INFECTION AND IMMUNITY, May 1994, p. 1920–1926
0019-9567/94/$04.00+0

Vol. 62, No. 5

Protection of C3H/HeN Mice from Challenge with *Borrelia burgdorferi* through Active Immunization with OspA, OspB, or OspC, but Not with OspD or the 83-Kilodalton Antigen

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Received 25 October 1993/Returned for modification 12 January 1994/Accepted 16 February 1994

Recent advances in the development of animal models for Lyme borreliosis have provided means of identifying potential targets for the design of a subunit vaccine to prevent this disease. The C3H/HeN mouse model was used to study several *Borrelia burgdorferi* antigens from a single isolate for their ability to elicit borreliacidal and protective antibodies. The *ospA*, *ospB*, *ospC*, *ospD*, and 83-kDa genes from a California isolate, SON 188, were cloned and expressed in *Escherichia coli* as proteins fused to the C-terminal end of maltose-binding protein. Active immunization of mice with these fusion proteins elicited high titers of antibodies that recognized the homologous SON 188 antigens upon immunoblotting. Antibodies generated to the OspA and OspB fusion proteins, but not to the OspC, OspD, and the 83-kDa fusion proteins, demonstrated in vitro borreliacidal activity. Challenge of all actively immunized mice with 10^7 SON 188 spirochetes resulted in infection in all mice receiving the OspD or 83-kDa immunogens but not in any mice receiving the OspA, OspB, or OspC fusion proteins. These results demonstrate the potential of OspA, OspB, and OspC as components of a subunit vaccine for the prevention of Lyme borreliosis.

Lyme borreliosis is a multisystemic disease transmitted by tick bite. The causative agent was identified as the spirochete *Borrelia burgdorferi* (6). The salient features of the disease in humans include early flu-like symptoms which, if untreated, may progress to arthritis, carditis, and neurological involvement (44). Lyme borreliosis is now the most prevalent vector-borne disease in North America (8). The clinical importance of Lyme borreliosis to veterinary and human medicine has prompted a concerted effort to develop a vaccine for its prevention (12). Recently, a killed whole-cell vaccine was approved for use in canines (9). However, the demonstration of cross-reactive epitopes among *B. burgdorferi* and neural tissue brings to issue the possible risk of autoimmune sequelae when using a whole-cell vaccine (42). A vaccine comprised of antigenic subunits or components of *B. burgdorferi* would potentially avoid this problem.

The development of a subunit vaccine for the prevention of Lyme borreliosis has been facilitated by the establishment of several laboratory animal models, including rodent, canine, and primate models (1, 2, 29, 32). The availability of these models, coupled with the recent cloning and sequencing of several outer surface proteins from *B. burgdorferi* (3, 17, 30, 35, 47), has been instrumental in identifying antigens with the potential of eliciting protective antibodies. Passive immunization with antibodies recognizing outer surface protein A (OspA) or OspB has proven effective in preventing infection in both the severe combined immunodeficiency and the C3H mouse models (37, 41). Active immunization using OspA or OspB was also successful in providing protection in immunocompetent C3H mice challenged with *B. burgdorferi* (13, 14, 45). More recently, a third antigen, OspC, was found to elicit protective antibodies in gerbils challenged with a European isolate of *B. burgdorferi* (33). These reports suggest that a number of *B. burgdorferi* antigens may prove important in the development of a subunit vaccine. In order to enhance our understanding of the antigens involved in the development of protective immunity to *B. burgdorferi*, we have evaluated the contribution of several immunologically relevant *B. burgdorferi* antigens derived from a single isolate.

The genes encoding OspA, OspB, OspC, OspD, and an 83-kDa protein from the *B. burgdorferi* isolate SON 188 were cloned, and the product of each gene was expressed as a fusion protein in *Escherichia coli*. The ability of each fusion protein to elicit borreliacidal and protective antibodies was evaluated by active immunization of C3H/HeN (C3H) mice and subsequent challenge with SON 188.

MATERIALS AND METHODS

Organisms. The *B. burgdorferi* isolate SON 188 originated from an *Ixodes pacificus* tick collected in Sonoma County, Calif., and was kindly provided by Paul Duffy of the California Department of Health Services. SON 188 was inoculated into and recovered by ear punch biopsy from C3H mice and then subsequently subcultured three times in Barbour-Stoenner-Kelly medium (BSK II) prior to use in these experiments. The 50% infectious dose by subcutaneous inoculation of SON 188 in 13-week-old C3H mice was 3×10^4 organisms (34).

Competent *E. coli* strain DH5α was obtained from Gibco/BRL (Gaithersburg, Md.).

Amplification of genes. The 83-kDa, *ospA*, *ospB*, *ospC*, and *ospD* genes were obtained by PCR using the primer pairs described in Table 1. Primer 2 of each set possesses a unique restriction enzyme site to facilitate cloning and a clamp (extra nucleotides 5′ to the restriction site) to increase cutting efficiency. The 100-μl amplification reaction consisted of 100 pmol of each primer, 200 ng of total DNA from SON 188, and 2.5 U of *Taq* polymerase (Promega, Madison, Wis.). The reaction buffer (Promega) was supplemented with $MgCl_2$ and deoxynucleotide triphosphates to a concentration of 1.5 and

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TABLE 1. Primer pairs used in the amplification of genes from SON 188

Gene	Primer 1[a]	Primer 2[a]	Reference
ospA	AAGCAAAATGTTAGCAGCTT	CGCG**GTCGAC**TTATTTTAAAGCGTTTTTAATTTC[b]	3
ospB	GCACAAAAAGGTGCTGAGT	CGCG**GAATTC**TTATTTTAAAGCGTTTTTAAGCT[c]	3
ospC	AATAATTCAGGGAAAGGT	GTCGCG**CTGCAG**TTAAGGTTTTTTTGGACTTTC[d]	17
ospD	GTTCATGATAAACAAGAATTA	CGCG**GAATTC**TTAAGTATTTAACAAGGCCACAACT[c]	30
83 kDa	CGCG**GGATCC**ATGAAAAAAATGTTACTAATC[e]	CGCG**CTGCAG**TTACTTAACTTTCTTTAAAGTATT[d]	31

[a] The direction of each sequence is from 5′ to 3′.
[b] The *Sal*I site is in boldface type.
[c] The *Eco*RI site is in boldface type.
[d] The *Pst*I site is in boldface type.
[e] The *Bam*HI site is in boldface type.

0.5 mM, respectively. Amplification was performed for 30 cycles under the following conditions: 2 min of denaturation at 94°C, 2 min of annealing at 45°C, and 4 min of extension at 72°C. The amplification products were purified with the Magic PCR Preps DNA purification system (Promega).

Cloning and expression of genes. The amplification products were prepared for cloning by treating with the Klenow fragment of DNA polymerase to form blunt ends, cutting with the appropriate restriction enzyme (Table 1), and phosphorylating with T4 polynucleotide kinase. The cloning vector pMal c2 (New England Biolabs, Beverly, Mass.) was digested with *Xmn*I and a restriction enzyme corresponding to the unique site engineered into the amplification product (Table 1). An exception to this was the cloning of the 83-kDa gene, in which the pMal c2 vector was cut with *Bam*HI and *Pst*I. Following digestion, the vector was dephosphorylated with calf intestinal alkaline phosphatase, mixed with the amplification product, and ligated overnight at 16°C. *E. coli* strain DH5α was transformed with the ligation mixture and cultured on Luria-Bertani plates containing ampicillin (100 μg/ml). Transformants were then screened on Luria-Bertani–ampicillin plates containing isopropylthiogalactoside (IPTG) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). White colonies were selected for further characterization by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting (immunoblotting).

Identity verification of cloned genes by dot blot hybridization. The plasmid constructs were purified from *E. coli* with the Wizard Miniprep DNA purification system (Promega). Ten nanograms of each plasmid construct was spotted onto a nylon membrane, and the membrane was probed with a digoxigenin-labeled synthetic oligonucleotide probe (Table 2). Hybridization was performed at 37°C with 5 pmol of each probe per ml. Labeling of the oligonucleotides with digoxigenin and detection of the hybridized probes were performed by using the Genius system (Boehringer Mannheim, Indianapolis, Ind.).

Purification and cleavage of the fusion proteins. Purification and cleavage of the fusion proteins were performed by following the manufacturer's instructions (New England Biolabs). Overnight cultures of transformed *E. coli* were inoculated into 500 ml of Luria-Bertani broth supplemented with 100 μg of ampicillin per ml and grown for 5 h at 37°C. IPTG was added to a concentration of 3 mM, and the cultures were incubated for an additional 2 h. The bacteria were harvested by centrifugation, washed in column buffer (10 mM Tris, 200 mM NaCl [pH 7.4]), and stored at −20°C. The bacterial pellet was thawed, sonicated for 2.5 min, and centrifuged to remove cellular debris. The sonicate was loaded onto 5 ml of amylose resin (New England Biolabs), and the resin was washed extensively in column buffer. The fusion proteins were then eluted in column buffer containing 10 mM maltose. The eluted fusion proteins were extensively diafiltrated into column buffer supplemented with 2 mM $CaCl_2$ by using Centricon 10 concentrators (Amicon, Beverly, Mass.). The protein concentration was quantitated by the BCA protein assay (Pierce, Rockford, Ill.).

For immunoblotting purposes (see below), the purified fusion proteins were cleaved into individual components by factor Xa. This was accomplished by combining 20 μg of purified fusion protein and 1 μg of factor Xa (New England Biolabs) in column buffer supplemented with 2 mM $CaCl_2$ and incubating for 16 h at 37°C. The completion of cleavage was confirmed by SDS-PAGE.

Immunization and protection studies. C3H mice (Simonsen Laboratories, Gilroy, Calif.), in groups of four, were immunized with one of the following seven immunogens: β-galactosidase (β-gal), 83-kDa, OspA, OspB, OspC, or OspD fusion protein; or heat-inactivated (56°C for 30 min) SON 188. Four-week-old female mice were primed intraperitoneally with 20 μg of each immunogen in 200 μl of complete Freund's adjuvant (Sigma, St. Louis, Mo.). Three boosts containing the same amount of immunogen in incomplete Freund's adjuvant were administered at 2, 5, and 8 weeks following the initial injection. Mice were bled from the tail vein 7 days after each injection. The serum from each blood sample was pooled by immunogen group and stored at −20°C. Only serum collected from the final blood sample was used in subsequent experiments.

Ten days following the final boost, mice received a subcutaneous inoculation containing 10^7 SON 188 organisms in 100 μl of BSK II medium. Three weeks later, the mice were sacrificed and washed in betadine–70% ethanol and the following tissues were aseptically removed: ear, bladder, and base of the heart. Each tissue was inoculated without further processing into 5 ml of BSK II medium and grown at 32°C for 14 days. Spirochete recovery was determined by evaluating 20 fields at 40× by dark-field microscopy.

Borreliacidal assay. The borreliacidal assay was performed as described by Ma and Coughlin (26). Briefly, twofold serial dilutions of serum from each immunogen group were prepared in 96-well microtiter plates containing BSK II supplemented

TABLE 2. Synthetic oligonucleotide probes used for identity confirmation of cloned *B. burgdorferi* genes

Gene	Sequence	Reference
ospA	CAGCGTTTCAGTAGATTTGC	3
ospB	GTGTTCTTAACAGATGGTAC	3
ospC	GAGTCTGCGAAAGGGCCTAA	17
ospD	AGGCGCAAATTCAAATTACG	30
83 kDa	AGCTGCCACCACCTTATCTGTA	31

with 120 μg of phenol red per ml, and 4×10^6 SON 188 spirochetes in BSK II were added to each well. Guinea pig complement (Gibco/BRL) was added to each well at a concentration of 5%, and the plates were sealed with tape and incubated for 7 days at 32°C. Serum dilutions were performed in triplicate. The absorbance value at each dilution was measured by taking the average A_{560} of the triplicate samples and subtracting the average absorbance of the control wells containing no serum. The titer was recorded as the highest dilution yielding an A_{560} at an optical density of >0.2.

SDS-PAGE and Western blotting. SDS-PAGE and the transfer of proteins to nitrocellulose were accomplished as previously described (21, 46). Proteins prepared in sample buffer (62.5 mM Tris, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol [pH 6.8]) were loaded onto 10 and 12% gels and electrophoresed for 45 min at 200 V with a Mini-Protean II gel apparatus (Bio-Rad, Richmond, Calif.). Proteins were transferred to a nitrocellulose membrane at 35 mA for 50 min with a semi-dry transfer apparatus (LKB, Bromma, Sweden).

Following transfer, membranes were blocked in Blotto (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, 5% nonfat dry milk [pH 7.5]) for 30 min. Mouse serum diluted in Blotto was incubated with the membrane for 1 h at room temperature. The membrane was washed in Tris-buffered saline containing 0.05% Tween 20 and incubated with alkaline phosphatase-labeled goat anti-mouse immunoglobulin G (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) for 1 h. The wash was repeated, and the membrane was developed for 10 min in bromochloroindolyl phosphate-nitroblue tetrazolium.

For serum titrations, 3-mm-wide membrane strips containing either SON 188 lysate at 5 μg/0.5 cm or factor Xa-cleaved recombinant proteins at 0.5 μg/0.5 cm of gel were probed with two-fold serial dilutions of mouse serum. The highest dilution producing a detectable band was considered the end point of the titration.

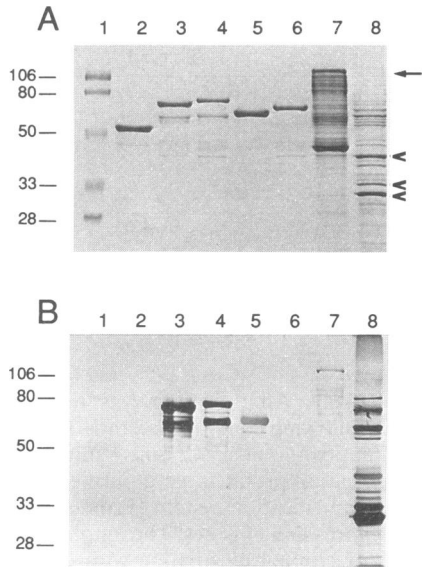

FIG. 1. SDS-PAGE and Western blotting of immunogens. (A) Coomassie blue-stained, 10% polyacrylamide gel containing molecular mass markers (in kilodaltons) (lane 1); 1 μg of β-gal (lane 2), OspA (lane 3), OspB (lane 4), OspC (lane 5), and OspD (lane 6) fusion proteins; 5 μg of the 83-kDa fusion protein (lane 7); and 10 μg of SON 188 lysate (lane 8). The arrow indicates the predicted size of the full-length 83-kDa fusion protein. The arrowheads indicate the locations in order of decreasing molecular mass of the flagella, OspB, and OspA proteins of SON 188 (lane 8). (B) Western blot of immunogens probed with mouse anti-SON 188 serum at a 1:20,000 dilution. The order of immunogens was as described above but with 0.5 μg of each fusion protein and 5 μg of SON 188 lysate loaded onto a 10% polyacrylamide gel and electrophoretically transferred to nitrocellulose.

RESULTS

Cloning and expression of fusion proteins. The vector pMal c2 was used for the cloning and expression of the 83-kDa, *ospA*, *ospB*, *ospC*, and *ospD* genes from SON 188. Insertion of each gene into the multiple cloning site of the pMal c2 vector positions the insert downstream and in frame with a portion of the *E. coli* gene (*malE*) encoding maltose-binding protein (MBP) and a nucleotide sequence encoding a factor Xa cleavage site, respectively. Subsequent expression of each construct in *E. coli* results in the production of a fusion protein consisting of MBP, a factor Xa cleavage site, and the protein encoded by the inserted gene. Factor Xa cleavage of the fusion protein potentially yields two products: MBP and the cloned gene product. Expression of the pMal c2 vector without an insert produces a fusion protein consisting of MBP and a portion of β-gal. This protein served as a negative control in all experiments.

Insertion of the amplified sequences representing the 83-kDa, *ospA*, *ospB*, *ospC*, and *ospD* genes into the pMal c2 expression vector was facilitated by the incorporation of a restriction site into primer 2 of each primer pair (Table 1). Problems in cloning the 83-kDa gene necessitated the addition of a restriction site (*Bam*HI) to primer 1 to promote ligation of this gene. The entire amino acid coding region of the 83-kDa gene was cloned, whereas the nucleotide sequences encoding the signal II peptidase leader sequence and N-terminal cysteine were deleted from the *osp* genes. Transformation of *E. coli* with each plasmid construct resulted in fusion protein expression levels representing 10 to 20% of the total bacterial protein content. In each case, the identity of the cloned gene possessed by each transformant was confirmed by specific hybridization of the plasmid construct with the appropriate synthetic oligonucleotide probe (Table 2) (data not shown).

The binding of each fusion protein to the amylose resin and subsequent elution resulted in significant enrichment of the fusion proteins. A single major band was observed on the Coomassie blue-stained SDS-PAGE gel for the β-gal and Osp fusion proteins (Fig. 1A). The estimated molecular masses of the β-gal, OspA, OspB, OspC, and OspD fusion proteins were 56, 69, 74, 66, and 68 kDa, respectively. Conversely, the 83-kDa fusion protein appeared as multiple bands ranging from 40 to 120 kDa. Coomassie blue staining of the 83-kDa fusion protein at quantities equivalent to the OspA and β-gal fusion proteins failed to produce a detectable band. Therefore, greater amounts of this protein were required for visualization (Fig. 1A, lane 7). Staining of the SON 188 lysate with Coomassie blue revealed a protein profile similar to that of the reference strain, B31.

Electrophoretic transfer of these samples to nitrocellulose and subsequent probing with mouse anti-SON 188 serum were performed to assess the immunoreactivity of the fusion proteins (Fig. 1B). The anti-SON 188 serum recognized bands of the expected molecular weight for each of the OspA, OspB, OspC, and 83-kDa fusion proteins. Immunoreactive bands of lower than expected molecular weights were also associated with each of these fusion proteins. The OspD and the β-gal fusion proteins, however, were not recognized by the anti-SON 188 serum. The spectrum of *B. burgdorferi* proteins recognized

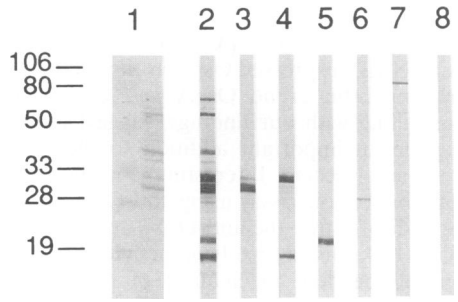

FIG. 2. Western blot analysis of mouse antiserum. A lysate of SON 188 was separated by SDS-PAGE on a 12% polyacrylamide gel and transferred to nitrocellulose. Nitrocellulose strips were either stained for total protein with Ponceau S (lane 1) or probed with a 1:20,000 dilution of serum from the following immunogen groups: SON 188 (lane 2), OspA (lane 3), OspB (lane 4), OspC (lane 5), OspD (lane 6), 83-kDa (lane 7), and β-gal (lane 8). The molecular mass markers (in kilodaltons) are also in lane 1.

TABLE 3. Titers of mouse sera generated to fusion proteins and heat-inactivated SON 188 expressed as reciprocal dilution values

Immunogen	Immunoblot[a]		Borreliacidal activity[b]
	Xa product[c]	SON 188[d]	
β-gal	NT[e]	<20,000	<50
SON 188	NT	2,048,000	6,400
OspA	512,000	2,048,000	1,600
OspB	256,000	1,024,000	1,600
OspC	256,000	256,000	<50
OspD	128,000	128,000	<50
83 kDa	256,000	256,000	<50

[a] Each value is the reciprocal of the dilution at which immunostaining remains detectable.
[b] Each value is the reciprocal dilution yielding an optical density of >0.2 at 560 nm.
[c] Xa product, reactivity with the *B. burgdorferi*-encoded portion of the fusion protein released following factor Xa cleavage.
[d] SON 188, total bacterial lysate.
[e] NT, not tested.

by this serum was demonstrated by the inclusion of SON 188 lysate on the Western blot (Figure 1B, lane 8).

Immunological analysis of serum from immunized mice. Following the final boost, the antibody response to each immunogen was measured by immunoblotting and borreliacidal assays. Figure 2 illustrates the reactivity of mouse serum from each immunogen group with nitrocellulose strips containing a lysate of SON 188. Antibodies generated against the OspA fusion protein reacted strongly with a 31-kDa band as expected and showed slight cross-reactivity with the 33-kDa OspB band. The OspB immunogen elicited antibodies recognizing a protein of 18 kDa and one of the expected molecular mass of 33 kDa. Faint cross-reactivity of this serum with the 31-kDa OspA band was also detected. Sera from mice immunized with the OspC and OspD fusion proteins generated bands having the expected molecular masses of 22 and 28 kDa, respectively. Antibodies to the 83-kDa fusion protein produced two bands on the nitrocellulose strip, one corresponding to the expected molecular mass of 83 kDa and the other corresponding to a protein of 60 kDa. Serum derived from mice immunized with heat-inactivated SON 188 recognized numerous proteins, the most predominant of which were OspA and OspB. Anti-β-gal serum did not bind any antigens on the nitrocellulose strip.

Antibody titers for each immunogen group were determined by immunoblot and defined as the highest dilution of sera yielding a detectable band. Immunoblot strips were prepared from two sources: factor Xa-cleaved fusion proteins and SON 188 lysate. Cleavage of the fusion proteins with factor Xa and subsequent separation of the products by SDS-PAGE and Western blotting allowed the titration of antibodies reactive with the *B. burgdorferi*-derived portion of the immunogens. Thus, this assay measured only immunoreactivity specific for the portion of the immunogen derived from *B. burgdorferi* and not to MBP. Titers exceeding 100,000 were observed for the 83-kDa and all four Osp immunogen groups, with the anti-OspA titer exceeding 500,000 (Table 3). While immunogen groups demonstrated similar titers against their respective cleaved proteins, titers against the SON 188 lysate were more varied (Table 3). A 16-fold difference in titer against the SON 188 nitrocellulose strips was observed for those mice immunized with the OspA fusion protein (titer, 2,048,000) compared with that for those mice receiving the OspD fusion protein (titer, 128,000). These differences in titers may reflect the relative difference in expression levels of these proteins by SON 188 (Fig. 2).

The borreliacidal activity of each serum group was also evaluated (Table 3). This assay measured spirochetal growth as a function of pH and was indicated by a change in medium color from red to yellow. These colorimetric changes were monitored at 560 nm. Spirochetal growth was detected as a decrease in A_{560}, whereas spirochetal death was measured as little or no decrease in absorbance. Antiserum against the heat-inactivated SON 188 immunogen demonstrated a high level of borreliacidal activity (titer, 6,400). Antisera to OspA and OspB yielded borreliacidal activities (titer, 1,600) that were 25% of that yielded by the anti-SON 188 sera. No borreliacidal activity was observed with sera from mice in any other immunogen groups.

Protection studies. Following the immunization series, the mice were challenged with 10^7 SON 188 organisms to ensure an infectivity rate of 100%. Three weeks after challenge, recovery of organisms from ear, bladder, and heart by culture in BSK II was attempted. Table 4 illustrates that organisms were recovered from the tissues of mice receiving the β-gal, OspD, and 83-kDa immunogens. No organisms were recovered from the mice receiving the heat-inactivated SON 188, OspA, OspB, and OspC immunogens.

DISCUSSION

The purpose of this investigation was to evaluate the contribution of several proteins from a single isolate of *B. burgdorferi* in eliciting a protective immune response. SON 188 was

TABLE 4. Recovery of spirochetes from tissues of immunized C3H mice challenged with *B. burgdorferi*

Immunogen	No. of mice infected/no. of mice immunized		
	Ear	Bladder	Heart
β-gal	4/4	3/4	3/4
SON 188	0/4	0/4	0/4
OspA	0/4	0/4	0/4
OspB	0/4	0/4	0/4
OspC	0/4	0/4	0/4
OspD	4/4	4/4	4/4
83 kDa	4/4	4/4	4/4

chosen for this study for the following reasons: (i) it is an infectious isolate of known titer (50% infectious dose, 3×10^4 organisms) (34), (ii) it represents the first isolate from the West Coast of North America evaluated in a protection study, (iii) SON 188 has been genetically characterized and found to be related to B31 (49), and (iv) unlike some isolates, it possesses the genetic information encoding all four Osps (A, B, C, and D), as well as the 83-kDa protein. Insertion of these genes into the pMal c2 vector provided a convenient means of expressing these genes in *E. coli* and purifying the expressed products as fusion proteins. Active immunization of C3H mice with these fusion proteins and subsequent challenge with SON 188 resulted in protection in those mice receiving OspA, OspB, and OspC but not in those receiving the OspD and 83-kDa fusion proteins.

The ability of OspA and OspB to elicit a protective immune response has been well documented by others (15, 43, 45). Our findings also demonstrate that OspA and OspB derived from SON 188 have this capability. In addition to eliciting protective antibodies, both OspA and OspB were found to elicit borreliacidal antibodies (titer, 1,600 for each), and the effect of using anti-OspA and anti-OspB sera together in a borreliacidal assay was observed to be additive (titer, 3,200). Conversely, serum derived from mice immunized with OspC, OspD, or the 83-kDa fusion protein did not result in enhanced borreliacidal activity when pooled with anti-OspA serum or anti-OspB serum (data not shown).

Immunoblot analysis of SON 188 lysate probed with anti-OspA serum or anti-OspB serum revealed slight cross-reactivity of each serum with the heterologous antigen. A similar observation has been made by Jaing et al. (18) with monospecific polyclonal antibodies. This cross-reactivity is not surprising, considering that the OspA and OspB proteins reported by Bergstrom et al. (3) were homologous over 53% of their deduced amino acid sequences. Interestingly, the anti-OspB serum generated in our study reacted strongly with two antigens of SON 188 by immunoblot analysis, one having the expected molecular mass (33 kDa) and the other of lower molecular mass (18 kDa). The 18-kDa antigen may represent a truncated form of the OspB protein described by Bundoc and Barbour (5). In contrast to our findings, Fikrig et al. (14) have demonstrated that a *B. burgdorferi* isolate (N40) possessing both full-length and truncated *ospB* genes can escape destruction in mice immunized with a full-length recombinant OspB protein derived from B31. Later studies by this group attribute the lack of protection to a truncation of the *ospB* gene and consequently the elimination of a protective epitope in a subpopulation of the N40 isolate (16). It is probable that as in the case of N40, a subpopulation of SON 188 possesses *ospB* genes with a premature stop codon. Our inability to reisolate SON 188 organisms following challenge of OspB-immunized mice may indicate that the subpopulation of organisms possessing the truncated OspB protein was too small, in contrast to that of N40, to establish infection. Alternatively, the truncated OspB expressed by SON 188 may still possess an epitope capable of eliciting a protective immune response. Verification of a truncated *ospB* gene in SON 188 would require cloning of the subpopulation possessing the truncated protein and subsequent sequencing of the *ospB* gene.

More recently, a third antigen, OspC, was shown to elicit protective immunity in gerbils after challenge with a European isolate of *B. burgdorferi* (33). In contrast to isolates derived from the midwest and East Coast of the United States, which appear relatively homogeneous genetically and phenotypically, West Coast isolates more closely resemble European isolates in exhibiting greater genetic and phenotypic heterogeneity (4, 22, 49). Moreover, a large number of California isolates express an abundance of the OspC protein (40). A clone of one such isolate, DN 127, expressed OspC as the predominant Osp, while expressing little or no OspA and OspB (20). These observations, along with our findings, suggest that the OspC antigen may be an important addition to the design of an efficacious subunit vaccine. Interestingly, no borreliacidal activity was observed in our assay using anti-OspC mouse serum. This finding indicates that the anti-OspC antibodies generated in this study lack direct cytolytic activity and suggests a complement-independent mechanism of protection such as enhancement of opsonization. An explanation for this lack of borreliacidal activity may be the failure of OspC to elicit an immunoglobulin isotype that efficiently activates complement. Indeed, Schmitz et al. (39) have attributed borreliacidal activity in hamsters to immunoglobulin primarily of the G2 isotype. Another possible explanation for this observation could be the relative paucity of OspC expressed in SON 188. However, attempts to demonstrate the borreliacidal activity of anti-OspC serum against an isolate (25015) that was strongly reactive upon immunoblotting and expressed OspC as the major Osp were unsuccessful (data not shown). Confirmation of this result by others may contraindicate the use of the borreliacidal assay in the serodiagnosis of Lyme borreliosis (7).

Immunization of mice with OspD elicited a strong antibody response, yet no borreliacidal or protective activity was observed. Norris et al. (30) have localized this protein to the outer surface on the basis of [^{3}H]palmitate labelling and surface proteolysis. We have confirmed the outer surface location of OspD, as well as those of OspA, OspB, and OspC, on SON 188 by surface proteolysis (data not shown), suggesting that OspD should theoretically present a target for antibodies. The inability of anti-OspD antibodies to protect mice from *B. burgdorferi* challenge may reflect low levels of expression of this protein by SON 188. This was supported by the observation that OspD was difficult to identify on a Coomassie blue-stained gel containing SON 188 lysate (Fig. 1A, lane 8) and demonstrated the lowest antibody titer to SON 188 of all the immunogens tested. Moreover, the failure of anti-SON 188 serum to recognize the OspD fusion protein further indicates that OspD expression levels are below that required to initiate an immune response. Interestingly, spirochetes isolated from mice immunized with the OspD fusion protein continued to express OspD as demonstrated by Western blotting (data not shown). This observation refutes the possibility that a clonal population of SON 188 lacking surface expression of the OspD protein was selected as a result of specific immunological pressures. Evidence supporting the possibility of an immunological selection process was demonstrated in a report describing the loss of OspB expression following cultivation of *B. burgdorferi* in the presence of bactericidal anti-OspB monoclonal antibodies (10).

Unlike the *osp* genes, the gene encoding the 83-kDa protein is chromosomally encoded and highly conserved among *B. burgdorferi* isolates (23). The stability of this gene and the potent immunostimulatory nature of the 83-kDa protein prompted its evaluation as a vaccine candidate (31). This protein was recently described as being identical to the 94- and 100-kDa antigens of *B. burgdorferi* (11). Like OspD, the 83-kDa protein elicited a strong antibody response but failed to produce borreliacidal activity and to protect mice. One possible explanation for this observation may be the destruction of important epitopes as a consequence of proteolysis of the 83-kDa fusion protein prior to immunization. Proteolysis of the 83-kDa fusion protein was observed by SDS-PAGE and Western blot (Fig. 1). In addition, the 60-kDa band recognized

by the anti-83-kDa serum on a Western blot of SON 188 lysate may also represent a degradation product. The heightened susceptibility of native 83-kDa protein and recombinant forms of this protein to degradation has been observed by Luft et al. (25). Alternatively, the lack of surface exposure demonstrated by Luft et al. provides a more likely explanation of the failure of the 83-kDa fusion protein to elicit protective immunity.

Recent reports describing possible mechanisms by which *B. burgdorferi* escapes immunological pressures suggest that a single vaccine component may not be sufficient to effectively prevent infection and thus reveal the need for a multicomponent vaccine (16, 28, 36). Moreover, the extensive genotypic variation observed among the Osps characterized thus far supports the argument for such a vaccine (19, 24, 27, 38). We have identified three antigenic components important in the design of a subunit vaccine for the prevention of Lyme borreliosis. We are currently investigating the ability of a vaccine consisting of OspA, OspB, and OspC to provide protection against a panel of genetically characterized isolates of *B. burgdorferi*. Such studies, we hope, will lead to the identification of variant Osps and their inclusion in the formation of a broadly protective vaccine.

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