

Selection of Variant *Borrelia burgdorferi* Isolates from Mice Immunized with Outer Surface Protein A or B

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A nonclonal population of *Borrelia burgdorferi* N40 (passage 3) that survived protective immunity following challenge inoculation of outer surface protein (Osp) A- or B-hyperimmunized mice were characterized for the molecular basis of evasion of immunity. Two of six *B. burgdorferi* isolates, cultured from OspA-immunized mice, had antigenic diversity in the carboxyl terminus of OspA and did not bind to the protective OspA monoclonal antibody designated IXDII. However, OspA-immunized mice challenged with these variants were fully protected. Moreover, *B. burgdorferi* isolates with a point mutation in *ospB*, which results in a truncated OspB that does not bind to protective OspB monoclonal antibody 7E6C, were frequently enriched after infection of OspB-immunized mice. These studies suggest that the incomplete efficacy of an OspA- or OspB-based vaccine may be partly due to immunomediated *in vivo* selective pressure, resulting in the persistence of some spirochetes that do not bind to protective antibodies.

Antibodies to outer surface proteins (Osps) A and B are important in the protective immune response to *Borrelia burgdorferi*, the agent of Lyme disease. Mice vaccinated with OspA or OspB or passively immunized with OspA or OspB monoclonal antibodies (MAbs) are protected from spirochete challenge infection (2–4, 23, 25). The degree of immunity and therefore the efficacy of an OspA- or OspB-based vaccine may depend upon many factors, including the immunogenicity of the vaccine preparation being tested, the experimental animal model used to test the vaccine, the route of spirochete challenge, and the quantity and virulence of the *B. burgdorferi* isolate used in the experimental study (2–6, 10–12, 19, 20).

Antigenic diversity among *B. burgdorferi* isolates may enable spirochetes to escape immune destruction in the vaccinated host. Immune serum prepared against one of the genospecies of *B. burgdorferi* is sufficient to protect mice or hamsters against challenge with the homologous strain of the spirochete but does not provide protection against challenge with organisms from different genospecies (12, 20). The molecular basis for this strain-specific immunity is beginning to be understood. For example, *B. burgdorferi* 25015 can survive in mice immunized with OspA from *B. burgdorferi* N40, and the lack of cross-protection is due to variability in the carboxyl terminus of strain 25015 OspA from that of strain N40 OspA, which results in an inability of protective strain N40 OspA antibodies to bind strain 25015 OspA (5). Furthermore, we have shown that a *B. burgdorferi* N40 mutant (N40ΔB) that lacks the carboxyl terminus of OspB, as a result of a premature stop codon in the *ospB* gene, can survive within a mouse immunized with full-length OspB (6). Immunofluorescence studies, using permeabilized and unpermeabilized spirochetes, have shown that epitopes within the carboxyl terminus of OspB are exposed on the surface of the organism, whereas some amino-terminal epitopes are embedded within the outer membrane. This indicates that the carboxyl terminus of OspB is most accessible to

antibody-mediated immune responses (6). In addition, steady-state and time-resolved fluorescence quenching techniques suggest that OspA epitopes involved in the antibody-mediated agglutination of spirochetes are located within alpha-helices on the bacterial surface (9). These studies suggest that antigenic diversity of the spirochete—in particular, changes that result in differences in the carboxyl terminus—can account for failure of a protective immunity.

The exposure of spirochetes to borrelicidal antibodies has been used to isolate mutant organisms *in vitro*, suggesting that antibodies can elicit selective pressure (1, 17). The growth of *B. burgdorferi* in the presence of anti-*B. burgdorferi* serum or antibodies to OspA or OspB results in the inhibition of growth of a majority of the spirochetes (1, 17). However, the outgrowth of spirochetes that do not express OspA or OspB, have mutations in the *osp* genes resulting in mutant Osps, or have lost the 49-kb plasmid that contains the *ospAB* operon occurs occasionally (17). The frequency of recovery of these variant strains has been estimated at a rate of from 1 in 10⁴ to 1 in 10⁷ spirochetes. In addition, limiting dilution cloning of *B. burgdorferi* isolates on semisolid medium led to the identification of spirochetes in which recombination within the *ospAB* operon has resulted in chimeric Osps that may not bind protective Osp antibodies; such recombination also occurs at a low frequency (15). These studies indicate that the growth of *B. burgdorferi* is impaired in the presence of borrelicidal antibodies and that this selection *in vitro* results in the expansion of spirochete populations that have a diminished capacity to bind borrelicidal antibodies.

The efficacy of an OspA- or OspB-based vaccine may be influenced by many factors that are related to the ability of the host to mount and maintain a protective immune response. In addition, antigenic variation among spirochetes due to selection pressure exerted by the OspA and OspB immune response *in vivo* must be considered when assessing a vaccine. In previous studies, OspA and OspB have been capable of eliciting a strong protective response in mice (2, 4, 14, 25). However, the dose of spirochete challenge could account for partial vaccine failure since *B. burgdorferi* can be recovered from OspA-immunized mice inoculated with high doses of spirochetes or OspB-vaccinated animals administered moderate doses of *B.*

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burgdorferi. Therefore, we have characterized the spirochetes recovered from vaccinated animals for the ability to bind protective OspA and OspB MAbs to determine the molecular basis of immune evasion.

MATERIALS AND METHODS

Mice. Three-week-old female C3H/HeN mice were obtained from the National Institutes of Health, Bethesda, Md. The mice were housed in filter frame cages and euthanized with CO₂ when needed.

***B. burgdorferi*.** A nonclonal population of *B. burgdorferi* N40 (passage 3) with proven infectivity and pathogenicity was used throughout the study (6). This inoculum contains at least two subpopulations of *B. burgdorferi* that express full-length OspB (N40-B) or that express a truncated form of OspB (known as N40ΔB) because of a stop codon in *ospB* resulting from a point mutation at nucleotide 577, as described previously (6). The 50% infective dose of N40-B and N40ΔB each is 10² spirochetes (6). The spirochetes were grown to the stationary phase in Barbour-Stoenner-Kelly II (BSK II) medium, and the concentration was adjusted to 10⁸ or 10³ spirochetes per ml for inoculation.

Amplification, cloning, and sequencing of the *osp* genes. The *ospA* and *ospB* genes were amplified from the recovered spirochetes by the PCR. The *ospA* and *ospB* primers corresponded to the first and last 23 nucleotides of the *B. burgdorferi* N40 *ospA* and *ospB* sequences, respectively (3, 6). Primers were flanked by *Bam*HI and *Eco*RI restriction enzyme digestion sites to facilitate subcloning. Ten microliters of a *B. burgdorferi* culture containing 10⁴ organisms was used as the template for PCR. PCR was performed for 30 cycles with an annealing temperature of 55°C for 1 min, an extension temperature of 72°C for 2 min, and a denaturing temperature of 94°C for 1 min, with *Taq* DNA polymerase (Stratagene, La Jolla, Calif.). Amplified DNA segments were purified by agarose gel electrophoresis, digested with *Bam*HI and *Eco*RI, and cloned into the pMX expression vector (a pGEX-2T-based vector with a modified polylinker) (22). The nucleotide sequences of *ospA* and *ospB* were determined by an assay based on the methodology of Sanger et al. (18) with the Sequenase version 2.0 kit (United States Biochemical, Cleveland, Ohio). Both strands of DNA were sequenced. When point mutations were identified, three independent clones were sequenced for verification.

Immunoblots. *B. burgdorferi* whole-cell lysates (3 μg) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in a 12.5% polyacrylamide gel. Proteins were transferred to nitrocellulose, and the sheets were probed with MAbs (1:100 dilution) in phosphate-buffered saline (PBS) with 5% bovine serum albumin (BSA) for 1 h. The OspA MAbs 8C4BC, CIII.78, and IXDII bind to OspA epitopes encompassing amino acids (aa) 1 to 143, 133 to 273, and 200 to 273, respectively, and the OspB MAbs 7E6C and B10 bind within aa 133 to 296 and 1 to 50, respectively, as described previously (5, 6, 22). The OspA MAbs CIII.78 (3) and IXDII (8) and OspB MAb 7E6C (6) are protective, whereas OspA MAb 8C4BC and OspB MAb B10 (6) are not protective in nature. The strips were washed three times with PBS and incubated with horseradish peroxidase-labeled goat anti-mouse immunoglobulin (Southern Biotechnology Associates, Inc., Birmingham, Ala.), diluted 1:100 in PBS with 5% BSA, developed with an enhanced chemiluminescence detection kit (Amersham, Arlington Heights, Ill.), and exposed to X-ray film.

Immunization studies. Three-week-old female C3H/HeN mice were actively immunized with 10 μg of recombinant *B. burgdorferi* N40 OspA or OspB in complete Freund adjuvant and boosted with the same antigen in incomplete Freund adjuvant at 2-week intervals (4). OspA and OspB were both expressed as full-length recombinant fusion proteins with glutathione transferase (GT), as described previously (3, 6). Two weeks after the last boost, OspA-immunized mice were challenged with 10⁷ or 10⁴ spirochetes, and OspB-immunized mice were challenged with 10² spirochetes, administered via an intradermal injection (0.1 ml). After 14 days, the mice were euthanized and the blood, spleen, bladder, and skin were cultured in BSK II medium for 14 days. The knees, tibiotarsi, and hearts were formalin fixed, paraffin embedded, and examined for histopathology. An animal was considered to have arthritis when at least one joint showed evidence of edema and synovial infiltration with neutrophils and lymphocytes. Carditis was characterized by aortitis, myocarditis, or atrial and ventricular pericarditis. The recovery of spirochetes by culture or histopathologic evidence of disease was indicative of infection.

RESULTS

We challenged OspA- or OspB-immunized mice with doses of *B. burgdorferi* that were shown previously to overcome the protective effect and examined the spirochetes recovered from these studies to determine if the *B. burgdorferi* organisms retained their ability to bind with protective OspA or OspB antibodies. *B. burgdorferi* organisms were recovered readily from OspA-immunized or control mice challenged with 10⁷ spirochetes (Table 1). Similarly, spirochetes were recovered

TABLE 1. Recovery of *B. burgdorferi* from mice immunized with OspA or OspB^a

| Immunization | <i>B. burgdorferi</i> inoculum size | No. of mice positive/total no. | | |
|--------------|-------------------------------------|--------------------------------|---------|-----------|
| | | Culture | Disease | Infection |
| OspA | 10 ⁷ | 6/6 | 6/6 | 6/6 |
| OspA control | 10 ⁷ | 6/6 | 6/6 | 6/6 |
| OspB | 10 ² | 6/10 | 7/10 | 7/10 |
| OspB control | 10 ² | 7/10 | 8/10 | 8/10 |

^a Mice were immunized with 10 μg of recombinant OspA or OspB in complete Freund adjuvant and boosted twice with the identical preparation in incomplete Freund adjuvant at 14-day intervals. Control mice were immunized with GT in the same fashion. Two weeks after the last boost, the mice were challenged with an intradermal inoculation of 10⁷ (OspA) or 10² (OspB) *B. burgdorferi* organisms. The mice were sacrificed after 14 days. Cultures of blood, spleen, bladder, and skin were incubated in BSK II medium for 2 weeks and examined by dark-field microscopy for spirochetes. Knees, tibiotarsi, and hearts were examined microscopically for evidence of inflammation. A mouse with disease and/or from which spirochetes were recovered by culture was considered infected.

from OspB-immunized mice or control mice challenged with 10² organisms.

The recovered spirochetes were examined by immunoblot for their ability to bind with OspA or OspB MAbs that recognize different regions of OspA or OspB, some of which have borreliacidal activity in vivo. All of the spirochetes recovered from OspA-immunized mice expressed OspA (Fig. 1A). When the spirochetes recovered from the GT-immunized (control) or OspA-immunized mice were probed with OspA MAbs, all six of the isolates bound the nonprotective OspA MAb 8C4BC that binds near the amino terminus of OspA (aa 1 to 143) (Fig. 1A). Similarly, the protective OspA MAb CIII.78 that binds a conformational epitope within aa 133 to 273 bound to all six isolates, although one isolate (N40-OspA4) (Fig. 1B, lane 11) had a decreased intensity of binding relative to that of the other spirochetes. In contrast, an additional protective OspA MAb designated IXDII that binds an epitope within amino acids 200 to 273 uniformly bound to all six of the isolates recovered from control mice but did not bind to two of the six isolates recovered from OspA-immunized mice (i.e., N40-OspA4 and N40-OspA6) (Fig. 1C, lanes 11 and 13).

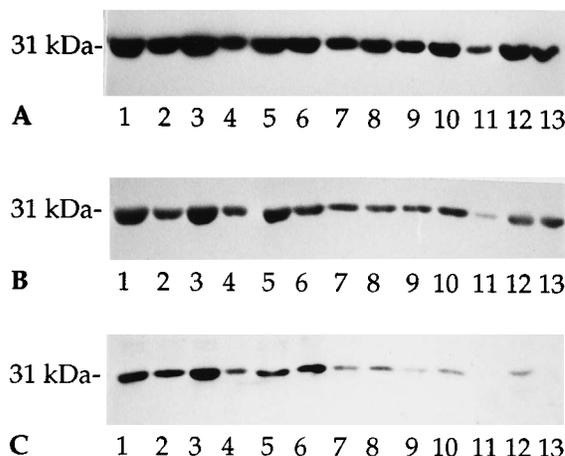


FIG. 1. Immunoblot of *B. burgdorferi* organisms recovered from OspA-immunized mice probed with OspA MAbs 8C4BC (A), CIII.78 (B), and IXDII (C). The designations OspA1 to OspA6 represent spirochetes cultured from OspA-immunized mice. The designations C1 to C6 represent spirochetes cultured from control (GT-immunized) mice. Lanes: 1, *B. burgdorferi* N40; 2 to 7, C1 to C6, respectively; 8 to 13, OspA1 to OspA6, respectively.

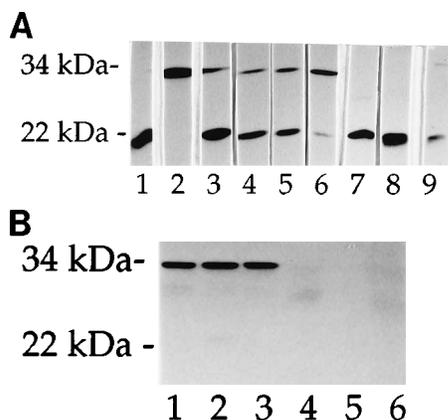


FIG. 2. Immunoblot of *B. burgdorferi* organisms recovered from OspB-immunized mice probed with OspB MAbs B10 (A) and 7E6C (B). The designations OspB1 to OspB3 represent spirochetes cultured from OspB-immunized mice. The designations C1 to C3 represent spirochetes cultured from control (GT-immunized) mice. (A) Lanes: 1, *B. burgdorferi* N40ΔB; 2, *B. burgdorferi* N40-B; 3, *B. burgdorferi* N40; 4 to 6: C1 to C3, respectively; 7 to 9: OspB1 to OspB3, respectively. (B) Lanes: 1 to 3, C1 to C3, respectively; 4 to 6: OspB1 to OspB3, respectively.

DNA from the two spirochetes that did not bind OspA MAb IXDII was amplified by PCR and sequenced to determine if sequence variability in *ospA* could be identified. N40-OspA4 (Fig. 1C, lane 11) had a TTC codon at nucleotides 742 to 744 (residue 248) rather than TAC, resulting in the substitution of Tyr by Phe in the published *B. burgdorferi* N40 sequence (3). In addition, the carboxyl-terminal sequence of N40-OspA6 (Fig. 1C, lane 13) from nucleotides 808 to 819, encoding protein residues 270 to 273, was AAG (Lys), GTG (Val), CTG (Leu), AGT (Ser) rather than ACC (Asn), GCT (Ala), TTA (Leu), AAA (Lys) as described for *B. burgdorferi* N40 (3).

OspA-immunized mice were then challenged with an inoculum of 10^4 N40-OspA4 or N40-OspA6 organisms and assessed for susceptibility to infection. Ten GT-immunized (control) mice were readily infected with N40-OspA4 (five of five) or N40-OspA6 (five of five). In contrast, none of the 10 OspA-immunized mice, 0 of 5 and 0 of 5, respectively, were infected with either isolate.

Spirochetes recovered from OspB-vaccinated mice were also examined for their ability to bind with selected OspB MAbs. Two populations of spirochetes (N40-B and N40ΔB) have been identified in the original *B. burgdorferi* N40 isolate; these two populations are similar except for a point mutation in *ospB* that results in a stop codon and a truncated OspB that lacks the carboxyl terminus (N40ΔB) (6). Both of these cloned subpopulations and the mixed, nonclonal population of N40 spirochetes are shown by immunoblot with MAb B10 that binds within the amino terminus of OspB (Fig. 2A, lanes 1 to 3). The initial challenge inoculum of *B. burgdorferi* N40 contained both N40-B and N40ΔB. All of the isolates recovered from control animals contained both subpopulations of organisms, those expressing the full-length OspB and those expressing the truncated form of OspB (Fig. 2A, lanes 4 to 6). Indeed, on the basis of the relative intensity of the staining of each band, approximately equivalent numbers of N40-B and N40ΔB were present in the isolates from control mice, which establishes that both forms are infectious to similar degrees. In contrast, in all six isolates from mice immunized with OspB, only the N40 spirochetes that expressed the truncated form of OspB (N40ΔB) were recovered, and three representative isolates are shown (Fig. 2A, lanes 7 to 9). Furthermore, all of the cultures of *B.*

burgdorferi recovered from control animals contained spirochetes that bound the OspB MAb 7E6C that is protective and binds a conformational epitope within the carboxyl terminus of OspB (Fig. 2B, lanes 1 to 3). In contrast, none of the spirochetes isolated from OspB-immunized mice bound MAb 7E6C (Fig. 2B, lanes 4 to 6), further indicating that these recovered spirochetes did not express the full-length OspB.

Mice immunized with both OspA and OspB and then challenged with 10^7 nonclonal N40 spirochetes were infected with *B. burgdorferi*. Spirochetes were readily recovered from all five control mice (four of five blood cultures, five of five bladder cultures, and four of five splenic cultures) and less efficiently from OspA- and OspB-immunized mice (none of five blood cultures, four of five bladder cultures, and three of five splenic cultures). Although the OspA and OspB responses prevented the recovery of spirochetes from the bloodstreams of experimental mice, *B. burgdorferi* organisms were cultured from at least one site in each of the immunized mice, indicating that all of the mice were infected.

DISCUSSION

These data show that the challenge dose of *B. burgdorferi* can result in a lack of vaccine efficacy and that the selective pressure of vaccine-induced immunity can affect the survival of spirochetes that do not bind with protective antibody. The influence of OspB immunity was clearly related to the ability of spirochetes with a truncated OspB to survive preferentially within the host. In contrast, the OspA-mediated immune response resulted only in the recovery of some spirochetes that did not react with the protective OspA antibody (IXDII) on immunoblot and with well-defined genetic differences in the 3' region of *ospA*. Immune-mediated *in vivo* selection of *B. burgdorferi* is therefore not the only factor that resulted in a lack of vaccine efficacy, because spirochetes without alterations in OspA were also recovered from the immunized mice. Indeed, OspA, OspB, and OspC do not vary in *B. burgdorferi* recovered from mice infected with a clonal population of spirochetes (13, 24). This suggests that the challenge dose is one factor that is important in assessing vaccine efficacy and that factors other than the ability of protective OspA immunity influence spirochete survival. Since *B. burgdorferi* isolates with variant OspA proteins may be small, it may not be possible to routinely identify the vaccine failures in experimental protocols with small challenge inocula of *B. burgdorferi*. In contrast, the relatively high frequency of *B. burgdorferi* isolates with a truncated OspB (N40ΔB) makes the selective recovery of the mutant spirochetes in vaccine studies more readily apparent.

The modest selection pressure that resulted in the increased populations of N40-OspA4 and N40-OspA6 isolated from OspA-immunized mice infected with a nonclonal population of *B. burgdorferi* N40 was not sufficient to fully account for the evasion of protective immunity. When N40-OspA4 and N40-OspA6 were cultured and used as the challenge inocula in murine protection studies, the animals were fully protected against infection, suggesting that antibodies that bind additional epitopes on OspA, such as the region defined by MAb CIII.78, may provide sufficient immunity. In contrast, when OspB-vaccinated mice were challenged with a nonclonal population of N40 *B. burgdorferi* organisms, all of the recovered spirochetes had a truncated OspB. These data suggest that antibodies directed towards at least several carboxyl-terminal OspA epitopes are protective and may have borreliaecidal activity against some spirochetes expressing variant OspA. In the initial studies, in which N40-OspA4 and N40-OspA6 were isolated from OspA-immunized mice challenged with 10^7 spiro-

chetes, we postulated that the quantity of protective antibodies may have been sequestered (21) or may not have been sufficient to bind with the large inocula, allowing for the preferential survival of the slightly variant organisms. When OspA-immunized mice were then challenged with a lower dose (10^4) of N40-OspA4 or N40-OspA6, the immune response may have been sufficient to afford full protection. In contrast, protection does not extend to N40 Δ B because these spirochetes lack the antigenic carboxyl terminus. These data indicate that immunity may extend to spirochetes with slightly variant Osps but not truncated Osps.

While the influence of OspA and OspB antibodies on the recovery of *B. burgdorferi* has been shown in vitro, this is the first study to show that immune pressures play a role in the selection of spirochetes in vivo (17). Since it has been shown that the efficacy of vaccine-mediated immunity against *B. burgdorferi* transmitted by tick bite may be greater than the efficacy of immunity provided by challenge with spirochetes transmitted by syringe inoculation, it remains to be determined how relevant the recovery of the variant *B. burgdorferi* isolate as a cause of vaccine failure will be in the natural setting of vector-transmitted disease (7, 8, 26). It is possible that variant spirochetes may be less infectious than parental strains or killed within the vector before transmission to the mammalian host (16). Indeed, in a limited study, OspA- or OspB-vaccinated mice were protected against *B. burgdorferi* transmitted by wild ticks collected from the isolated island of Nantucket, even though the naturally infected ticks contained an antigenically heterogeneous population of *B. burgdorferi*, including spirochetes with the OspB truncation (26). However, more comprehensive studies of ticks containing diverse populations of spirochetes from different geographic locations indicate that vaccine failures due to antigenic heterogeneity can occur, even though they may be less common than previously expected (8). Moreover, the current study shows that immunization with both OspA and OspB was not sufficient to protect mice against challenge with an inoculation of 10^7 uncloned *B. burgdorferi* organisms, indicating that spirochetes can persist in the presence of both OspA and OspB antibodies. Furthermore, OspB-vaccinated mice are protected from challenge with 10^3 (but not 10^4) *B. burgdorferi* organisms expressing full-length OspB, and OspA-immunized mice are protected from infection with 10^5 (but not 10^6) *B. burgdorferi* organisms expressing wild-type OspA (data not shown), showing that immunization against a clonal population of spirochetes is also dependent upon the challenge dose. Therefore, we postulate that during tick-borne infection, a population of antigenically heterogeneous spirochetes may be transmitted to the host (27) and that the spirochetes that persist in the immune host during the evolution of infection and the development of chronic disease are more likely to be partially resistant to borreliacidal immune responses.

This report describes the ability of OspA and OspB antibodies to cause the in vivo selection of *B. burgdorferi* organisms with subtle genetic alterations that result in the expression of OspA or OspB which do not bind to, or weakly bind with, antibodies that are protective in nature. These data suggest a potential reason for the lack of complete efficacy of an Osp-based Lyme disease vaccine. Over extended periods of time, the administration of an OspA- or OspB-based vaccine to hosts that are involved in the natural life cycle of the spirochete may result in the expansion of variant *B. burgdorferi* isolates within ticks at a higher frequency than would normally be found in the general population. If this selection pressure was to be maintained, the number of variant spirochetes could rise to a sig-

nificant level, such that the efficacy of a monovalent OspA- or OspB-based vaccine could be impaired in the future.

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