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Demonstration of OspC Type Diversity in Invasive Human Lyme Disease Isolates and Identification of Previously Uncharacterized Epitopes That Define the Specificity of the OspC Murine Antibody Response

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Outer surface protein C (OspC) of the Lyme disease spirochetes is an important virulence factor that has potential utility for vaccine development. Of the 21 OspC types that have been identified, it has been postulated that types A, B, I, and K are specifically associated with invasive infections. Through an analysis of isolates collected from patients in Maryland we found that OspC types C, D, and N are also associated with invasive infections. This observation suggests that there is greater diversity in the group of OspC types associated with invasive infection than has been previously suggested. Detailed knowledge of the antigenic structure of OspC is essential for vaccine development. To determine if the antibody response to OspC is type specific, recombinant proteins of several different OspC types were immunoblotted and screened with sera from mice infected with isolates having known OspC types. These analyses revealed a high degree of specificity in the antibody response and suggested that the immunodominant epitopes of OspC reside in the variable domains of the protein. To localize these epitopes, OspC fragments were generated and screened with serum collected from infected mice. These analyses led to identification of previously uncharacterized epitopes that define the type specificity of the OspC antibody response. These analyses provide important insight into the antigenic structure of OspC and also provide a basis for understanding the variable nature of the antibody response to this important virulence factor of the Lyme disease spirochetes.

Lyme disease is transmitted to humans through the bite of *Ixodes* ticks infected with *Borrelia burgdorferi*, *Borrelia garinii*, or *Borrelia afzelii*. Outer surface protein C (OspC) is thought to be an important virulence factor that is involved in the transmission process and possibly in the establishment of early infections in mammals (12, 24, 29). OspC is a variable, ~22-kDa, surface-exposed, plasmid-encoded lipoprotein (7, 17, 27). Crystal structures have been determined for three OspC proteins (4, 15). The protein is largely helical and has five alpha helices connected by variable loops. The loops have been postulated to form ligand binding domains (4, 15). Evidence suggests that OspC may facilitate translocation of spirochetes from the tick midgut by serving as an adhesin that binds to unidentified receptors in the salivary gland (24). Orthologs of OspC have been identified in several species belonging to the relapsing fever group, raising the possibility that the OspC-related proteins have a similar role in other *Borrelia* species (18, 19). OspC expression is environmentally regulated and is induced by tick feeding, and OspC is a dominant antigen during early infection in mammals (2, 29, 31). Transcription is regulated, at least in part, by the RpoN/S regulatory network (14). It should be noted that there are conflicting reports regarding the precise details of the temporal nature of OspC expression during transmission and during early infection (23, 29).

OspC exhibits significant genetic and antigenic diversity (33, 34). Twenty-one OspC phyletic groups (referred to below as OspC types) have been delineated (30, 36). OspC types are differentiated by letter designations (types A through U). Analysis of several hundred OspC amino acid sequences that are in databases indicated that the divergence between OspC types can be as high as 30%, while within a type the divergence is generally less than 6%. Seinost et al. hypothesized that there is a correlation between *ospC* types A, B, I, and K and invasive infections in humans (30). Lagal et al. also reported that specific *ospC* variants, as defined by single-strand conformation polymorphism analysis, correlate with invasive human infections (16). However, a recent study by Alghaferi and colleagues has called into question the strength of this correlation (1). The influence of the OspC type or sequence on function and the host-pathogen interaction is an important and fertile area of investigation. OspC has been investigated for use in Lyme disease vaccine development (3, 8, 9, 25, 34, 37). However, OspC variation and our limited knowledge of the antigenic structure of OspC have complicated these efforts. OspC has protective capability, but only against the same strain (3, 9, 10, 25, 37). This suggests that the protective epitopes reside in regions of the protein that have highly variable sequences.

The goals of this study were severalfold. First, we sought to further assess the putative correlation between OspC types and invasive infection by determining the OspC types of invasive

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TABLE 1. Bacterial isolates, source information, and OspC types

B. burgdorferi isolate	Source	OspC type
B31MI	Tick	A
5A4	Clone derived from B31MI	A
LDP56	Human blood	A
LDP61	Human blood	A
LDP60	Human blood	A
LDP80	Human blood	A
LDP76	Human blood	A
LDS106	Human skin	A
LDP73	Human blood	B
LDS79	Human skin	H
LDS101	Human skin	H
LDP84	Human blood	C
LDP63	Human blood	N
LDC83	Human cerebrospinal fluid	N
LDP120	Human blood	N
LDP74	Human blood	K
LDS81	Human skin	K
LDS88	Human skin	K
LDP89	Human blood	K
LDP116	Human blood	D

and noninvasive isolates recovered from a defined patient population in Maryland. Second, in an attempt to better understand the antibody response to OspC, we sought to determine if this response is type specific. Finally, we sought to define the antigenic structure of OspC by identifying epitopes that elicit an antibody response during infection in mice. The data presented here indicate that the number of OspC types associated with invasive infection is greater than previously postulated (30). In addition, we identified two previously uncharacterized epitopes and demonstrated that the antibody response to OspC appears to be type specific. These analyses provide important information that enhances our understanding of the role of OspC in Lyme disease pathogenesis and that will facilitate construction of an OspC-based vaccine.

MATERIALS AND METHODS

Bacterial isolates, cultivation, and generation of infection serum. Lyme disease isolates recovered from human patients in Maryland were employed in these analyses (Table 1). Patients provided informed consent prior to the study, as approved by the John Hopkins Medicine Institutional Review Board. The spirochetes were cultivated in BSK-H complete media (Sigma) at 33°C, monitored by dark-field microscopy and harvested by centrifugation. Clonal populations were generated for some isolates by subsurface plating as previously described (32). To determine the *ospC* types of individual colonies, the *ospC* gene was PCR amplified and sequenced, and comparative sequence analyses were performed (as described below). To generate antisera against a series of clonal populations expressing OspC proteins of known types, 10^3 spirochetes were washed in phosphate-buffered saline and needle inoculated into C3H-HeJ mice subcutaneously between the shoulder blades (Jackson Labs). Infection of the mice was confirmed by real-time PCR of ear punch biopsies at week 2 or 4 postinoculation using primers targeting the *flaB* gene as previously described (39). Blood was collected from each mouse at 0, 2, 4, and 8 weeks by tail snipping, and the infection serum was harvested. Additional antisera and infection serum used in these analyses have been described previously (21).

DNA isolation, OspC typing, and computer-assisted structural analyses. To determine the OspC type, total DNA was isolated from each strain as previously described (17) and used as a template for PCR with the OspC20(+)LIC and OspC210(−)LIC primers (Table 2). PCR was performed using Expand High Fidelity polymerase (Roche) with the following cycling conditions: initial denaturation at 94°C for 2 min; 94°C for 15 s, 50°C for 30 s, and 68°C for 60 s for 10 cycles; 94°C for 15 s, 50°C for 30 s, and 68°C for 60 s with an additional 5 s added to each of the last 20 cycles; and final elongation at 68°C for 7 min. The amplicons were recovered using a QiaQuick PCR purification kit (QIAGEN), treated with T4 DNA polymerase to generate single-stranded overhangs, annealed into the pET-32 Ek/LIC vector (Novagen), and transformed into *Escherichia coli* NovaBlue(DE3) cells (Novagen). The methods used for these procedures were used as described by the manufacturer. Colonies were selected for ampicillin resistance (50 μg ml^{-1}) and were screened for the *ospC* insert by PCR. Selected colonies were transferred into LB broth (Fisher) and cultivated at 37°C with shaking (300 rpm), and the plasmids were isolated using QiaFilter midi plasmid isolation kits (QIAGEN). The *ospC* inserts were sequenced on a fee-for-service basis (MWG Biotech). The sequences determined were translated and aligned using ClustalX (35) with default parameters. To determine the OspC type, a neighbor-joining tree was created, and bootstrap values were calculated (1,000 trials). The resultant phylogram was visualized with N-J Plotter. Additional OspC sequences available in the databases were included in the analysis. Structural models for OspC were generated using the NCBI molecular modeling database files 1GGQ, 1F1M, and 1G5Z (4, 15) and the CN3D software available at http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml.

Generation of recombinant proteins. To generate full-length OspC and truncations of OspC, primers were designed based on the type A *ospC* sequence of *B. burgdorferi* B31MI (6). The primers had tail sequences that allowed annealing into the pET-32 Ek/LIC vector (Novagen), a ligase-independent cloning and expression vector. All ligase-independent cloning procedures were performed as previously described (13). To verify the sequence of all constructs, recombinant plasmids were purified from *E. coli* NovaBlue(DE3) cells using QiaFilter midi plasmid purification kits (QIAGEN), and the inserts were sequenced (MWG Biotech).

TABLE 2. PCR primers employed in this study

Primer	Sequence[a]
ospC 20(+) LIC	GACGACGACAAGATTAATAATTCAGGGAAAGATGGG
ospC 40(+) LIC	GACGACGACAAGATTCCTAATCTTACAGAAATAAGTAAAAAAAT
ospC 60(+) LIC	GACGACGACAAGATTAAAGAGGTTGAAGCGTTGCT
ospC 80(+) LIC	GACGACGACAAGATTAAAATACACCAAAATAATGGTTTG
ospC 100(+) LIC	GACGACGACAAGATTGGAGCTTATGCAATATCAACCC
ospC 130(+) LIC	GACGACGACAAGATTTGTTCTGAAACATTTACTAATAAATTAAAAG
ospC 136(+) LIC	GACGACGACAAGATTAATAAATTAAAAGAAAAACACACAGATCTTG
ospC 142(+) LIC	GACGACGACAAGATTCACACAGATCTTGGTAAAGAAGG
ospC 151(+) LIC	GACGACGACAAGATTACTGATGCTGATGCAAAAGAAG
ospC 171(+) LIC	GACGACGACAAGATTGAAGAACTTGGAAAATTATTTGAATC
ospC 191(+) LIC	GACGACGACAAGATTCTTGCTAATTCAGTTAAAGAGCTTAC
ospC 130(−) LIC	GACGACAAGCCCGGTTTAACATTTCTTAGCCGCATCAATTTTTTC
ospC 150(−) LIC	GACGACAAGCCCGGTTTAAACACCTTCTTTACCAAGATCTGT
ospC 170(−) LIC	GACGACAAGCCCGGTTTAAGCACCTTTAGTTTTAGTACCATT
ospC 190(−) LIC	GACGACAAGCCCGGTTTACATCTCTTTAGCTGCTTTTGACA
ospC 200(−) LIC	GACGACAAGCCCGGTTTAGCTTGTAAGCTCTTTAACTGAATTAGC
ospC 210(−) LIC	GACGACAAGCCCGGTTTAAGGTTTTTTTGGACTTTCTGC

[a] LIC tail sequences are underlined.

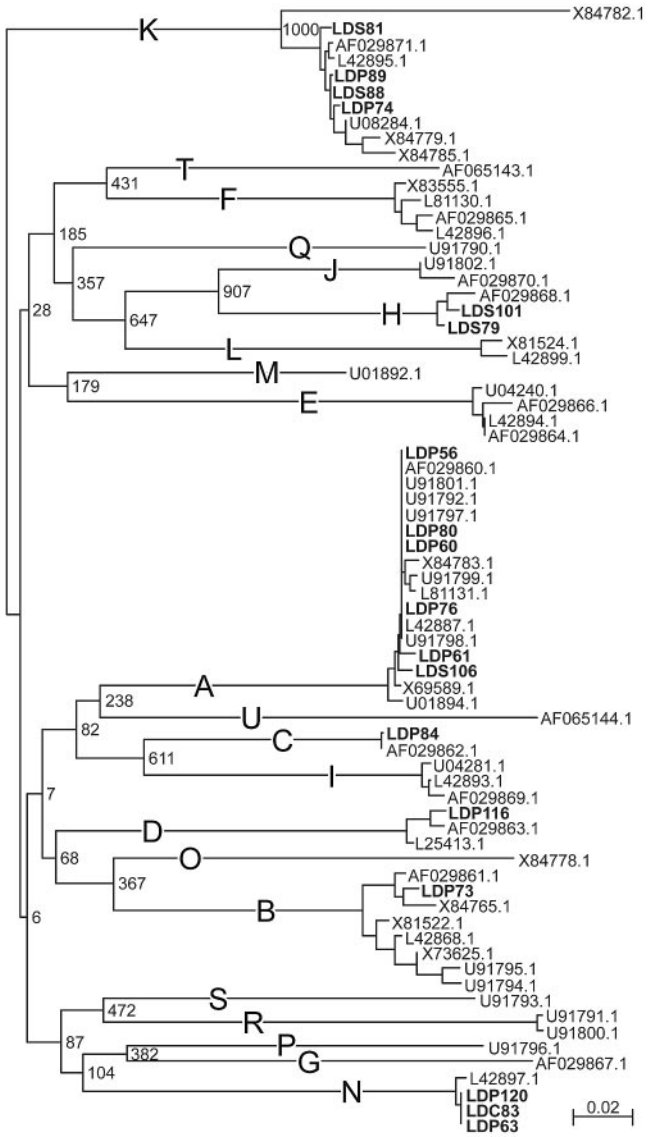

FIG. 1. Evolutionary relationships of OspC sequences derived from human patients in Maryland: OspC type identification. *ospC* genes were PCR amplified and sequenced, and a phylogram was constructed. Database sequences representative of the 22 OspC types were included in the analysis (accession numbers are indicated). The type designation assigned to each phyletic group is indicated by a capital letter on the branch. Bootstrap values (1,000 trials) are indicated at the nodes critical for group differentiation.

SDS-PAGE and immunoblot analyses. Proteins were separated in 12.5% Criterion precast gels (Bio-Rad) by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and were immunoblotted onto polyvinylidene difluoride membranes (Millipore) as previously described (26). Expression of recombinant proteins was confirmed using S-Protein–horseradish peroxidase (HRP) conjugate (Novagen), which detected the N-terminal S-Tag fusion that was carried by all recombinant proteins employed in this study. The HRP-conjugated S-Protein was used at a dilution of 1:10,000. For immunoblot analyses, serum collected from infected mice was used at a dilution of 1:1,000. HRP-conjugated goat anti-mouse immunoglobulin (IgG) served as the secondary antibody (Pierce) and was used at a dilution of 1:10,000. The general immunoblot methods used have been described previously (22).

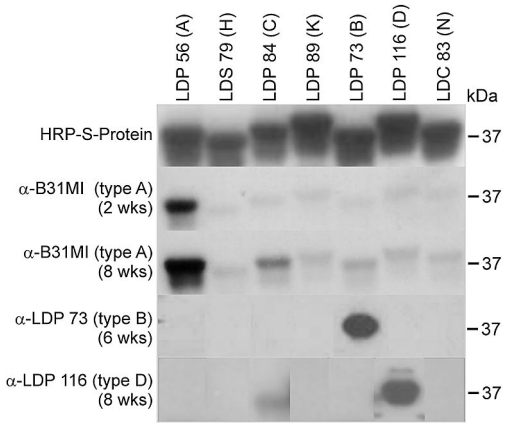

FIG. 2. Demonstration that the antibody response to OspC during infection is predominantly OspC type specific. Recombinant OspC proteins of several OspC types (indicated at the top) were generated, separated by SDS-PAGE, immunoblotted, and screened with HRP-conjugated S-Protein or serum collected from mice infected with clonal isolates having known OspC types, as indicated on the left.

RESULTS

OspC typing analysis of isolates recovered from human Lyme disease patients in Maryland. *ospC* was successfully amplified from each of the isolates analyzed that were recovered from human Lyme disease patients from Maryland. The sequence of each amplicon was determined and translated, and comparative sequence analyses were performed to determine the OspC type (Fig. 1). Representatives of several different OspC types, including types A ($n = 6$), B ($n = 1$), C ($n = 1$), D ($n = 1$), H ($n = 2$), K ($n = 4$), and N ($n = 3$) were identified. It has been reported previously that only OspC types A, B, I, and K are associated with invasive infections in humans (30). In that study, invasive isolates were defined as isolates that were recovered from blood, organs, or cerebrospinal fluid, whereas noninvasive isolates were defined as isolates that were recovered from the skin but were not found at other body sites (30). However, here we found that some isolates expressing OspC types C, D, and N were recovered from blood (LDP84, LDP63, LDP116, and LDP120) or cerebrospinal fluid (LDC83) and hence are invasive. This observation suggests that the correlation of specific OspC types with invasive infection may not be a strict one and that the strength of the correlation requires reevaluation.

Analysis of the type specificity of the antibody response to OspC during infection in mice. To determine if the antibody response to OspC elicited during infection is type specific, type A, B, C, D, H, K, and N recombinant OspC proteins were generated for use as test antigens. The recombinant proteins were immunoblotted and screened with serum collected from mice infected with *B. burgdorferi* clonal populations having the A, B, or D OspC type (determined as described above) (Fig. 2). Expression of the recombinant proteins in *E. coli* and equal loading of protein were confirmed by screening one immunoblot with HRP-conjugated S-Protein, which recognized the S-Tag in the N-terminal fusion. When screened with anti-*B. burgdorferi* B31MI antiserum (type A OspC) collected at week 2 of infection, strong reactivity was detected only with the type

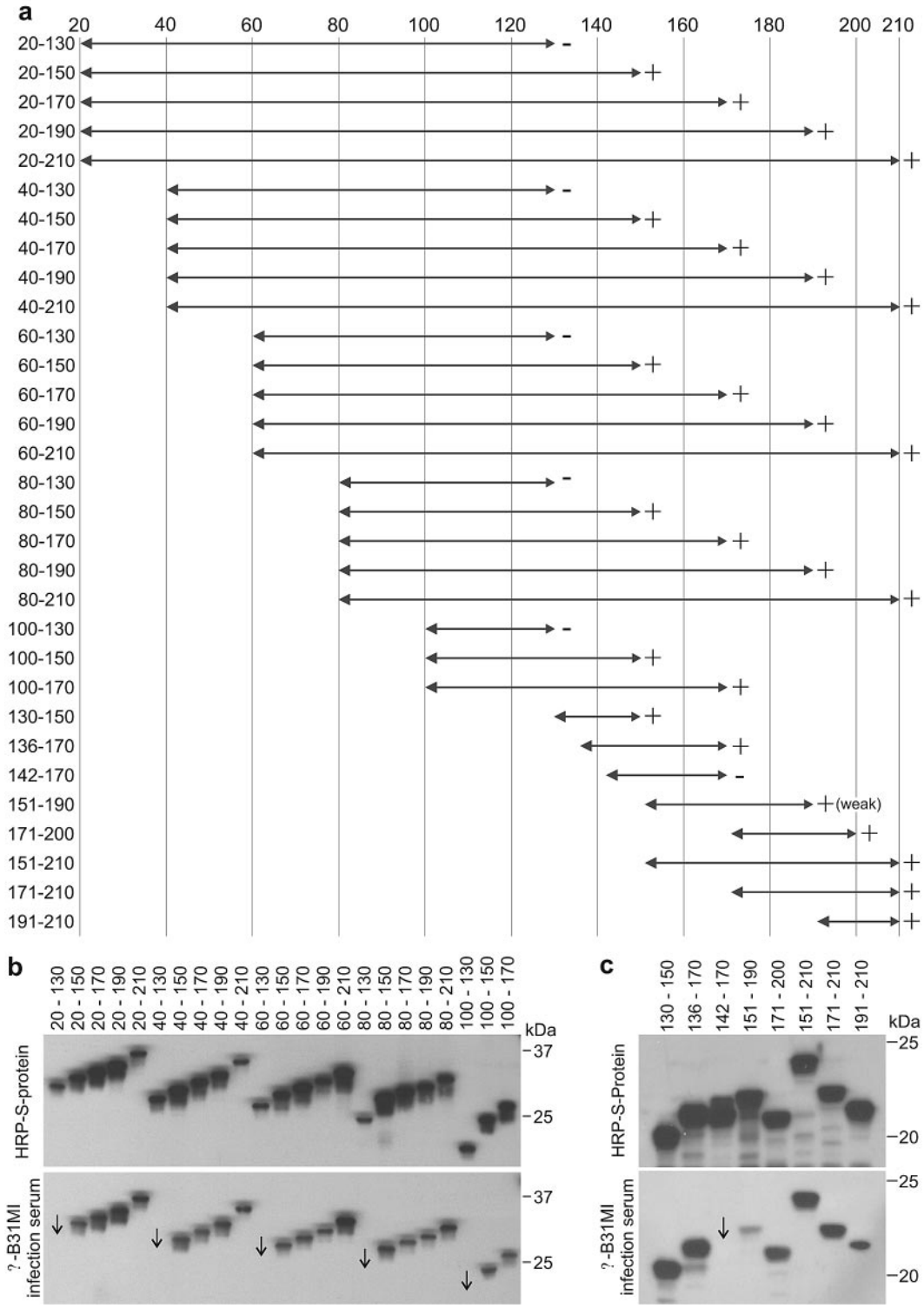

FIG. 3. Localization of the immunodominant epitopes of type A OspC. Truncations of type A OspC were generated as S-Tag fusion proteins and expressed in *E. coli*. (a) Schematic diagrams of the OspC truncations. The numbers reflect the residue numbers of *B. burgdorferi* B31MI OspC. The ability of each truncated protein to bind infection antibody is indicated on the right (+ or −). The numbers on the left indicate the amino acid residues that comprise each truncation. (b and c) Immunoblots of the recombinant proteins screened with HRP-conjugated S-Protein to verify expression and loading or with serum from a mouse infected with *B. burgdorferi* B31MI (α-B31MI infection serum), a type A OspC-producing strain. For reference, the arrows in panels b and c indicate the migration positions of recombinants that were not immunoreactive with the anti-B31MI infection serum. The positions of molecular mass markers are indicated on the right for each immunoblot.

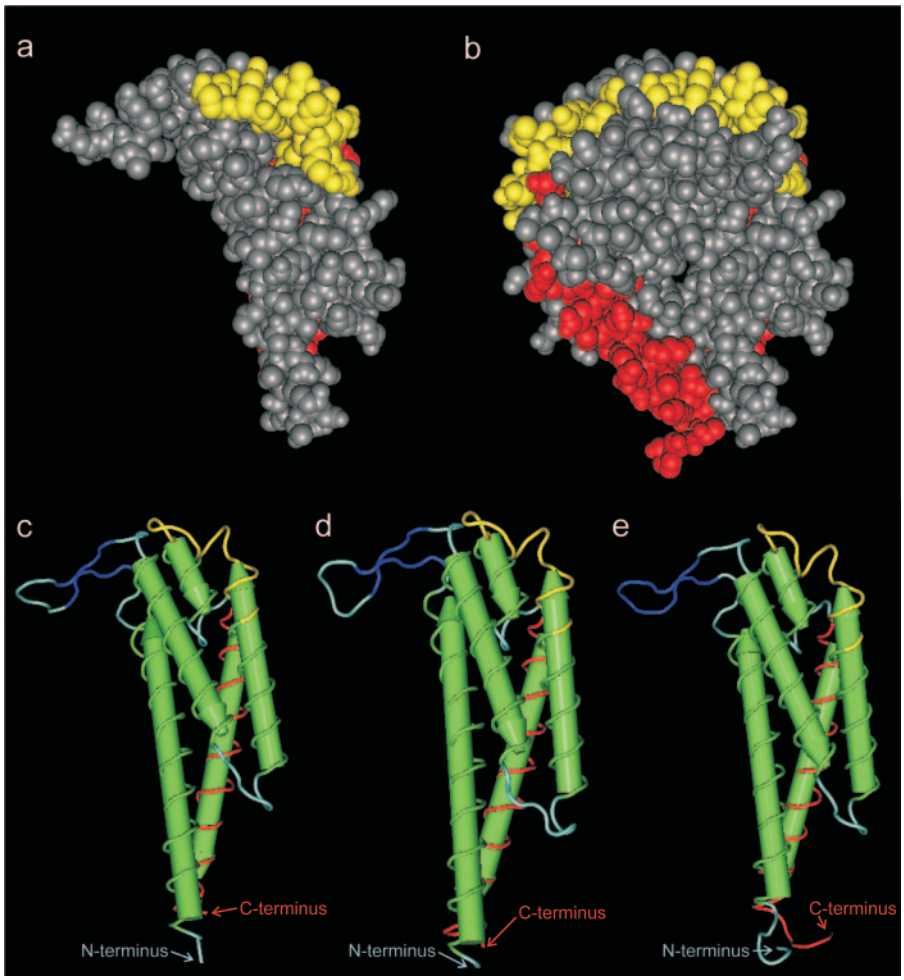

FIG. 4. Localization of the loop 5 epitope on the mono- and dimeric forms of OspC. Modeling was conducted as described in the text. The upper diagrams show the space fill models for both the monomeric (a) and dimeric (b) forms of the B31MI type A OspC. Diagrams c, d, and e are ribbon models for OspC types A, I, and E, respectively. In all diagrams, the residues that comprise loop 5 are indicated by yellow, and the residues that constitute the alpha 5 domain are indicated by red. Note that portions of both the N- and C-terminal domains are truncated since they either could not be modeled or were not part of the recombinant protein used in the structure determination.

A protein. The strong and early IgG response to OspC is consistent with previous reports (34, 38). Sera collected at week 8 of infection also reacted predominantly with type A OspC, but weak cross-immunoreactivity with other OspC types was observed. The antibody response to OspC in mice infected with LDP116 and LDP73 (OspC type D and B isolates, respectively) was also type specific. We concluded that there is a significant degree of type specificity in the antibody response to OspC and that this specificity implies that the in vivo immunodominant epitopes are located within the type-specific domains of the protein.

Localization of the OspC linear epitopes that elicit an antibody response during infection in mice. To identify the linear epitopes of type A OspC that elicit an antibody response during infection, several recombinant OspC fragments were generated and screened with anti-*B. burgdorferi* B31MI infection serum (week 8) (Fig. 3). B31MI is an OspC type A-producing strain. The expression of the recombinant proteins was confirmed by immunoblotting with the HRP-conjugated S-Protein. To localize the linear epitopes of OspC, immunoblots of the OspC fragments were screened with infection serum. Two domains containing one or more epitopes were localized, one in the C-terminal half of the protein between residues 168 and 203 of alpha helix 5 and the other between residues 136 and 150 of helix 3 and loop 5 (referred to as the alpha 5 and loop 5 epitopes, respectively, below). These epitopes have not been characterized previously.

***ospC* sequence analyses and computer modeling of OspC structure.** To determine where the loop 5 and alpha 5 epitopes spatially reside on the OspC protein, the coordinates determined by X-ray crystallographic analyses (4, 15) were accessed, and ribbon and space fill models were generated for monomeric and/or dimeric forms of type A OspC (Fig. 4). Monomeric forms of type I and E OspC proteins were also modeled. These analyses revealed that the loop 5 epitope is surface exposed on both the monomeric and dimeric forms of the type A, E, and I OspC proteins. In the original X-ray crystallographic analyses, portions of both the N and C termini either were not part of the recombinant protein or could not be modeled. In any event, the structures determined indicate that

TABLE 3. Comparative analysis of the loop 5 and alpha 5 epitopes at the inter- and intratype levels

OspC type	Loop 5 epitope		Alpha 5 epitope(s)	
	Sequence (amino acids 136 to 150)	No. of sequences	Sequence (amino acids 168 to 203)	No. of sequences
A	NKLKEKHTD-LGKEG--V	53	KGAEELGKLFESVEVLSKAAKEMLANSVKELTSPVV	42
	.........-...R.--.	3	..............................N...	1
	.........SF....--.	1		
B	TKLKDNHAQLG-IQG--V	24	KGVEELEKLSGSLESLSKAAKEMLANSVKELTSPVV	17
	......Q..R.-...--.	1		
C	KKLKEKHTDLG-KKD--A	4	KGAAELEKLFESVENLAKAAKEMLSNSVKELTSPVV	3
D	KKLSDNQAELG-IEN--A	7	KGAEELVKLSESVAGLLKAAQAILANSVKELTSPVV	5
E	NKLKSEHAVLG-LDN--L	13	KGAAELEKLFKAVENLSKAAQDTLKNAVKELTSPIV	13
F	NKLKNGNAQLG-LAA--A	11	KGAKELKDLSDSVESLVKAAQVMLTNSVKELTSPVV	9
G	KKLADSNADLGVAAG-NA	5	KGGKELKELSEAVKSLLKAAQAALANSVQELTSPVV	3
H	GKLKNEHASLG-KKD--A	7	KGAKELKDLSDSVESLVKAAKEMLTNSVKELTSPVV	3
I	AKLKGEHTDLG-KEG--V	12	KGADELEKLFESVKNLSKAAKEMLTNSVKELTSPVV	9
J	KKLKDSRAELG-KKD--A	2	KGAKELKELFEAVESLSKAAKEML	3
	......H....-...--.	1		
K	KKLEGEHAQLG-IEN--V	20	KGAAELEKLFKAVENLAKAAKEMLANSVKELTSPIV	12
	N..........-T..--.	3	................P...................	6
			......A.........PQ..................	3
L	DKLKSENVALG-KQD--A	7	KGAKELKELSESVETLLKAAKEMLANSVKELTSPVV	10
	.......A........--.	3		
M	DKLKSSHAELG-IANGAA	5	KGAQELEKLFESVKNLSKAAQETLNNSVKELTSPVV	4
N	KKLQSSHAQLG-VAGGAT	8	KGADELEKLFKSVESLAKAAQDALANSVNELTSPVV	7
	...KD..QE..-..N..A	2	...E.......A....S.....M.T...K.......	2
			......G...E.........KEM.....K.......	1
O	TKLKSSNAQLN-QAN--A	3	KGAEELVKLAESVAGLFKVAQEMLNNSVKELTSPVV	3
P	NKLKNSHAELGVAGNGAT	6	KGAKELKELFESVESLAKAAKESLTNSVKELTSPVV	3
	.......V..........	1	......................A.S.............	3
	............N....	1	................L...Q.A.........T...	1
Q	DKLKNEHASLG-KKD--A	4	KGADELIKLSGSLESLSKAAQAILANSVKELTSPVV	1
R	NKLKTSHAQLG-AANGGA	4	KGVTELEELFKSVEGLAKAAKEASANSVKELTSPVV	4
	....N...E..V......	3	................P...................	2
S	KKLKDNNAQLG-IQN--V	5	KGAKELKELFESVESLAKAAQAALANSVQELTNPVV	5
	......H..F.-...--.	2		
T	NKLKSGHAELG-PVGGNA	3	KGAKELKDLSESVEALAKAAQAMLTNSVKELTSPVV	3
U	KKLSESHADIG-IQA--A	3	KGAEELDKLFKAVENLSKAAKEMLANSVKELTSPVV	1

the N and C termini are in close proximity to one another and are proximal to the cell membrane.

To assess sequence variation within the loop 5 and alpha 5 epitopes at the intratype level, 227 OspC sequences were aligned. These analyses revealed that both the loop 5 and alpha 5 epitopes are highly variable at the intertype level but remarkably highly conserved within a type. Table 3 shows the loop 5 and alpha 5 domain sequences for each OspC type and indicates the frequency with which each specific sequence was detected in the OspC sequences analyzed. As evidence for the conservation of loop 5 at the intratype level, a comparison of 57 type A loop 5 epitope sequences revealed that 53 were identical, with the outlying sequences differing at only one or two residues. A similar observation was made for the alpha 5 epitopes. Of 43 type A OspC sequences, 42 were identical between residues 168 and 203. Note that fewer alpha 5 epitope sequences were analyzed, since in many cases the sequences available in the databases were partial and lacked various amounts of the C terminus.

Demonstration that the antibody response to the loop 5 epitope is not unique to an individual mouse. In view of the intratype conservation of loop 5 and its relatively short length, the loop 5 epitope might be an excellent candidate for use in the development of a chimeric OspC loop 5-based vaccinogen. To verify that the antibody response to the loop 5 epitope occurs commonly during infection and was not unique to an individual mouse, immunoblots of the loop 5 fragment containing residues 130 to 150 were screened with sera from several additional mice infected with the type A OspC-producing strains B31MI, LDP56, and 5A4. In all cases, we detected antibody that recognized this epitope (Fig. 5). While the response to loop 5 was weaker for the infection serum from LDP56-infected mouse 2, longer exposure clearly revealed that loop 5 was antigenic in this animal. This demonstrates that the immune response mounted to these epitopes is not unique to an individual animal and provides further support for the possibility that it could be used in vaccine development.

DISCUSSION

OspC has been clearly established as an important contributor to Lyme disease pathogenesis (12, 24, 29). There is strong evidence that it plays an important role during the transit of the Lyme disease spirochetes from the midgut to the salivary gland (24). In addition, it is selectively expressed during early infection, is an immunodominant antigen (5, 28, 38), and has been hypothesized by other investigators to be a key determinant in the dissemination capability of Lyme disease isolates

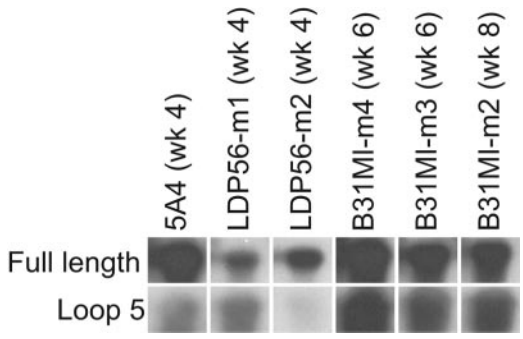

FIG. 5. Demonstration of a loop 5 antibody response in multiple animals infected with different type A OspC-producing strains. Immunoblots of either full-length type A OspC or loop 5 containing the fragment consisting of residues 130 to 150 were screened with infection sera. The strain used to generate the infection sera and the specific mouse (m) from which the sera were collected are indicated above each panel. The times during infection when the sera were collected are also indicated. An equal amount of protein was immunoblotted for each preparation, and all preparations were exposed to film for the same amount of time.

(30). The goals of this study were to test the potential correlation between the OspC type and invasive infection, to determine if the antibody response to OspC is type specific, and to further define the antigenic structure of OspC by localizing the linear epitopes that are presented during infection.

Sequence analyses of OspC have delineated 21 distinct OspC types (30), and it has been postulated that only four of these (types A, B, I, and K) are associated with invasive infections in humans (30). However, a recent study has called into question this putative correlation (1). To address this further, the OspC types of invasive and noninvasive Lyme disease isolates recovered from human patients in Maryland were determined. To accomplish this, the full-length *ospC* gene was PCR amplified and sequenced, and comparative sequence analyses were performed. These analyses revealed that the OspC types associated with invasive human infections in this patient population also included types C, D, and N. While it has been suggested that type I OspC-producing strains are a dominant type associated with invasive human infections (30), none of the invasive isolates identified in the Maryland patient population carried a type I *ospC* gene. Similarly, Alghaferi et al. also did not detect type I OspC-producing strains (1). Collectively, these two studies identified 18 invasive isolates in the greater Baltimore area, with the following breakdown: type A, 5 isolates; type B, 2 isolates; type C, 1 isolate; type D, 1 isolate; type H, 1 isolate; type K, 3 isolates; and type N, 6 isolates. Hence, in this geographic area it appears that OspC type A- and N-producing invasive isolates predominate. These data argue against the hypothesis that only four OspC types are associated with invasive infections in humans. Additional analyses of isolates recovered from larger patient populations from different geographic regions are necessary to further assess the validity of the OspC type-invasive infection correlation and to determine if there are differences in the prevalence of specific OspC types in defined geographic regions.

The variable protection offered by vaccination with OspC in conjunction with the delineation of distinct OspC types (30) raises the possibility that the antibody response could be type specific. This hypothesis is supported by the fact that vaccination with OspC has been found to provide protection only against the same strain (3, 9, 25). Until this report, the type specificity of the antibody response to OspC during infection had not been directly assessed. To address this, a series of full-length recombinant type A, B, C, D, H, K, and N OspC proteins were screened with infection serum generated in mice with clonal populations expressing known OspC types. The use of infection serum is important as it allows focused assessment of the antibody response to epitopes that are specifically presented by the bacterium in vivo. These analyses revealed that in spite of strong sequence conservation in the N- and C-terminal domains of OspC, the antibody responses to the OspC types analyzed were type specific. For example, serum from mice infected with type A or D strains was immunoreactive in a type-specific manner, and there was little or no cross-immunoreactivity with other OspC types. Although the antibody responses to all 21 OspC types were not analyzed, the data presented above suggest that the conserved domains are not immunodominant and that the linear epitopes of OspC presented by the bacterium during infection are contained in the variable domains (i.e., type-specific domains) of the protein.

To date, there have been only a few studies that have sought to localize or identify the epitopes of OspC. Both linear and conformational epitopes have been identified. Gilmore and Mbow demonstrated that independent N-terminal deletions beyond the leader peptide as short as six residues and C-terminal truncations of 13 residues abolish the binding of monoclonal antibody B5 (10, 11). From this finding it was concluded that the B5 monoclonal antibody recognizes a conformationally defined epitope (10). The precise residues that comprise the antibody recognition site within this conformationally defined epitope were not identified. In contrast to the findings obtained with monoclonal antibody B5, our analysis of the polyclonal antibody response to cell-associated, native OspC revealed that deletion of the last 10 C-terminal residues of OspC or of extended regions of the N terminus did not abolish recognition of OspC by IgG elicited during infection. The difference in the results is presumably a reflection of the focus on polyclonal versus monoclonal antibodies. Our data, which certainly do not preclude the existence of conformational epitopes, clearly demonstrate that there are linear epitopes in OspC as well. In a previous study, Mathiesen et al. also reported on a linear epitope in OspC (20). They found that the C-terminal seven residues of OspC constitute a linear epitope that is recognized by IgM in serum collected from European neuroborreliosis patients. While IgM binding was not assessed in this study, deletion of the C-terminal 10 residues of OspC did not abolish IgG binding. Epitopes that are recognized by infection-induced IgG appear to be localized at several sites in the protein. However, this does not suggest that a C-terminal epitope does not exist or is not recognized by antibody elicited during infection; rather, it suggests that there are additional epitopes that are located elsewhere in OspC.

Immunblot analysis of shorter OspC fragments allowed more precise localization of OspC epitopes. The antigenic regions of OspC were localized to two regions. One of these regions spans residues 136 to 150, and the other spans residues 168 to 210. Structural models generated using coordinates from X-ray diffraction analyses placed residues 136 to 150

largely within a surface-exposed loop, termed loop 5 (15). Loop 5 is surface exposed in both the mono- and dimeric models of OspC and is located within a prominent bend. While it has been demonstrated that recombinant OspC does in fact form dimers, it has not yet been determined if native OspC forms dimers or larger oligomers in vivo. The dimeric model for OspC indicates that there is a significant buried interface that comprises more than 30% of the protein. A buried interface of this extent suggests that there is a tight interaction between the monomers and is considered to be an indication that the dimeric form of the protein is the biologically active form. In the OspC dimer, residues within loop 5 are predicted to be part of a putative conformationally defined ligand binding pocket that may have biological significance. This charged pocket is lined by amino acids containing carbonyl groups, such as glutamate and aspartate. Crystal structures for representative proteins of types A, I, and E have been determined (4). In the type A and I proteins, the solvent structures of the putative binding pocket are remarkably well conserved. The accessibility of loop 5 to antibody in infection serum supports the postulate that this domain may be surface exposed and potentially available for ligand binding. In spite of strong intertype structural conservation of loop 5 and the putative ligand binding pocket, the sequence of this domain is highly variable at the intertype level. The sequence of the alpha 5 domain spanning residues 168 to 210 is also variable at the intertype level, with the exception of the last 20 residues, which are highly conserved. To determine if there is sufficient conservation at the intratype level to allow construction of a chimeric OspC vaccine consisting of a series of type-specific epitopes, OspC sequences were aligned and a dendrogram was constructed. Through these analyses the OspC type was determined for 227 sequences (data not shown). Both the loop 5 and alpha 5 epitopes were found to be well conserved at the intratype level. For example, the loop 5 epitopes of type A OspC proteins were identical in 53 of 57 sequences, while the alpha 5 epitope was conserved in 42 of 43 type A sequences. Significant conservation of these domains in the other OspC types was noted as well, with types C through I, M, T, and O exhibiting absolute intratype conservation within the loop 5 and alpha 5 epitopes.

In this study we demonstrated that there is greater OspC diversity among invasive isolates than has previously been recognized. In addition, we demonstrated that the antibody response to OspC in mice is largely type specific and is defined by previously uncharacterized loop 5 and alpha 5 epitopes. Previous studies and the data presented here clearly demonstrate that a single OspC protein does not or is not likely to convey protection against diverse strains (3). One possible vaccination approach is to exploit the epitopes identified in this report in the development of a recombinant chimeric OspC vaccinogen. The loop 5 epitope or a combination of loop 5 and alpha 5 epitopes may offer the most promise if they also prove to be consistently antigenic in humans. These epitopes are relatively short, linear, and highly conserved at the intratype level. In light of these features it should prove to be technically feasible to construct a loop 5-alpha 5 chimeric vaccinogen that can provide protection against highly diverse Lyme disease isolates. Efforts are now under way to construct such a vaccine and to assess the antibody response to loop 5 and helix 5 in human Lyme disease patients.

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