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Roles of OspA, OspB, and Flagellin in Protective Immunity to Lyme Borreliosis in Laboratory Mice

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Vaccination with recombinant outer surface protein A (OspA) has been shown to protect mice from infection with *Borrelia burgdorferi*, the Lyme disease agent. To determine whether antibodies to *B. burgdorferi* proteins other than OspA are involved in protective immunity, antibodies to OspA were removed from protective anti-*B. burgdorferi* serum; the residual serum was still protective. Absorption of OspA and OspB antibodies from anti-*B. burgdorferi* serum eliminated the protective effect. Therefore, active immunization experiments were performed to determine the roles of OspB and flagellin in protective immunity and to determine whether protective immunity induced by OspA is dose dependent. Active immunization with recombinant OspA protected mice from infection with an inoculum of 10^4 spirochetes, but this protection could be overcome with a challenge of 10^7 spirochetes; OspB protected mice from infection with an inoculum of 10^3 spirochetes but was insufficient to fully protect against 10^4 organisms; and immunization with flagellin had no protective effect. These studies suggest that OspA and OspB, but not flagellin, play roles in protective immunity to spirochete infection.

Lyme borreliosis is caused by the spirochete *Borrelia burgdorferi*, which is transmitted by *Ixodes* ticks and can result in musculoskeletal, cardiac, and neurologic diseases (20). Various animal models, including rats, hamsters, and mice, have been developed to study experimental Lyme disease (5, 6, 14). The C3H/He mouse is a superior model since mature, immunocompetent animals develop arthritis and carditis when challenged with *B. burgdorferi*, partially mimicking human disease (5). By 14 days after intradermal infection with small numbers of spirochetes, all mice develop polyarthritis and carditis (4). As in humans, the disease regresses and is exacerbated over the course of several months, and spirochetes may be cultured from the blood, spleen, skin, and other organs of the mice up to 1 year after infection (7).

Three major immunodominant proteins, outer surface proteins A and B (OspA and OspB) and flagellin, occur at or near the surface of *B. burgdorferi* spirochetes and are therefore logical candidates for vaccination strategies to prevent *B. burgdorferi* infection. OspA, a 31-kDa protein, is the major outer surface protein of the spirochete (3). We have shown that OspA is a vaccine candidate for Lyme disease, since active immunization of C3H/He mice with subcutaneous injections of recombinant OspA in complete Freund's adjuvant or intraperitoneal injections of live *Escherichia coli* organisms expressing OspA protects the animals against challenge with an intradermal inoculum of 10^4 *B. burgdorferi* spirochetes (12). Furthermore, passive administration of polyclonal and monoclonal antibodies to OspA, as well as polyclonal anti-*B. burgdorferi* serum, protects both immunocompetent and *scid* mice from infection (12, 18). OspB is also located on the surface of the spirochete; the OspB gene is on the same 49-kb linear plasmid as OspA, and both genes are transcribed from a common promoter (8). In addition, a 41-kDa flagellar protein named flagellin has been identified, cloned, and sequenced (2, 13). Antibodies to flagellin appear early in both human and animal infections, whereas antibodies to OspA and OspB appear early in murine infections and later in human disease (5, 10). The roles of these antibodies in protective immunity are unclear, although in a recent review it has been suggested that monoclonal and polyclonal antibodies to flagellin do not protect *scid* mice from infection and that monoclonal antibodies to OspB partially delay the development of infection and disease in *scid* mice (19). The purpose of this study was to determine whether OspA, OspB, and flagellin *B. burgdorferi* proteins evoke protective immunity.

MATERIALS AND METHODS

Mice. Random-sex, virus antibody-free C3H/HeJ (C3H) mice were obtained from the Jackson Laboratory, Bar Harbor, Maine. They were shipped in filtered crates and housed in microisolator cages. Food and water were provided ad libitum. Mice were killed with carbon dioxide gas.

B. burgdorferi. Low-in vitro-passage *B. burgdorferi* isolates B31 and N40, with previously proven infectivities and pathogenicities in C3H mice, were utilized. The spirochetes were grown to log phase in modified Barbour-Stoenner-Kelly (BSK II) medium and counted with a hemocytometer under dark-field microscopy (1).

Amplification and cloning of the OspA, OspB, and flagellin genes and expression of the recombinant antigens. Recombinant OspA was expressed and purified as a fusion protein with glutathione transferase as previously described (12), and recombinant OspB and flagellin were cloned, expressed, and purified in a similar manner. Ten nanograms of *B. burgdorferi* N40 DNA was used as a template for the polymerase chain reaction to amplify OspA and flagellin. Plasmid pTRH46, a pBR322-based plasmid which contains the *B. burgdorferi* B31 OspB (OspB-B31) gene in an inverted

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position with respect to the promoter, was used to amplify OspB-B31. The oligonucleotide primers used for amplification were based on the known nucleotide sequences of OspA, OspB, and flagellin and were flanked by restriction enzyme digestion sites to facilitate cloning and expression (8, 13). The polymerase chain reaction-amplified DNA segments were isolated by agarose gel electrophoresis and purified by electroelution with a DEAE membrane. The purified DNA was digested with *Eco*RI and *Bam*HI, and the genes for OspA-N40, OspB-B31, and flagellin-N40 were ligated into plasmid pGEX-2T (Pharmacia) in frame with the glutathione transferase gene. pGEX-2T contains the glutathione transferase gene of *Schistosoma japonicum* under control of the *tac* promoter, which is inducible with isopropyl-β-D-thiogalactopyranoside (IPTG). The recombinant plasmids were used to transform *E. coli* DH5α.

Recombinant fusion protein production was induced with 1 mM IPTG for 3 h. Cells were washed in phosphate-buffered saline (PBS), suspended in 1/100 volume of PBS with 1% Triton, and lysed by sonication. The lysate was centrifuged at 13,000 × *g*, and the supernatant containing the recombinant fusion proteins was placed over a glutathione Sepharose 4B column (Pharmacia). The OspA and OspB fusion proteins were eluted with 5 mM glutathione. The flagellin fusion protein was insoluble in PBS with 1% Triton and located in the cell extract. It was solubilized in 0.3% sodium dodecyl sulfate (SDS) and diluted into 0.03% SDS, and it corresponded to more than 50% of the total solubilized protein; however, *E. coli* contaminants were also present. The flagellin protein could not be purified over a glutathione column.

The OspB-B31 gene was also ligated into plasmid 197-12 and expressed in *E. coli* as the native OspB protein by using the original ATG codon, with a protocol previously described for OspA (12). Plasmid 197-12, obtained from Biogen, Inc., in Cambridge, Mass., expresses recombinant antigens under control of the p_L promoter and the phage λ *c*I857 thermolabile repressor (12).

Absorption of rabbit anti-*B. burgdorferi* serum with recombinant OspA and OspB. Rabbit anti-*B. burgdorferi* N40 serum was prepared as previously described (12). A 2-mg sample of recombinant OspA fusion protein, OspB fusion protein, or glutathione transferase was bound to Sepharose 4B by CNBr activation. Specifically, 0.8 g of CNBr-activated Sepharose 4B (Pharmacia catalog no. 17-0430-01) was washed with 200 ml of 1 mM HCl and then equilibrated with coupling buffer (0.1 M $NaHCO_3$–0.5 M NaCl, pH 8.3). A 2-mg sample of recombinant antigen in coupling buffer was added to the gel, and the mixture was shaken end over end at room temperature for 2 h. The gel was washed twice with coupling buffer, and then 4 ml of ethanolamine (pH 9.0) was added to the gel, which was then incubated for 2 h to block any unbound sites. The gel was washed three times with 50 ml of 0.1 M sodium acetate–0.5 M NaCl, pH 4.0, and then equilibrated in PBS and stored at 4°C in PBS with 0.01% sodium azide. A 5-ml sample of rabbit anti-*B. burgdorferi* N40 serum was passed over the OspA column twice to remove antibodies to OspA. The serum was diluted 1:50 (1.4 mg/ml) for immunization experiments and 1:100 to 1:10,000 for immunoblots. Serum which had been passed over the OspA column was then passed over an OspB column four times to remove antibodies to OspB. This serum was also diluted to a concentration of 1.4 mg/ml for passive immunization. As a control, serum was passed over the glutathione column in a similar fashion.

Vaccination and challenge of C3H mice. Mice were actively immunized with 10 μg of recombinant OspA, OspB, or flagellin glutathione transferase fusion protein in complete Freund's adjuvant and given booster injections of the same amount of protein in incomplete Freund's adjuvant on days 14, 28, and 42 after immunization. Other groups of mice were passively immunized with 0.1 ml of rabbit anti-*B. burgdorferi* serum that had been passed over a recombinant OspA column, over both a recombinant OspA and a recombinant OspB column, or over a glutathione transferase column. Control mice were actively immunized with glutathione transferase or passively immunized with normal rabbit serum in a similar manner.

Immunized mice were challenged with an intradermal inoculation of 10^7, 10^4, 10^3, or 10^2 *B. burgdorferi* B31 or N40 spirochetes in 0.1 ml of medium 14 days after the last boost and were sacrificed 14 days later, at which time the disease manifestations are most pronounced (5). Passively immunized mice were challenged with N40 spirochetes at 24 h and also sacrificed 14 days later. The joints and hearts were formalin fixed, paraffin embedded, sectioned, and examined microscopically for evidence of inflammation. Both tibiotarsal joints were examined, and an animal was considered to have arthritis when at least one joint showed evidence of periorbital edema and synovial infiltration with neutrophils and lymphocytes. Arthritis was blindly graded on a scale from 0 to 3: grade 0 represents a lack of inflammation, grades 1 and 2 indicate mild inflammation, and grade 3 signifies severe inflammation. Animals which had grade 0 lesions were considered free of disease. Carditis was characterized by aortitis, myocarditis, or atrial and ventricular pericarditis. Blood and spleens from experimental animals were collected aseptically, homogenized in BSK II medium (spleens), and cultured in BSK II medium. Cultures were incubated in BSK II medium for 2 weeks and examined by dark-field microscopy as previously described (12). Briefly, 20 high-power fields per culture were scanned. Positive cultures had between 1 and 100 spirochetes and typically contained 15 organisms, while negative cultures had no organisms. Mice were considered infected when at least one culture was positive.

Serum collection, immunoblots, and protein sequencing. Thirty micrograms of a protein extract of *B. burgdorferi* N40 or recombinant OspA and OspB fusion proteins was separated by SDS-polyacrylamide gel electrophoresis (PAGE) with a Laemmli buffer system (15). The samples were suspended in SDS sample buffer, heated to 95°C for 5 min, and separated by SDS-PAGE with a Hoeffer apparatus and a 12% polyacrylamide gel. The transfer of protein from SDS-PAGE to nitrocellulose and the incubation of the blots with antibody were performed as modifications of the protocols of Towbin et al. (21) and Renart et al. (17). The protein was transferred to nitrocellulose overnight at 4°C in blotting buffer (14.5 mM glycine, 50 mM Tris, 20% methanol, 0.01% SDS) with a Hoeffer transfer apparatus. Mouse sera obtained 7 days after the last boost of recombinant antigen or anti-*B. burgdorferi* serum which was absorbed with recombinant OspA and OspB were diluted from 1:100 to 1:64,000 and incubated with the nitrocellulose strips. The strips were washed, incubated with a 1:5,200 dilution of alkaline phosphatase-labeled goat anti-mouse immunoglobulin G, and developed with nitroblue tetrazolium and 5-bromo-4-chloro-indolyl phosphate (Stratagene).

Thirty micrograms of a protein extract of *E. coli* expressing OspB-B31 in the 197-12 plasmid was separated by SDS-PAGE and transferred to an Immobilon polyvinylidene difluoride membrane. The amount of protein loaded on the

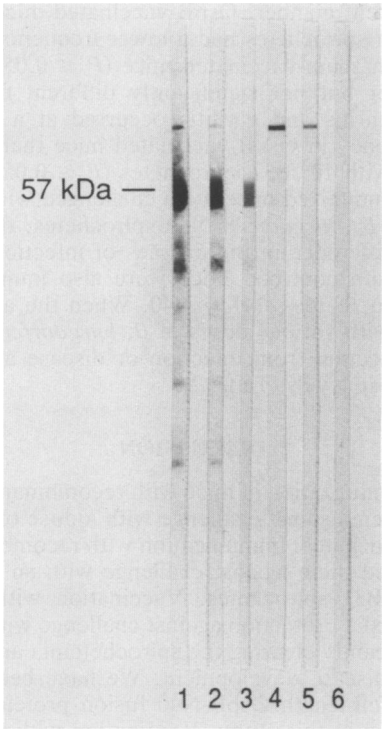

FIG. 1. Immunoblot of recombinant OspA probed with dilutions of rabbit anti-*B. burgdorferi* serum which has been passed over an OspA or glutathione transferase column. The band at 57 kDa represents the recombinant OspA fusion protein, and the band above 57 kDa is an *E. coli* product that is reactive with the rabbit antiserum. Lanes 1 through 3, antisera (1:100, 1:1,000, and 1:10,000, respectively) passed over a glutathione transferase column; lanes 4 through 6, antisera (1:100, 1:1,000, and 1:10,000, respectively) passed over an OspA column.

gel was determined by the method developed by Bradford (9). The protein was probed with a 1:100 dilution of an OspB-N40 monoclonal antibody, designated OspB Mab 7 B10.36 and produced at Yale, or stained with Coomassie brilliant blue. The bands which showed binding to the OspB monoclonal antibody were excised from the membrane stained with Coomassie brilliant blue and directly sequenced at the N terminus at the Yale Protein Chemistry Laboratory.

RESULTS

To determine whether antibodies to proteins other than OspA play roles in protective immunity to spirochete infection, rabbit anti-*B. burgdorferi* serum was twice passed over a recombinant OspA column. The removal of anti-OspA antibodies appeared to be complete, since the eluted serum showed no binding with the recombinant OspA fusion protein in the immunoblot (Fig. 1). We cannot exclude, however, the possibility that antibodies which react with conformationally dependent epitopes did not bind to OspA on the column or immunoblot and were therefore not removed. The eluted serum, lacking antibodies to OspA, was then used at 1:50 dilutions to passively immunize C3H mice. The OspA antibody-negative serum still protected the animals from challenge with 10^4 N40 spirochetes, indicating that antibodies to proteins other than OspA were involved in protective immunity (Table 1). As a control, mice were immunized with

TABLE 1. Protection of mice from infection with *B. burgdorferi* by passive immunization with absorbed rabbit anti-*B. burgdorferi* serum

Rabbit anti-*B. burgdorferi* serum	No. of positive results/total[a] by:		
	Culture	Histopathologic evaluation	
		Arthritis	Carditis
Whole	0/5 (4/4)	0/5 (5/5)	0/5 (5/5)
With OspA antibodies absorbed	0/5 (4/4)	0/5 (5/5)	1/5 (5/5)
With OspA and OspB antibodies absorbed	5/5 (5/5)	5/5 (5/5)	2/5 (5/5)

[a] Results for control animals (normal rabbit serum) are indicated in parentheses.

anti-*B. burgdorferi* serum which had been passed over a glutathione column. To determine whether antibodies to proteins other than OspA and OspB are involved in protective immunity, anti-*B. burgdorferi* serum was passed over both an OspA and an OspB column. The residual sera did not react with OspA or OspB on the immunoblot (data not shown). Mice passively immunized with the residual sera developed infections when challenged with 10^4 spirochetes, suggesting that OspA and OspB are major determinants in protection.

Previous work has indicated that mice vaccinated with OspA are protected from challenge with an intradermal inoculation of 10^4 spirochetes (12). To determine whether the protective effect of OspA was dose dependent, the studies were repeated with a larger dose of spirochetes. Mice were protected against challenge with 10^4 spirochetes, but this protective effect was removed by challenging the mice with an inoculum of 10^7 organisms (see Table 2). Even at this challenge dose, the degrees of periarticular edema and synovial proliferation seen on microscopic examination of the joints were less severe in vaccinated mice (grade 1) than in control animals (grade 3).

To determine the roles of OspB and flagellin in protective immunity, animals were actively immunized with the recombinant proteins. Mice immunized with recombinant OspB made antibodies to OspB which were detectable on immunoblot at a dilution of 1:5,000 (Fig. 2). Antibodies in sera from mice immunized with recombinant OspB did not bind to native OspA on immunoblot, suggesting that the majority of Osp antibodies are not cross-reactive. This corroborates our previous results, which showed that the sera from mice immunized with recombinant OspA bound native OspA but not native OspB on immunoblot (12). The sera containing OspB antibodies also bound to a 17-kDa *B. burgdorferi* protein. It is likely that this smaller protein is a fragment of OspB, because when the gene for OspB-B31 was expressed in *E. coli* by using the 197-12 plasmid and probed with an OspB monoclonal antibody on immunoblot, binding to both a 34-kDa and a 17-kDa protein was observed. Two bands of identical size were identified on a *B. burgdorferi* immunoblot with the same monoclonal antibody. The 17-kDa fragment in *E. coli* is N terminal, since terminal protein sequencing of these two bands in *E. coli* expressing OspB revealed identical sequences corresponding to the NH_2-terminal proteins deduced from the known nucleotide sequence of OspB-B31: Met-X-Leu-Leu-Ile-Gly-Phe-Ala-Leu-Ala-Leu-Ala-Leu-Ile-Gly-X-Ala-Gln-Lys-Ala-Glu-Ser-Ile. Neither the 34-kDa nor the 17-kDa *Borrelia* protein was amenable to direct sequencing, suggesting that the proteins were blocked at the amino

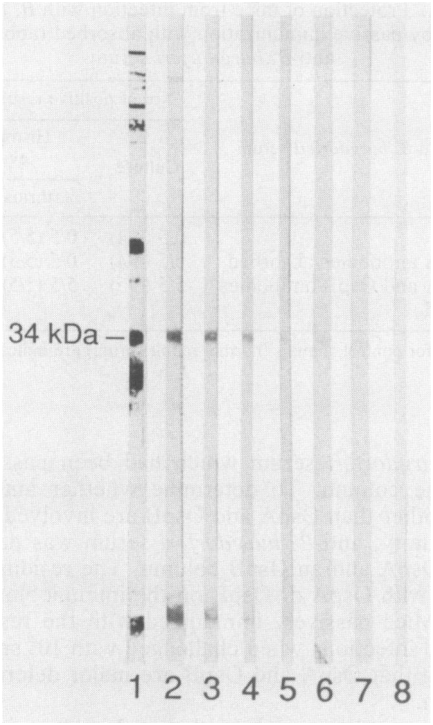

FIG. 2. Immunoblots of *B. burgdorferi* probed with dilutions of sera from mice immunized with recombinant OspB. Lane 1, anti-*B. burgdorferi* serum (1:100). Lanes 2 through 7, dilutions of mouse anti-OspB serum, as follows: lane 2, 1:100; lane 3, 1:1,000; lane 4, 1:5,000; lane 5, 1:10,000; lane 6, 1:30,000; and lane 7, 1:60,000. Lane 8, normal mouse serum (1:100).

terminus. Mice subcutaneously immunized with the cell extract containing 10 μg of recombinant flagellin produced antibodies to flagellin which could be detected at a dilution of 1:6,000 on immunoblot.

Mice immunized with OspB were challenged intradermally with 10^4, 10^3, or 10^2 *B. burgdorferi* B31 or N40 spirochetes and evaluated for protection (Table 2). The animals were protected from infection and disease in a dose-dependent manner. OspB-vaccinated mice challenged with 10^4 B31 spirochetes had a lower frequency of positive cultures than sham-vaccinated mice ($P \leq 0.05$ by χ^2 test), with a lower but not significantly different frequency of disease. Arthritis and carditis occurred at a significantly lower frequency in OspB-vaccinated mice than in controls challenged with 10^3 B31 spirochetes ($P \leq 0.05$ by χ^2 test). When the immunized mice were challenged with inocula of 10^3 and 10^4 *B. burgdorferi* N40 spirochetes, there was no significant difference in the degrees of infection or disease compared with controls. Mice were also immunized with flagellin from *B. burgdorferi* N40. When the animals were challenged with various doses of *B. burgdorferi* N40, there was no protection from infection or disease at any of the challenge dose levels (Table 2).

TABLE 2. Protection of mice from infection with *B. burdorferi* by active immunization with recombinant OspA-N40, OspB-B31, or flagellin-N40 fusion protein

Immunogen	*B. burgdorferi* strain (dose)	No. of positive results/total[a] by:		
		Culture	Histopathlogic evaluation	
			Arthritis	Carditis
OspA-N40	N40 (10^4)	0/5 (4/5)	0/5 (5/5)	0/5 (5/5)
	N40 (10^7)	4/5 (5/5)	5/5 (5/5)	5/5 (5/5)
OspB-B31	B31 (10^4)	5/14 (12/13)	7/14 (12/13)	3/14 (6/13)
	B31 (10^3)	0/5 (2/4)	0/5 (4/5)	0/5 (4/5)
	B31 (10^2)	0/8 (3/8)	0/10 (3/8)	1/10 (3/8)
	N40 (10^4)	2/5 (5/5)	5/5 (5/5)	4/5 (5/5)
	N40 (10^3)	2/5 (3/5)	4/5 (5/5)	1/5 (5/5)
Flagellin-N40	N40 (10^4)	8/9 (7/10)	9/9 (10/10)	6/9 (8/10)
	N40 (10^3)	2/3 (3/5)	3/3 (5/5)	3/3 (5/5)

[a] Results for control animals (normal rabbit serum) are indicated in parentheses.

DISCUSSION

Active immunization of mice with recombinant OspA-N40 protected them against challenge with a dose of 10^4 but not 10^7 N40 spirochetes. Immunization with recombinant OspB-B31 protected them against challenge with an inoculum of 10^2 or 10^3 B31 spirochetes. Vaccination with OspB-B31 provided partial protection against challenge with 10^4 organisms, significantly clearing the spirochetemia and minimally influencing disease development. We have been unable to generate a full-length OspB-N40 fusion protein, so OspB-B31 was used in this study. Vaccination with flagellin-N40 had no protective effect against challenge with *B. burgdorferi* N40. It appears, therefore, that OspA remains the major antigen involved in eliciting a protective immune response but that the immune response to OspB probably also plays a role. We hypothesize that the differences in protective immunity may be due to the quantities or locations of OspA and OspB proteins on the surface of the organism. In addition, it cannot be excluded that other antigens which elicit antibodies in low titers or induce a cellular immune response may also play roles in protective immunity.

The passive-immunization data indicate that anti-*B. burgdorferi* N40 serum which lacks OspA antibodies is able to protect mice from challenge with N40 spirochetes. When this serum was passed over an OspB-B31 column, the residual serum was no longer protective. This suggests that OspB-N40 antibodies produced upon immunization with *B. burgdorferi* are capable of eliciting a protective immune response and that these antibodies were removed by passage over the OspB-B31 column. However, active immunization with recombinant OspB-B31 did not protect mice from challenge with N40 spirochetes. We hypothesize that this difference could arise if the antibodies elicited by immunizing with recombinant OspB-B31 fusion protein or native OspB in an extract of *B. burgdorferi* differ qualitatively or quantitatively. For example, neutralizing OspB-N40 antibodies could bind to the OspB-B31 fusion protein, whereas immunization with the recombinant OspB-B31 fusion protein may not elicit the production of high titers of neutralizing antibodies which protect against N40 spirochetes. It is also possible that passage over an OspA column did not remove conformational OspA epitopes, which were then eliminated by passage over the OspB column.

The degree of heterogeneity of OspA and OspB among different isolates of *B. burgdorferi* has not yet been fully defined but remains an important issue for vaccine development. Work on the taxonomic classification of the organisms indicates that *B. burgdorferi* can be subdivided into at least two groups on the basis of DNA-DNA hybridization and

rRNA gene restriction patterns, although it is not yet known whether there are differences in OspA or OspB between the groups (16). OspA is a strong candidate for a vaccine, as the nucleotide sequences of three different isolates in the United States and Europe show nearly identical homology (12). Furthermore, there is some degree of cross protection, since animals immunized with OspA from *B. burgdorferi* N40 are protected against challenge with three strains of the spirochete, namely, N40, B31, and CD16 (12). The present report shows that vaccination with OspB from strain B31 is sufficient to protect against challenge with 10^3 B31 spirochetes but not against 10^3 N40 spirochetes, suggesting that OspB protection may be somewhat strain specific. Indeed, initial sequencing data indicate that the OspB-N40 gene differs from the OspB-B31 gene by at least nine nucleotides, which corresponds to six amino acid differences (11). It is not known whether strains B31 and N40 are in the same taxonomic group; however, OspA and OspB from both strains of the spirochete migrate at identical positions on SDS-PAGE, and the nucleotide sequences of the OspA-B31 and OspA-N40 genes are highly conserved (12). Once recombinant OspB-N40 is obtained, experiments will determine whether vaccination with OspB-N40 protects mice from challenge with *B. burgdorferi* N40.

It is also possible that the degrees of virulence of the N40 and B31 spirochetes may play roles in the lack of cross protection. *B. burgdorferi* N40 is more virulent than B31, as the control animals challenged with N40 generally have more disease manifestations. It can be hypothesized that the immune response to OspB is able to protect against a moderately virulent strain of *Borrelia* but not against a highly pathogenic organism. Further work with different strains of *B. burgdorferi* which are found to be infectious in the C3H mouse will address this question. It must also be determined whether the dose of spirochetes delivered via an intradermal injection is qualitatively and quantitatively different from that administered by a tick bite. If the number of spirochetes introduced by a tick bite is fewer than 1,000 organisms and no qualitative differences exist in tick inoculations, then OspA, OspB, or hybrid molecules would afford full protection. Overall, the present experiments indicate that OspA and OspB are both vaccine candidates for Lyme disease.

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