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An OspB Mutant of *Borrelia burgdorferi* Has Reduced Invasiveness In Vitro and Reduced Infectivity In Vivo

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Most *Borrelia burgdorferi* strains have two major surface proteins, OspA and OspB. In the present study, we selected from a clonal population of infectious *B. burgdorferi* an OspB escape mutant, identified the genetic basis for this phenotype, and evaluated its functional activities. Selection with the anti-OspB antibody H614 was performed in vitro in medium and extended in vivo in *scid* mice. Mutants with a truncated OspB protein were selected at a frequency of 1×10^{-5} to 3×10^{-5}. After no major rearrangements in DNA were detected, sequence analysis of the mutant's *ospAB* locus revealed a single base change in the consensus ribosomal binding sequence for *ospB* and a single nucleotide deletion in the *ospB* gene itself. The effect of these mutations was reduced expression of a truncated OspB protein. When functional abilities of the wild type and mutant were compared, the mutant had a threefold-lower capacity to penetrate a human endothelium umbilical vein cell monolayer. Infectivity of wild-type and mutant cells for *scid* mice was evaluated by culturing different organs, and the median infectious dose was calculated. The inoculum of mutant cells for infecting the mice was 30- to 300-fold higher than that of wild-type cells. This study shows that reduced size and expression of OspB are associated with lowered virulence of *B. burgdorferi*. Selection of mutants that to some degree remain infectious is one approach to defining the role of different surface proteins in the pathogenesis of Lyme disease.

Lyme disease is a multisystem disorder caused by at least two species of the spirochete genus *Borrelia* (1). In North America, *Borrelia burgdorferi* is the cause of Lyme disease (47). Almost all *B. burgdorferi* isolates have two major surface proteins, OspA and OspB (8, 10, 27). Much has been learned about the biochemical and genetic structure, immunogenicity, and vaccine potential of these proteins (12–14, 21, 22, 26, 39). However, the functional importance of these proteins, their role in infectivity, and their contribution to the microorganism's ability to survive in the host are still unknown. In the present study, our goals were to (i) select for an OspB escape variant of an infectious *B. burgdorferi* strain, (ii) determine the genetic basis for this phenotype, and (iii) evaluate the functional activities of this mutant in vitro and in vivo in comparison with the wild type.

There were two major reasons for undertaking this study. One rationale was to follow up on findings on the spontaneous occurrence of antibody-resistant mutants in *B. burgdorferi* (16, 35). We had identified four phenotypic classes of antibody escape mutants in high-passage populations of *B. burgdorferi* (35). One of these classes was similar to mutants with truncated or absent OspB proteins that had been described previously (15, 33, 42). Although there was evidence that such antigenic changes occur in the host (20, 45), we did not know whether in vitro antibody selection could be used with a low-passage infectious isolate. Other studies had indicated that OspB may have a role in invasion by *B. burgdorferi* into and through the cells (19). These findings concerning a possible function of the OspB protein in *B. burgdorferi* pathogenesis served as a second basis for undertaking this study.

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MATERIALS AND METHODS

Strains and culture conditions. The study was performed with the low-passage *B. burgdorferi* strain Sh.2 with known infectivity and virulence in laboratory mice (43–45). Isolate R2 of strain HB19 lacked the 49-kb plasmid and did not have the *ospAB* operon (35). Borrelias were grown in BSK II broth or on solid medium, harvested by methods described previously (2, 15, 25), and cloned at least once by single-colony plating (35). Cells were counted in a Petroff-Hauser chamber by phase-contrast microscopy.

MAbs. The origins of the OspA-specific monoclonal antibody (MAb) H5332 (10) and the OspB-specific MAbs H614, H68 (15), and 84C (17) have been given. These MAbs had been found to recognize Sh.2 cells by Western blot (immunoblot) (33, 34). The H68 MAb binds an epitope in the N-terminal half of OspB (15), and the 84C MAb binds an epitope at the C-terminal end of OspB (46). The growth-inhibiting and killing abilities of the H614 MAb for Sh.2 cells were demonstrated by a growth inhibition assay as described previously (37).

Mutant selection. Conditions for mutant selection with antibodies in sealed, 96-well, flat-bottom microtiter plates were essentially as described before (35). The initial inoculum of Sh.2 spirochetes in each well varied between 10^2 and 10^6. The ascitic fluid with selecting MAb was heat inactivated (56°C for 30 min) and passed through a 0.2-μm filter before use. The concentration of the selecting MAb was 10 times the minimal concentration required for growth inhibition (37). Growth of the borrelias at 34°C in a 1% CO_2 atmosphere was monitored visually for changes in the color of the phenol red indicator and by phase-contrast microscopy. The mutants were cloned by single-colony plating, with selecting antibody present in the top layer of agarose (35).

PAGE and Western blot analysis. Whole-cell lysates were subjected to polyacrylamide gel electrophoresis (PAGE)

with 15% acrylamide as described previously (4, 9). In some experiments, cleavage of surface-exposed proteins of borrelias with proteinase K was carried out prior to PAGE, essentially as described previously (35). Briefly, 490 μl of a suspension of 5×10^8 cells in phosphate-buffered saline (PBS) was incubated with 10 μl of an aqueous solution of proteinase K (20 mg/ml) for 40 min at 22°C. The reaction was stopped by the addition of phenylmethylsulfonyl fluoride (15). For Western blot analysis, proteins were transferred to nitrocellulose membranes, which were then blocked with 3% (wt/vol) dried nonfat milk in 10 mM Tris-HCl (pH 7.4)–150 mM NaCl (milk/TS) for 2 h as described before (35). After the membranes were washed with milk/TS, they were incubated with MAb ascitic fluid diluted 1:100 in milk/TS. Alkaline phosphatase-conjugated recombinant protein A/G (Immunopure; Pierce Chemical Co., Rockford, Ill.) served as the second antibody. The blots were developed with nitroblue tetrazolium chloride–5-bromo-4-chloro-3-indolylphosphatase *p*-toluidine salt (Pierce Chemical Co.).

Nucleic acid isolation and Northern (RNA) blot analysis. Extractions of total or plasmid-enriched DNA from borrelias and agarose gel electrophoresis of DNA were performed as described previously (3, 5, 6, 15). Restriction enzymes were obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind., and used according to the manufacturer's recommendations. Total RNA was extracted from freshly harvested borrelias and subjected to Northern blot analysis as described previously (15, 26). The gel was stained with acridine orange to visualize the 23S and 16S rRNA bands (29). The probe for the *ospAB* operon was recombinant plasmid pTRH46, which contains an entire *ospB* gene and the 3′ end of the *ospA* gene (13). The probe DNA was labeled with [α-^{32}P]dATP by nick translation, using a commercial kit (Bethesda Research Laboratories, Gaithersburg, Md.). Hybridization fluid contained 50% formamide; the blot was incubated with the probe at 37°C overnight and washed at 64°C with 15 mM NaCl–1.5 mM sodium citrate–1 mM EDTA–0.1% sodium dodecyl sulfate.

DNA sequencing. The *ospAB* locus was amplified by polymerase chain reaction essentially as described previously (32, 33) and directly sequenced by using the dideoxy method and a double-stranded DNA Cycle Sequencing kit (Bethesda Research Laboratories). Polymerase chain reaction primers represented nucleotides 1 to 20 and 1896 to 1915 (opposite strand) of the *ospAB* locus sequence (13) (GenBank accession number X14407). Total borrelial DNA, 100 ng, was amplified for 20 cycles under the following conditions: 94°C for 1 min, 50°C for 0.5 min, and 70°C for 2 min. Buffer conditions were as recommended by Perkin-Elmer-Cetus. Before sequencing, polymerase chain reaction fragments were purified by precipitation in 2 M ammonium acetate with an equal volume of isopropanol. Twenty nanograms of amplified DNA was used per sequencing reaction; sequencing primers were located approximately every 200 nucleotides throughout the *osp* operon and were labeled by T4 kinase with ^{32}P. Nucleic acid sequences were deposited in the GenBank data base under accession number L19701.

Cell adherence and penetration assays. Assays for adherence and penetration of intrinsically labeled spirochetes with and through human umbilical vein endothelium (HUVE) cells were carried out essentially as described previously (18, 19, 51). All assays were done four times. For assessing HUVE cell adherence, borrelias were intrinsically radiolabeled with ^{35}S-methionine as described before (51), washed with PBS, and resuspended to a density of 1.7×10^8 cells per ml in medium 199 with 20% fetal calf serum. Aliquots, 300 μl, of radiolabeled spirochetes were added to confluent HUVE cell monolayers grown in 24-well plates. After a 4-h incubation at 4°C, monolayers with associated organisms were washed, solubilized, mixed with scintillation cocktail, and counted by scintillation (51). For penetration experiments, HUVE cell monolayers were grown on 3-μm-pore-size filter units (Millipore Corp., Bedford, Mass.) mounted in wells of 24-well tissue culture plates (Costar, Cambridge, Mass.). For each assay, 200-μl samples containing 10^8 borrelias were added to the upper portions of the chambers above the monolayers. After a 6-h incubation at 37°C in 5% CO_2 in air, aliquots from beneath the filters were removed, and spirochetes were quantitated by dark-field microscopy and amount of radioactivity in the lower chamber. Differences between borrelia populations in adhesion and invasion were analyzed by Student's *t* test.

Mouse infectivity. Four- to 6-week-old strain CB-17 *scid* mice (homozygous for the *scid* mutation) were bred and maintained at the University of Texas Health Science Center at San Antonio under specific-pathogen-free conditions. For in vivo mutant maintenance, mice were injected intraperitoneally with an inoculum of 10^6 borrelias in 100 μl of PBS; 2 h prior to infection, mice had been injected intraperitoneally with 250 μl of selecting MAb in PBS at 10 times the minimal concentration required for growth inhibition. For determination of the median infectious dose (ID_{50}), mice were inoculated intradermally at the base of the tail with serial 10-fold dilutions of spirochetes in 100 μl of PBS in the range of 10^1 to 10^5. The accuracy of inoculum estimates was confirmed by subsurface plating in triplicate of the lowest dilutions of spirochetes. Twelve days following the injection, mice were bled by retroorbital sinus puncture, and 100 μl of plasma was directly plated in duplicate on solid medium by adding it to an equal volume of BSK II containing 1% low-gelling-temperature agarose (SeaPlaque; FMC Corp., Rockland, Maine) (25). Plates were evaluated for growth at 10 days. Mice were euthanized 14 days following the injection. Plasma (0.5 ml) obtained from citrated blood, the whole bladder, macerated heart, cross-cuttings of both tibiotarsal joints, and both ears were added to BSK II medium cultured at 34°C. Cultures were examined for the presence of motile spirochetes by phase-contrast microscopy on days 7 and 14 of cultivation; they were scored as negative when no motile spirochetes were seen in 20 high-power fields. For each group of mice, the ID_{50}, expressed as $\log_{10}$ of borrelias injected, was determined (31).

RESULTS AND DISCUSSION

Selection of escape variant. The Sh.2 strain was chosen for this study for these reasons. (i) It has a low tendency to autoagglutinate, a feature that allows for precise counts of borrelias in animal infections. (ii) Sh.2 also has a high efficiency of plating in semisolid medium. (iii) In a preliminary study, the ID_{50} of Sh.2 by intradermal inoculation in *scid* mice was only 10 to 20 spirochetes (34). The Sh.2 population subjected to selection was derived from a single colony. For mutant selection, we used MAb H614, which is specific for the OspB protein of several *B. burgdorferi* strains including Sh.2 (15, 34). In the growth inhibition assay (37), the purified MAb H614 was inhibitory for growth of high-passage B31, high-passage HB19, and low-passage Sh.2 isolates at minimal concentrations of 2.4, 9.6, and 9.6 μg/ml, respectively (34, 37). We have shown previously that this MAb could be applied to isolate OspB escape mutants of strains HB19 and B31 (35).

Mutants resistant to antibody H614 in microtiter plates were present in the clonally derived Sh.2 population at a frequency of 1×10^{-5} to 3×10^{-5}, as calculated with Poisson distribution tables (35). Of five escape mutants examined by PAGE, all had similar protein profiles and appeared to lack an OspB (data not shown). Next, an arbitrarily chosen mutant with this phenotype was cloned again by single-colony plating. One clone was grown in liquid BSK II, and an inoculum of 10^6 cells was administered intraperitoneally to each of three *scid* mice. All mice became infected as assessed by blood culture. A blood culture from one of the mice was subjected to further evaluation. During both in vitro and in vivo selection, mutant cells were kept under the selective pressure of MAb H614 by plating the cells in the presence of MAb and by treating the mice with MAb before infection.

Biochemical characterization of the infectious mutant. Whole-cell lysates of wild-type and mutant cells were subjected to PAGE and Western blot analysis with the anti-OspA MAb H5332 and anti-OspB MAbs H614, H68, and 84C. Results are shown in Fig. 1. The only difference between the wild-type cells and the antibody-resistant mutant was that mutant cells apparently lacked the full-length OspB at 32 kDa. The mutant instead presented in the PAGE a faint band at 18 kDa which in Western blot was bound by anti-OspB MAb H68, an antibody known to bind truncated OspB proteins (15). Probing with MAb H68 also showed that a faint band of the same size was also present in the cloned Sh.2 population (Fig. 1). These data suggested that wild-type cells were producing a very small amount of truncated OspB protein as well. We also observed this phenomenon in Sh.2 cells after a second round of single-colony plating, as well as in several other cloned low-passage *B. burgdorferi* isolates, such as N40 and HB19 (34). Neither MAb H614, which was used for selection, nor MAb 84C, which binds an epitope at the carboxy-terminal end of OspB (46), reacted with mutant cells. Both the wild type and the mutant had a full-length OspA at 29 kDa that was bound by MAb H5332.

We investigated whether the 18-kDa band was cleaved from intact cells by proteinase K, as has been shown for other *B. burgdorferi* surface proteins (15). Intact wild-type and mutant cells treated with proteinase K were examined by PAGE and Western blot. Proteinase K cleaved the 18-kDa band as well as other Sh.2 surface proteins. No bands were detectable with MAb H68 by Western blot in lysates of proteinase K-treated wild-type and mutant Sh.2 cells (data not shown). Thus, the phenotypic characteristics of the infectious escape variant were consistent with class II antibody-resistant mutants described in reference 35 and resembled the spontaneous $OspB^-$ variants observed by Schwan and Burgdorfer (42), Bundoc and Barbour (15), and Coleman et al. (16).

Genetic characterization of the infectious mutant. To determine the basis for the scant, truncated OspB, we looked first for major DNA rearrangements. The plasmid contents of the mutant and wild type were compared by low-voltage constant field electrophoresis in a 0.2% agarose gel of plasmid-enriched DNA; there were no detectable differences between the two isolates in plasmid profiles (data not shown). We also performed total DNA extractions from wild-type and mutant Sh.2 cells and separated *Eco*RI-digested total DNA fragments on a 1% agarose gel. Both wild-type and mutant patterns of *Eco*RI-digested total DNA appeared the same (data not shown). These findings indicated that the mutant did not feature large DNA rearrangements. Next, Northern blot analysis was used to determine whether the absence of the full-size OspB was the result of failure or premature termination of *ospB* transcription. Both wild-type and mutant cells contained hybridizable transcripts of about 2.2 kb, representing the *ospAB* operon (Fig. 2) (26), a finding that indicated that the altered OspB was not due to premature termination of transcription of the *ospAB* operon.

Alternatively, local changes in a regulatory sequence, such as the consensus ribosomal binding site between the end of the *ospA* and the start of the *ospB* genes, could affect translation of the *osp* transcript. In such cases, the length of the *ospAB* transcript would likely be unchanged and could

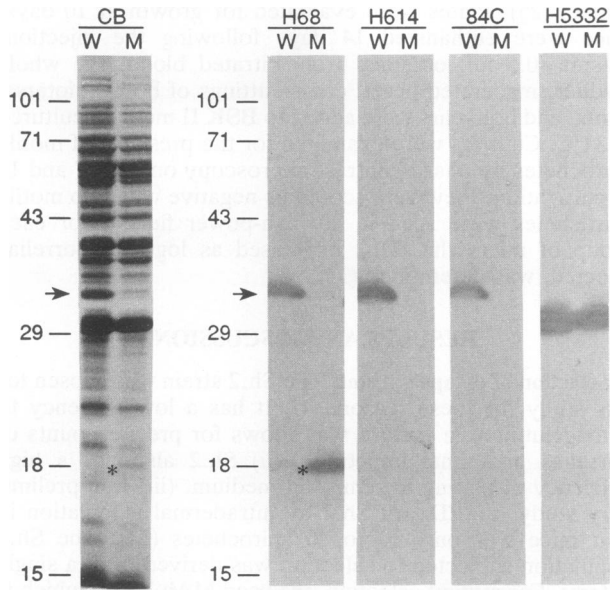

FIG. 1. Coomassie blue-stained 15% polyacrylamide gel (CB) and Western blot analysis of Sh.2 wild-type (W) and mutant (M) cells. The OspA-specific MAb H5332 and OspB-specific MAbs H68, H614, and 84C were used for the Western blot. The arrowhead indicates the OspB of wild-type cells; the asterisk identifies the 18-kDa protein in mutant cells. The locations of the molecular weight standards (10^3) bovine serum albumin (71), ovalbumin (43), carbonic anhydrase (29), and β-lactoglobulin (18), from Bethesda Research Laboratories, are shown on the left.

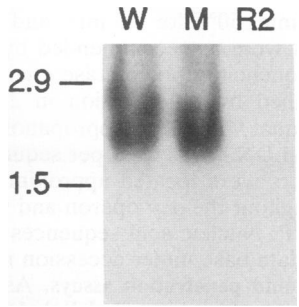

FIG. 2. Northern blot analysis of mRNA from *B. burgdorferi* Sh.2 wild-type (W) and mutant (M) cells. Isolate R2 of strain HB19 lacking the *ospAB* operon plasmid (35) served as a negative control. Total RNA was separated in a 1.2% agarose gel under denaturing conditions and transferred to the membrane. The blot was probed with ^{32}P-labeled plasmid pTRH46 (13). The positions of 16S and 23S rRNA bands, corresponding to 1.5 and 2.9 kb, respectively, revealed by staining with acridine orange, are indicated on the left.

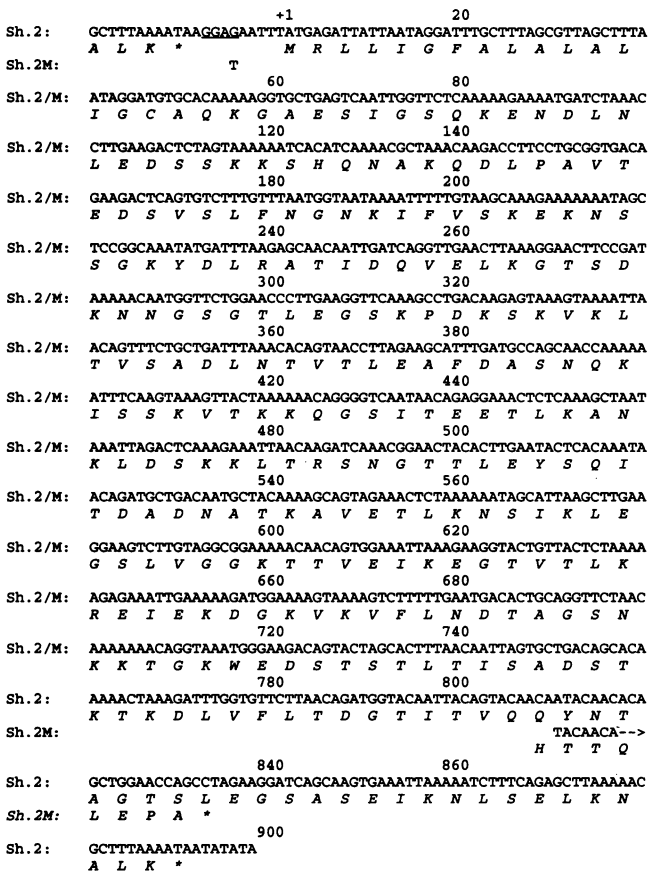

FIG. 3. Nucleotide and deduced amino acid sequences of *ospB* genes of Sh.2 wild type and mutant (Sh.2M). The uppermost line shows the termination of the *ospA* gene of Sh.2, the presumed ribosomal binding sequencing (underlined), and the start of the *ospB* open reading frame at position +1; the deduced amino acid sequence in one-letter code and italics is shown below. Only differences between Sh.2 and Sh.2M are shown. At position −6 there is a T-to-G transversion. At position 811 there is a deletion of the A in Sh.2M, thereby producing in Sh.2M a frame shift of 1 base and a premature stop codon (TAG) at position 832 in the *ospB* reading frame.

only be detected by nucleotide sequencing of DNA. Accordingly, we sequenced the mutant *ospAB* locus and compared the sequence with that of the wild-type cells (33). No differences between the *ospA* loci were found. In the mutant *ospB* locus, there were a single base change at position −6 (G to T) in the consensus ribosomal binding sequence for *ospB* and a deletion of the base, an A, at position 810, thereby altering the reading frame and terminating OspB seven amino acids downstream (Fig. 3). Absence of even a few amino acids from the end of OspB might destabilize the protein and render it more susceptible to proteases. This may account for the fragment of approximately 18 kDa instead of the predicted polypeptide of 29.7 kDa. Although the role of the consensus ribosomal binding site on the mRNA transcript of *ospAB* has not been defined, the G-to-T transversion at base −6 in the consensus ribosomal binding sequence would be expected to reduce expression of OspB (49). This is consistent with the observations by PAGE.

Two mutations in the *ospAB* locus could explain the lower (1×10^{-5} to 3×10^{-5}) frequency of the Sh.2 mutant compared with strain B31 and HB19 antibody-resistant variants, which had a single base change in their *osp* sequences (35). The frequency of the latter mutants in the population was $\sim 10^{-4}$ (35). However, the faint 18-kDa band detectable only by Western blot in the wild-type population of Sh.2 and other strains suggests that nonsense mutations at one or more points in the 3′ end of the *ospB* gene are cumulatively frequent. Such mutations, in fact, have been observed previously in a noninfectious Sh.2 mutant in which a G-to-A transition in the *ospB* gene produced a nonsense mutation (33). It has also been shown that sera from mice immunized with recombinant full-length OspB protein bound not only to a 32-kDa band but also to a 17-kDa band of *B. burgdorferi*; bands of these sizes were also detected when the same purified recombinant OspB-B31 was probed with anti-OspB MAb (23). These findings suggest that alteration of the conformation of the protein or changes even in the terminal amino acid sequence lead to proteolytic cleavage at a more internal site. Thus, wherever in the 3′ end of the *ospB* gene the nonsense mutation might occur, fragments of about 20 kDa seem to be the common degradation products (15, 16, 42). Selective degradation of abnormal proteins in *Escherichia coli* was first recognized by Pine (30) and later characterized by Goldberg (24). Proteins that may be structurally normal in one organism may be viewed as foreign and abnormal when produced from cloned DNA in *E. coli* (50).

Functional characterization of the mutant in vitro. Mutant and wild-type cells were compared in adherence and penetration assays in vitro. Adherence of radiolabeled *B. burgdorferi* populations to HUVE cell monolayers was measured after 4 h at 4°C. At this temperature borrelias do not detectably enter endothelial cells (19). Also, adherence of cells becomes maximal by 4 h (19). The penetration of HUVE cells by the wild-type and mutant cells was quantitated after 6 h at 37°C by dark-field microscopy and scintillation spectrophotometry. To improve the accuracy of quantitation of penetration, the borrelias subjected to the penetration assay were intrinsically radiolabeled with a higher specific activity than those used for the adherence assay. The results are given in Table 1. Adherence and penetration assays were each repeated four times, with agreement between replicate experiments. The difference between wild-type and mutant cells in the level of maximal adherence to HUVE cells at 4 h was not significant. In contrast, the relative penetration of the mutant population through the HUVE cell monolayers was only 37% of that of wild-type borrelias, a difference that was significant ($P < 0.001$). We already have characterized a flagellum-less, nonmotile mutant of another *B. burgdorferi* strain, HB19, in which the highly reduced penetration of endothelial cells was associated with the loss of flagellin protein (36). In the case of the Sh.2 mutant, a reasonable explanation for the significantly poorer penetration of endothelial cells is that the deleted portion of the OspB molecule, or, very possibly, an intact whole OspB protein, has an important role in *B. burgdorferi* invasiveness.

Functional characterization of the mutant in vivo. Results of the preceding experiments prompted a comparison of the mutant with wild-type cells in an in vivo model of infection. The reduced ability of the Sh.2 mutant to penetrate HUVE cells suggested that infectivity of the mutant may also be lower than that of the wild-type cells. Immunodeficient *scid* mice were chosen for use, because the question at this point was infectivity, not evasion of the immune response, and because the utility of this animal model of Lyme disease had been demonstrated (38, 40). The goal in this experiment was to determine the minimal infectious dose for each isolate,

TABLE 1. Adherence to and penetration of HUVE cells by wild-type and mutant Sh.2[a]

Expt	Cell population	Adherence[b]		Penetration[c]	
		Mean cpm adhered ± SEM[d]	Mean % of inoculum adhered	Mean cpm in lower chamber ± SEM[e]	Mean % of inoculum in lower chamber
I	Wild type	3,643 ± 36	10.4	83,010 ± 3150	11.7
	OspB mutant	3,580 ± 78	10.2	36,320 ± 280	4.3
II	Wild type	12,722 ± 491	9.9	69,720 ± 1704	10.6
	OspB mutant	12,429 ± 605	9.7	27,106 ± 990	4.1
III	Wild type	8,547 ± 218	11.3	75,297 ± 1252	9.8
	OspB mutant	8,611 ± 355	11.4	30,903 ± 725	4.0
IV	Wild type	7,692 ± 192	10.6	68,472 ± 1487	11.5
	OspB mutant	7,473 ± 278	10.3	29,553 ± 1096	5.0

[a] The specific activities of inocula for adherence assays in experiments I to IV were 3.5×10^4, 12.8×10^4, 7.6×10^4, and 7.3×10^4 cpm, respectively; the specific activities of inocula for penetration assays in experiments I to IV were 7.1×10^5, 6.6×10^5, 7.7×10^5, and 6.0×10^5 cpm, respectively.
[b] Measured following incubation for 4 h at 4°C (19).
[c] Assessed as described previously (18, 19).
[d] Radioactivity bound to host cells following incubation and washing, expressed as the mean of three samples.
[e] Radioactivity associated with bacteria which penetrated through the host cell monolayers in a dual-chamber system; the inoculum was applied to the upper chamber above the monolayer.

and for that we used the intradermal route of infection (11, 21). Results are presented in Table 2. Overall, about 100-fold, or 2 $\log_{10}$, more mutant cells than wild-type cells were required to produce detectable infection of a given organ or body fluid. When individual sites were examined, the ID_{50}s for the mutant ranged between 30- and 300-fold higher than those for wild-type cells. It appeared that fewer mutant cells were needed to produce an infection of blood than of organs. The greatest difference between the mutant and the wild type was in infection of the tibiotarsal joints: approximately 300-fold more mutant cells were required for infection of the least-vascularized tissue of all those examined. These in vivo data are consistent with the in vitro findings of reduced penetration of mutant cells.

The goal of this study was to evaluate infectivity, not virulence, of mutant cells; therefore, no histopathologic data were obtained. Nevertheless, reddened joints were observed in mice infected with both wild-type and mutant cells, a finding that suggests that mutants with altered OspB protein can still be responsible for disease manifestations. To be confident that the cells that grew in the mice were not revertants to the wild-type phenotype, positive cultures from mice given the minimal infectious inoculum of the mutant were subjected to PAGE. All of these cultures retained the same phenotype of a scant, truncated OspB protein (data not shown).

The abilities of wild-type and mutant cells to survive and proliferate in the bloodstream were also examined. Aliquots of 100 μl of plasma from each mouse were plated in duplicate on solid BSK II medium. Current procedures for cultivation of even a low passage of *B. burgdorferi* in solid medium yield a plating efficiency of 80 to 100% (35). The ability of mutant cells to grow on solid medium in terms of plating efficiency was evaluated in a preliminary study and was shown to be undiminished. In addition, the mutant cells did not clump any more or less than wild-type cells. Consequently, we assumed that CFU on the plates should closely approximate the numbers of both types of borrelias in the blood. Data are given in Table 3. There were approximately 10^3 borrelias per ml of plasma in mice with the wild-type isolate; the corresponding number of mutant borrelias in those mice with positive blood cultures was approximately 2×10^3 per ml. Although the ability of the mutant cells to enter the bloodstream from an intradermal site was reduced by approximately 30-fold and had to be at least 10^3 cells, once in the bloodstream, the mutant borrelias appeared to adapt themselves to the host as well as the wild type did. It is also possible that mutant cells, being diminished in endothelial cell penetrating abilities and, therefore, presumably less capable of leaving the blood for the tissues, remain mostly in the bloodstream. This could be the explanation for the higher numbers of mutant than wild-type cells in the plasma. Some other virulence characteristics such as time to appearance of borrelias in the blood after intradermal inoculation or extent

TABLE 2. Infectivity of the wild type and the OspB mutant of *B. burgdorferi* Sh.2 in *scid* mice

Sample from:	Wild-type infectivity (no. of mice infected/total tested)					Log_{10} ID_{50}[b]	OspB mutant infectivity (no. of mice infected/total tested)					Log_{10} ID_{50}[b]
	10^5[a]	10^4	10^3	10^2	10^1		10^5	10^4	10^3	10^2	10^1	
Plasma	3/3	3/3	6/6	6/6	2/6	1.5	3/3	3/3	3/6	0/6	0/6	3.0
Bladder	3/3	3/3	6/6	5/6	2/6	1.6	3/3	3/3	2/6	0/6	0/6	3.5
Heart	3/3	3/3	6/6	4/6	2/6	1.8	3/3	3/3	2/6	0/6	0/6	3.5
Ear	3/3	3/3	5/6	1/6	0/6	2.8	3/3	0/3	0/6	0/6	0/6	4.8
Joint	3/3	3/3	6/6	3/6	0/6	2.0	3/3	1/3	0/6	0/6	0/6	4.5

[a] Inoculum.
[b] Calculated number of borrelias in inoculum needed to detectably infect different organs (31).

TABLE 3. CFU of wild-type and mutant Sh.2 in blood of infected mice

Cell population	CFU/ml of plasma ± SEM[a] with given inoculum				
	10^5	10^4	10^3	10^2	10^1
Wild type	1,917 ± 214 (3)[b]	1,100 ± 125 (3)	1,400 ± 255 (6)	702 ± 280 (6)	800 ± 35 (2)
OspB mutant	2,567 ± 27 (3)	2,100 ± 245 (3)	1,900 ± 336 (3)	0	0

[a] Evaluated by plating 100 μl of plasma from each mouse on solid BSK II medium.
[b] Number of plasma samples is given in parentheses.

of borrelia dissemination in the skin might be other appropriate measures of altered functions.

The proximate cause for the reduced invasiveness and infectivity of the mutant cells in this case appears to be the changes in *ospB* sequence. Alternatively, the reduced invasiveness and infectivity of mutant cells may be an effect of an undetected and unrelated second mutation in another gene. The latter explanation is unlikely, because the selection was on the OspB protein itself, but it cannot be excluded. For a cause-effect relationship to be established, the *ospB* mutation would have to be successfully transferred as a well-defined DNA packet to an infectious *B. burgdorferi* strain, thereby resulting in the altered OspB protein and the same functional characteristics as the Sh.2 mutant. As transformation or transduction is not yet possible with borrelias, the relationship remains an association by precise definition.

Circumstantial evidence implicating OspA and OspB in pathogenesis in vertebrates is the finding that all vertebrate and human isolates of *B. burgdorferi* from North America expressed these surface proteins (7, 41, 48). Some strains without OspA and OspB proteins were isolated in California, but these strains were recovered from ticks, and the *ospAB* operon was still present (28). Further circumstantial evidence of an association between OspB expression and infectivity was provided by the data of Schwan and Burgdorfer showing that the uncloned infectious Sh.2 isolate lost full-length OspB and its virulence at about the same time during in vitro cultivation (42).

In this study, we concentrated on the functional role of OspB and found that changes in the OspB sequence resulting in reduced expression and truncation of this protein diminished the penetration capability and infectivity of the mutant cells. In this mutant, the adherence level and the ability to survive once inside the host appeared to be unchanged. This suggests that this type of mutation, while diminishing initial infectivity, might confer an advantage for avoiding host immune response. Once the borrelia with the full-length surface proteins has gained access to certain niches in the host, such as the nervous system or synovial space, the pressure for further migration may diminish; consequently, antibody-resistant mutants would fare better over the long term. It has been shown that protection with recombinant OspA protein can be overcome with an inoculum of 10^7 spirochetes, and only 10^4 spirochetes were necessary to infect mice immunized with recombinant OspB protein (23). These results should be considered when evaluating the efficacy of potential Lyme disease vaccines that are based on the immune response against a single surface protein of a particular *B. burgdorferi* strain.

An infectious mutant of *B. burgdorferi* was first selected and then characterized structurally and functionally. Among *Borrelia* spp., mutants for these purposes had not hitherto been available. The present study encourages further use of antibody-resistant mutants of infectious isolates to examine the role of surface proteins and different portions of these molecules in *B. burgdorferi* pathogenesis as well as immunity to infection.

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