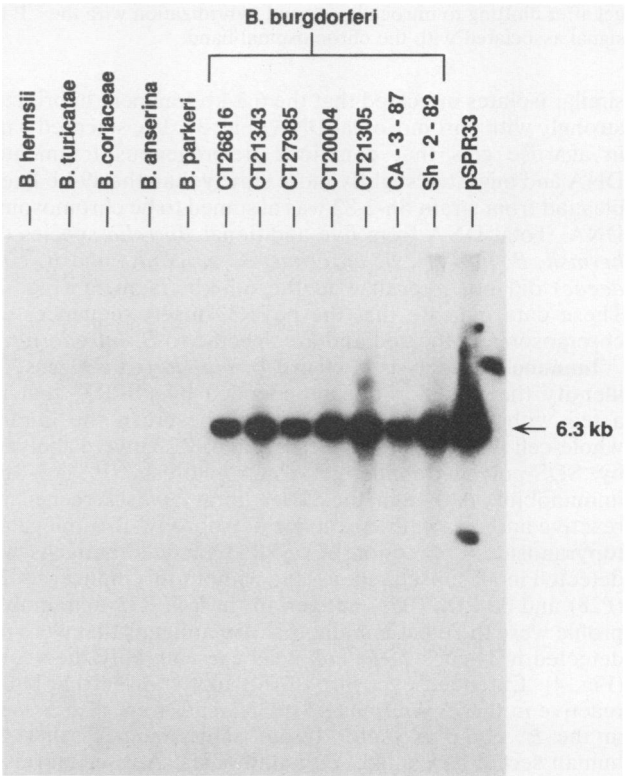

FIG. 2. Autoradiograph showing hybridization of ^{32}P-labeled insert DNA from pSPR33 with total DNA digested with *Eco*RI from seven isolates of *B. burgdorferi* and five other *Borrelia* species. The right lane contained pSPR33 digested with *Eco*RI. A linear molecular size marker is indicated on the right.

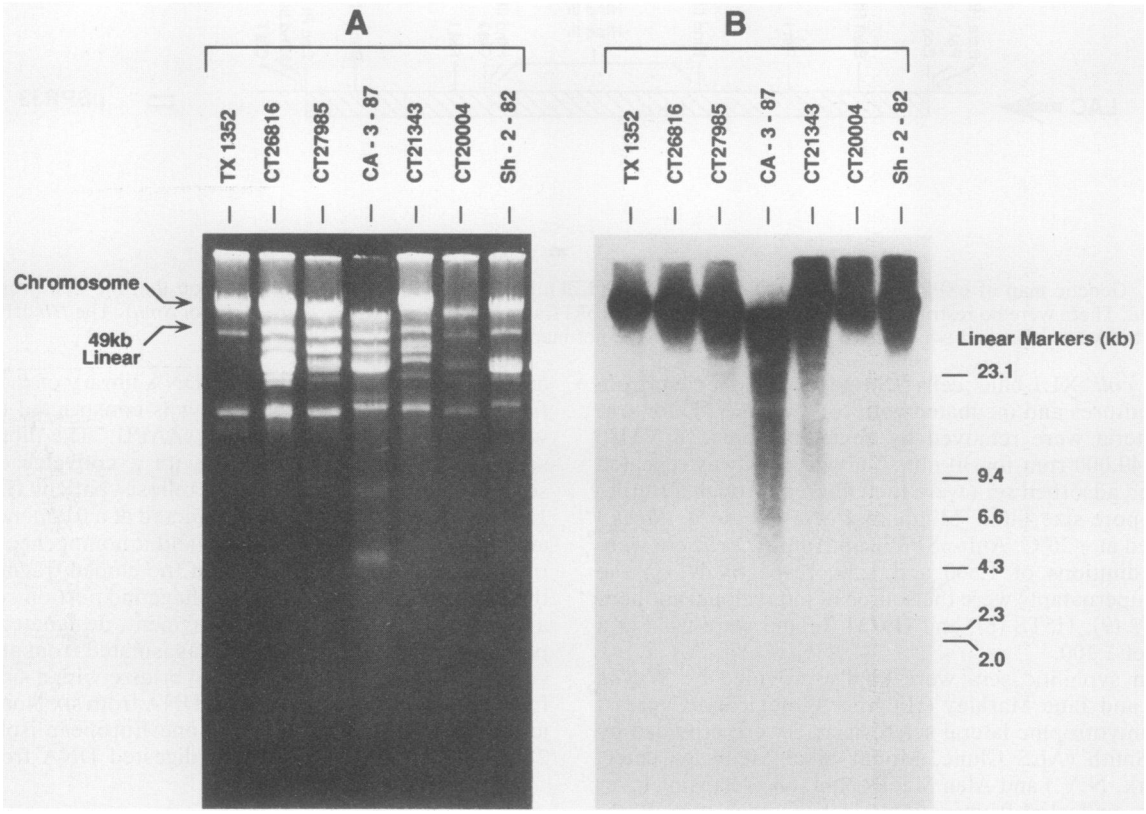

FIG. 3. (A) Ethidium bromide-stained gel of undigested total DNA from seven isolates of *B. burgdorferi*. (B) Autoradiograph of the same gel after blotting to nitrocellulose and hybridization with the ^{32}P-labeled 6.3-kb *Eco*RI fragment from pSPR33. Note the strong hybridization signal associated with the chromosomal band.

similar isolates indicated that the 6.3-kb fragment hybridized strongly with chromosomal DNA (Fig. 3). The smeared band in agarose gels that contained heterogenous fragmented DNA and migrated slightly more slowly than the 49-kb linear plasmid from strain Sh-2-82 was assumed to be chromosomal DNA. Total DNA from five additional *Borrelia* species (*B. hermsii*, *B. parkeri*, *B. anserina*, *B. turicatae*, and *B. coriaceae*) did not hybridize to the 6.3-kb fragment (Fig. 2). These data indicate that the pSPR33 insert sequences are chromosomally located and are specific to *B. burgdorferi*.

Immunoblot analysis of cloned *B. burgdorferi* antigens. To identify the specific antigens encoded by pSPR33 that reacted with the human serum used to screen the library, whole-cell lysates of *E. coli* carrying pSPR33 were analyzed by SDS-polyacrylamide gel electrophoresis (PAGE) and immunoblot. Although the DNA library was screened for reactive antigens in the presence of isopropyl-β-D-thiogalactopyranoside, expression of pSPR33-encoded antigens was detected in all subsequent assays without this inducer. A 28- (P28) and 39-kDa (P39) antigen in the pSPR33 immunoblot profile were the most immunoreactive antigens that were not detected in lysates of *E. coli* cells carrying only the vector (Fig. 4). Extended exposure of this blot showed that bands reactive in the *E. coli*-plus-pSPR33 lysate were also present in the *E. coli*-plus-vector lysate. This indicates that the human serum had anti-*E. coli* antibodies. Antiserum raised to whole-cell lysates of *E. coli* carrying only the vector (anti-*E. coli* serum) did not react with P28 or P39 at a dilution of 1:50 but did react with the other bands that bound human antibodies (data not shown). P28 and P39, therefore, are antigenically unrelated to native *E. coli* components and appear to be encoded by the cloned *Borrelia* sequences. P28 and P39 could not be observed in SDS-PAGE gels stained with either Coomassie blue or silver nitrate (data not shown) because they comigrated with other, more abundant *E. coli* antigens.

Antigens similar in size to P28 and P39 were detected by immunoblot (Fig. 4) in cell lysates of *B. burgdorferi* Sh-2-82, suggesting that P28 and P39 are expressed by this strain. To determine whether the 28- and 39-kDa *Borrelia* antigens seen in whole cell lysates were identical to the gene products P28 and P39, respectively, antiserum generated to cells carrying pSPR33 (anti-pSPR33 serum) was incubated with Western-blotted whole-cell lysates of 1 European and 19 North American *B. burgdorferi* isolates and compared with a lysate of *E. coli* producing P28 and P39 (Fig. 5). All of the 20 *Borrelia* isolates expressed a 39-kDa antigen that comigrated with P39. A 28-kDa antigen was also detected, but considerably less antibody bound this antigen than that which bound P39. P39 produced by pSPR33 also reacted with sera from five white-footed mice (*Peromyscus leucopus*) experimentally infected with *B. burgdorferi* Sh-2-82 but did not react with the preimmune sera from these animals or with sera from mice infected only with *E. coli* (data not shown). Other *Borrelia* species did not produce detectable amounts of P28, P39, or any other antigenically related components under the conditions employed (Fig. 5). Extended exposure (>24 h) of autoradiographs revealed weak bands with molecular masses other than 28 and 39 kDa in all *Borrelia* profiles, but these are attributed to nonspecific binding. Our data, including the fact that DNA from other *Borrelia* species lacked sequences with close identity to those that

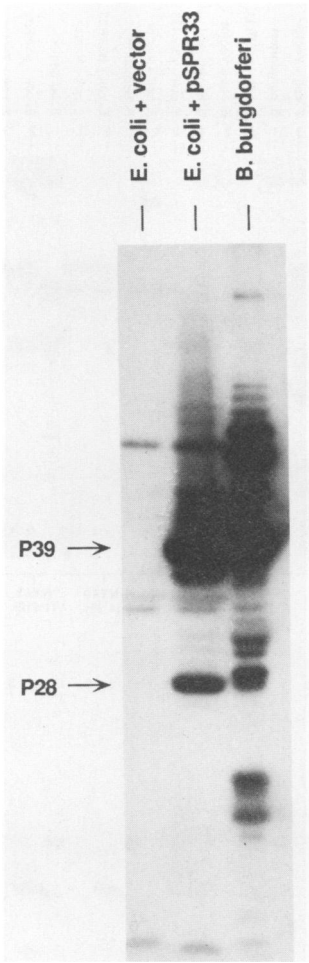

FIG. 4. Immunoblot analysis of proteins expressed by pSPR33. Whole-cell lysates of *B. burgdorferi* Sh-2-82, *E. coli* carrying pSPR33, and *E. coli* carrying only vector were immunoblotted with the human Lyme borreliosis serum used to screen the DNA library of *B. burgdorferi*.

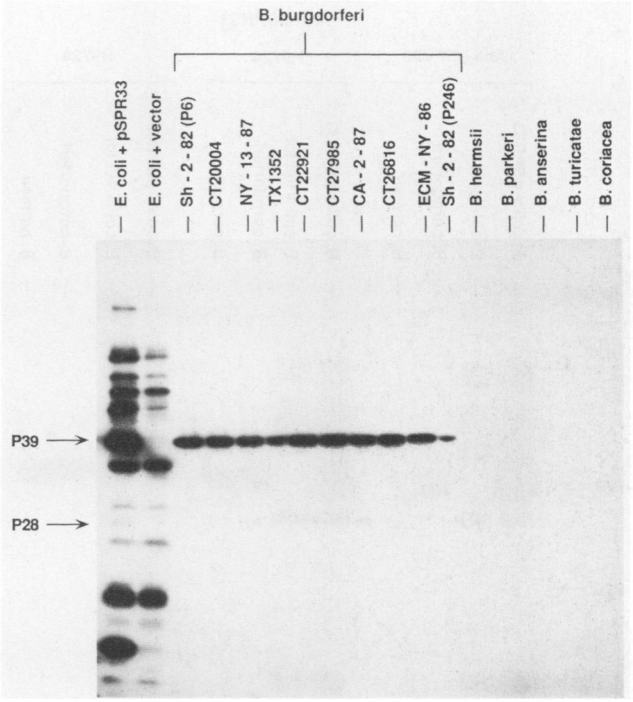

FIG. 5. Specificity of P28 and P39 expression in *B. burgdorferi*. Whole-cell lysates of different *B. burgdorferi* strains [including low (P6) and high (P246) in vitro passages of strain Sh-2-82] and isolates representing five additional *Borrelia* species were immunoblotted with anti-pSPR33 serum. Lysates of *E. coli* that expressed P28 and P39 (*E. coli* + pSPR33) and that did not (*E. coli* + vector) were also immunoblotted as positive and negative controls, respectively. Not all of the 20 *B. burgdorferi* isolates tested are shown (Table 1).

encode P28 and P39 (Fig. 2), show that P28 and P39 are antigens specific to *B. burgdorferi*. Furthermore, anti-pSPR33 did not react with the *B. burgdorferi* antigens OspA (31 kDa), OspB (34 kDa), or the 41-kDa flagellin (Fig. 5 and 6), suggesting that these antigens are immunologically unrelated to P28 and P39. To confirm this, we showed that the monoclonal antibodies H5332, H5TS (data not shown), and H9724 (Fig. 6), which bind specifically to OspA (9), OspB (8), and flagellin (6), respectively, did not bind to P28 or P39 produced by either pSPR33 or strain Sh-2-82. The specificity of monoclonal antibody H9724 for *Borrelia* flagellin is evident in Fig. 6, as it bound only a 41-kDa band in the *B. burgdorferi* profile and a 39-kDa band, which corresponds to its flagellin (6), in the *B. hermsii* profile. Furthermore, as revealed by electron microscopy and colloidal gold staining, monoclonal antibody H9724 bound to endoflagella from *B. burgdorferi*, whereas anti-pSPR33 did not (data not shown).

Immunoreactivity of Lyme borreliosis sera with cloned *Borrelia* antigens. To test the possibility that P28 and P39 are recognized by the sera of Lyme borreliosis patients, 94 serum specimens collected from patients clinically diagnosed as having Lyme borreliosis were tested for reactivity with cloned P28 and P39 at a dilution of 1:100. Whole-cell lysates were electrophoresed in SDS-PAGE gels and Western blotted. The nitrocellulose was cut into equal strips (five per gel) such that each strip contained lanes for *E. coli* carrying pSPR33, *E. coli* carrying only the vector, and *B. burgdorferi* Sh-2-82. Each strip was incubated with a different human serum, except one strip from each gel was incubated with anti-pSPR33 serum. This strip served as a marker for the positions of P28 and P39. All of 33 serum specimens with IFA titers of $\geq$1:256 (100%), 13 of 17 serum specimens (76%) with IFA titers of 1:128, and 14 of 44 serum specimens (32%) with titers of $\leq$1:64 reacted with P39 (Table 2). Examples of immunoblots for human sera reacting with P39 are shown in Fig. 7 (arrow 1). A strongly reacting 58- to 64-kDa band was observed in the *B. burgdorferi* profile (Fig. 7, band A) for all sera that reacted with P39, but since anti-pSPR33 serum did not react to a band in this region of the gel (Fig. 5), P39 and the 58- to 64-kDa protein(s) are presumably unrelated. Although P28 appeared to react strongly to some sera (Fig. 7B, band B), for other, less reactive sera, it was not clear if the sera reacted to P28 or to some other protein. This was because these sera also reacted with comigrating *E. coli* antigens that were detected with a longer autoradiographic exposure (Fig. 7A, band B). Therefore, although it is not clear to what extent P28 actually reacts with human Lyme borreliosis sera, it appears that antibody to P39 was detected in 100% of all sera that had IFA titers of $\geq$1:256. Notably, many sera reactive to P39 did not appear to react with the 41-kDa flagellin (Fig. 7). Yet, all sera that reacted within the 41-kDa band also reacted with P39, although the latter antigen was often obscured in *B.*

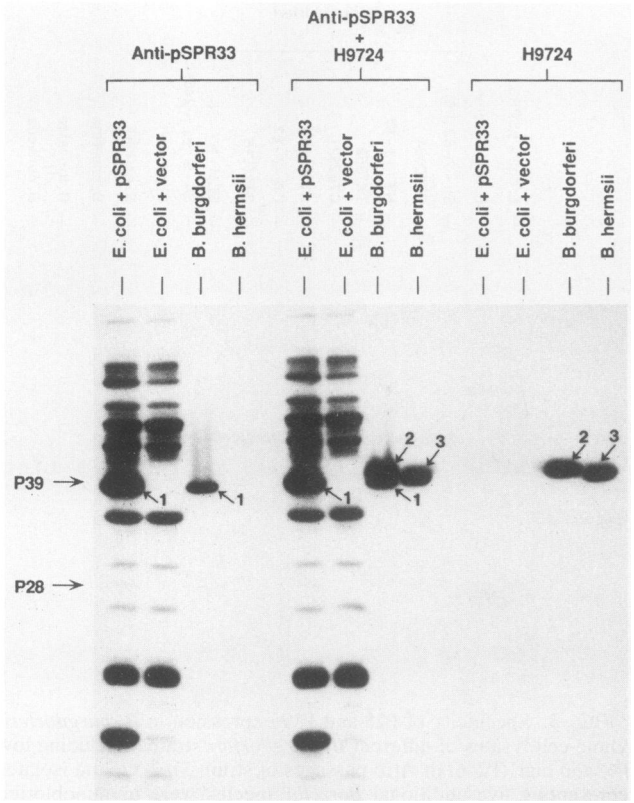

FIG. 6. Comparison of reactivity of anti-pSPR33 and monoclonal antibody H9724. Components in whole-cell lysates of *E. coli* plus pSPR33, *E. coli* plus vector only, *B. burgdorferi* Sh-2-82, and *B. hermsii* HS1 were separated by SDS-PAGE. After Western blotting, the nitrocellulose was cut into three strips such that each portion contained a lane representing one of the four whole-cell lysates. The three strips were incubated with anti-pSPR33, anti-pSPR33 plus H9724, or H9724. Arrows: 1, P39; 2, 41-kDa flagellin from *B. burgdorferi*; 3, 39-kDa flagellin from *B. hermsii*.

burgdorferi profiles by the signal associated with the 41-kDa band. In view of this, antibody to P39 could be mistaken as antibody to flagellin in tests of human sera by immunoblot with whole-cell lysates of *B. burgdorferi*. Because P39 was shown to be specific to *B. burgdorferi* by immunoblot, it is not surprising that control sera, which included sera from 5 amytrophic lateral sclerosis patients, 5 syphilitic patients, 5 relapsing fever patients, and 10 normal individuals who showed no symptoms of clinical disease, did not react to the

TABLE 2. Summary of human Lyme borreliosis sera tested for reactivity with P39

IFA titer	No. of sera		% Sera positive
	Tested	Reacting with P39	
≥1:2,048	5	5	100
1:1,024	8	8	100
1:512	9	9	100
1:256	11	11	100
1:128	17	13	76
1:64	10	4	40
1:32	9	5	55
≤1:16	25	5	20

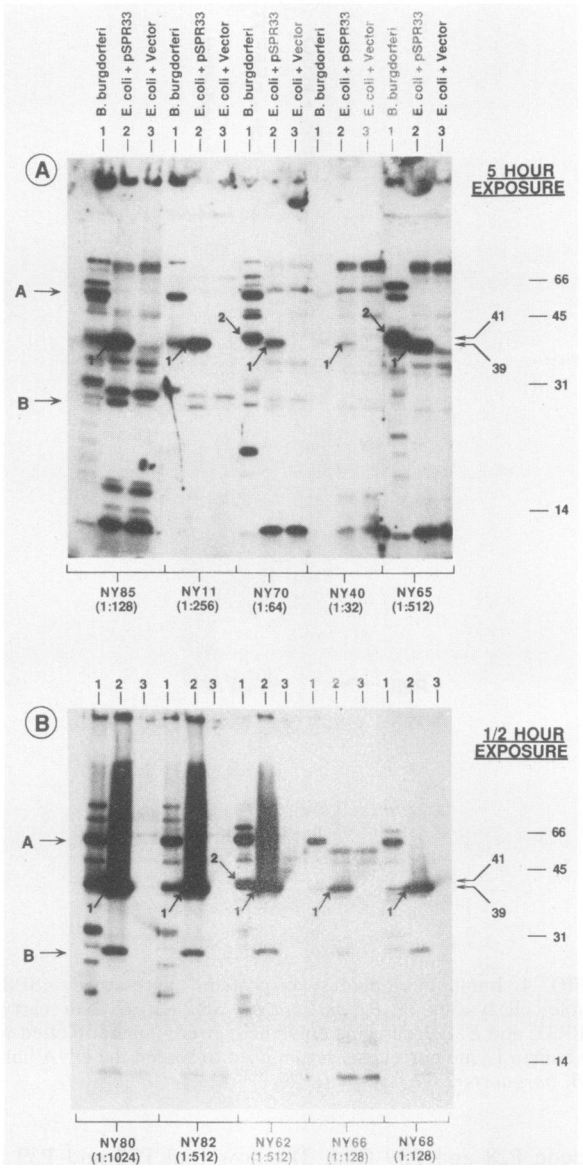

FIG. 7. Immunoblot analysis of 10 human Lyme borreliosis sera and their reactivity with P28 and P39. Whole-cell lysates of *B. burgdorferi* Sh-2-82 (lanes 1), *E. coli* carrying pSPR33 (lanes 2), and *E. coli* carrying only vector (lanes 3) were immunoblotted with human Lyme borreliosis sera (NY). Anti-pSPR33 reactivity, which identified the position of the band corresponding to P39, is not shown. IFA Lyme borreliosis titers for each human serum are indicated below their designations. Autoradiographs exposed for 5 h (A) represented sera having weaker reactivity than those exposed for 0.5 h (B). Arrows: 1, P39; 2, a 41-kDa antigen. Band B corresponds to the position of P28, and band A is a 58- to 64-kDa antigen that bound all sera that reacted with P39. Molecular mass markers (in kilodaltons) are indicated on the right of each panel.

cloned P39 protein at a dilution of 1:50 (Table 3). Immunoblot findings for the syphilitic sera are shown in Fig. 8. These data suggest that P39 has antigenic specificity for sera collected from patients with Lyme borreliosis. This is despite the fact that both the syphilitic and relapsing fever sera tested had significantly high IFA Lyme borreliosis titers (Table 3) and therefore most likely contained cross-reacting antibodies directed at other *B. burgdorferi* antigens.

TABLE 3. Summary of IFA titers for control sera that did not react with P39

Serum sample	IFA titer		
	Lyme	Relapsing fever	Rapid plasma, reagin test[a]
Syphilitic			
1	1:128	1:256	1:128
2	1:256	1:1,024	1:128
3	1:1,024	1:2,048	1:128
4	1:512	1:1,024	1:64
5	1:128	1:1,024	1:32
Relapsing fever			
1	1:1,024	1:1,024	ND
2	1:32	1:5,12	ND
3	1:128	1:5,12	ND
4	1:64	1:5,12	ND
5	1:64	1:1,024	ND
Amytrophic lateral sclerosis			
1	1:16	1:64	ND
2, 3, 4	<1:16	<1:16	ND
5	1:16	1:16	ND
Normal			
1, 2, 3, 4	<1:16	1:16	ND
5, 6, 7, 8	<1:16	<1:16	ND
9, 10	<1:16	1:32	ND

[a] Reference 31. ND, Not done.

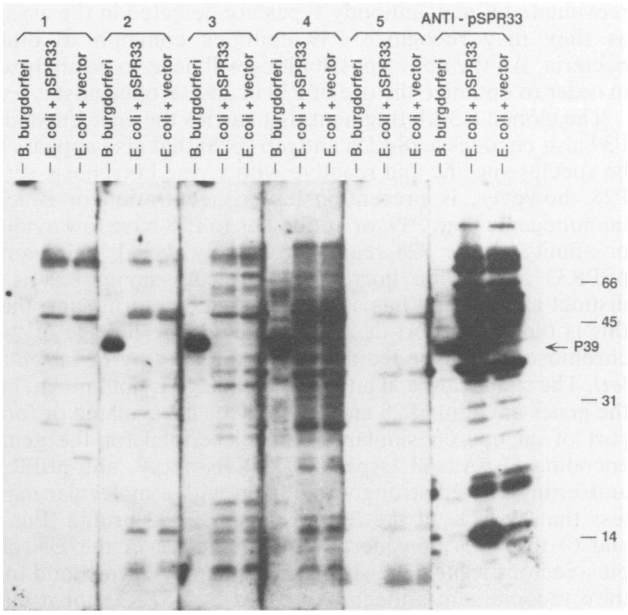

FIG. 8. Immunoblot analysis of syphilitic sera. Whole-cell lysates of *B. burgdorferi* Sh-2-82, *E. coli* carrying pSPR33, and *E. coli* carrying only vector were immunoblotted with five syphilitic sera (lanes 1 to 5) or anti-pSPR33. Molecular mass markers (in kilodaltons) are indicated on the right. Note the absence of P39 in pSPR33 lanes reacted with syphilitic sera, which contrasts with a strongly reactive 41-kDa antigen in three of the five *B. burgdorferi* lanes. Exposure was 5 h.

DISCUSSION

The difficulty in diagnosing cases of Lyme borreliosis in humans, especially in nonhyperendemic areas, has created a need for improved serodiagnostic tests. In this regard, the identification of *B. burgdorferi* antigens reacting specifically with sera from patients with Lyme borreliosis will help to derive new and better diagnostic reagents. OspA, OspB, and flagellin have been reported as immunodominant *B. burgdorferi* antigens (3). Although antibodies to these antigens have been detected in Lyme borreliosis sera, their use as serodiagnostic antigens can be questioned due to their lack of antigenic specificity (22) or antigenic stability (7, 8, 33). We describe a 39-kDa antigen (P39) that is specific to *B. burgdorferi* and strongly serologically reactive. Monoclonal antibodies H5332, H5TS, and H9724 permitted the identification of the bands in SDS-PAGE profiles that corresponded to OspA, OspB, and flagellin, respectively. Thus, the position of the band corresponding to P39 could be distinguished from these three antigens, including the closely migrating flagellin. Polyclonal antisera to P39 did not react with these other antigens in immunoblots (Fig. 5 and 6) and did not bind to endoflagella from *B. burgdorferi* in electron microscopy studies. These observations indicate that P39 is unrelated to OspA, OspB, or endoflagella.

The specificity with which P39 bound antibody from Lyme borreliosis sera was demonstrated by its nonreactivity with sera from several control groups, including syphilitic and relapsing fever patients. This was despite the fact that these sera had relatively high concentrations of antibodies that cross-reacted with whole cells of *B. burgdorferi* in IFAs (Table 3). Many of these antibodies were probably binding to epitopes common to spirochetal flagellins (Fig. 8), illustrating the problem of cross-reactivity encountered when endoflagella (22) and whole cells (27; L. A. Magnarelli, Editorial, J. Am. Med. Assoc. **262**:3464–3465, 1989) are used as antigens in serological tests. All sera from Lyme borreliosis patients with IFA titers of ≥1:256 were positive for anti-P39 antibodies. This IFA Lyme borreliosis titer is generally considered the lowest dilution that can be considered a true positive, and therefore, P39 reactivity correlates 100% with positive IFAs. This, taken together with the specificity P39 has for Lyme borreliosis sera, is suggestive that cloned P39 could be a good antigen for use in a serodiagnostic test. Conceivably, a patient that has detectable antibodies to P39 could be considered by this more specific definition to have Lyme borreliosis, notwithstanding any atypical clinical or epidemiological data.

Unfortunately, the absence of anti-P39 antibodies does not eliminate the possibility that the patient is infected with *B. burgdorferi*. Patients that appear to lack a significant humoral response to *B. burgdorferi* infections have been documented (17) and may be represented here by the sera from Lyme borreliosis patients that failed to react with P39. Alternatively, these sera may have been collected so early in the infection that anti-*B. burgdorferi* antibodies, including anti-P39, had not accumulated to detectable concentrations. This possibility could not be tested in the present study, as data pertaining to the stage of the disease when the sera were collected were not available for analysis. Notably, protein A binds well only to several subclasses of immunoglobulin G in humans. Because antibodies reactive with P39 were detected in our study using labeled protein A, it can be assumed that not all antibody classes or subclasses have been evaluated for P39 reactivity. Therefore, the sensitivity of a P39-based Lyme borreliosis test may be improved if antibodies not detected in this study (e.g., immunoglobulins M and E) are shown to react with P39. The specificity with which P39 reacts with Lyme borreliosis sera, however, will need to be

reevaluated if new antibody types are detected in the assay, as they may recognize P39 epitopes common to other bacteria. All the above possibilities will need to be analyzed in order to optimize the use of P39 in any serodiagnostic test.

The cloned 6.3-kb fragment that carries the gene encoding P39 also encodes a 28-kDa antigen (P28) that also appears to be species specific and reactive with Lyme borreliosis sera. P28, however, is present in less concentration or is less immunogenic than P39, or antibodies to P28 have low avidity or affinity, since P28 reacted relatively weakly with anti-pSPR33 and Lyme borreliosis sera. Assuming P28 is a distinct antigen and has a unique genetic determinant, then this is the first report describing the genetic linkage of two chromosomally encoded antigens expressed by *B. burgdorferi*. The significance, if any, of this linkage is not known, but the genes encoding P28 and P39 may be coregulated or form part of an operon similar to that described for the genes encoding OspA and OspB (23, 24). Notably, anti-pSPR33 antiserum reacted strongly to a band with a molecular mass less than 28-kDa in the *E. coli*-plus-pSPR33 profile (Fig. 5 and 6) that was considerably less reactive in the *E. coli*-plus-vector profile. The stronger signal may correspond to a third recombinant antigen expressed by pSPR33, or it may merely reflect variability in the expression of an *E. coli* antigen. The absence of a similar-size antigen in *B. burgdorferi* and the fact that cultures of *E. coli* carrying pSPR33 do not always produce profiles showing the strongly reactive band (data not shown) favor the latter possibility.

Numerous studies have evaluated antigenic components of *B. burgdorferi* for their reactivities with Lyme borreliosis sera by Western blotting bacterial whole-cell lysates (2, 5, 14, 16, 29). Yet, the presence of P39, which we show reacts with a significant percentage of human Lyme borreliosis sera, has not been previously described. Although several reports (19, 26) have noted antibodies in Lyme borreliosis sera reactive with a 39-kDa band, the frequency with which they were detected was considerably lower than what we report for anti-P39 antibodies. This may, in part, be because anti-P39 antibodies are best detected with the recombinant P39 protein rather than with whole-cell lysates of *B. burgdorferi*. It is also possible that the human sera examined in our study represent a group of Lyme borreliosis patients distinct from those used in other studies or that the presence of P39 was masked by the 41-kDa band corresponding to flagellin in many studies. The large number of sera tested in our study makes the former possibility unlikely. However, although the clear separation of P39 from the flagellin band by SDS-PAGE was demonstrated in this study, the separation of these two antigens may not always be evident and may explain why P39 has not been observed in some studies. In addition, anti-P39 antibodies may have been mistaken for anti-flagellin antibodies, as many of the P39-reactive Lyme borreliosis sera failed to react with flagellin (Fig. 7). Thus, anti-P39 but not anti-flagellin antibodies were present in these Lyme borreliosis sera. The possibilities that antibodies raised to the flagellin of *B. burgdorferi* during infection are not as prevalent in Lyme borreliosis sera as first thought and that P39 is a more immunodominant antigen will need to be examined more closely.

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