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Mapping Antibody-Binding Domains of the Major Outer Surface Membrane Protein (OspA) of *Borrelia burgdorferi*

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The major outer surface membrane protein of *Borrelia burgdorferi*, OspA, is one of several antigens recognized by sera from some patients in the chronic phase of Lyme borreliosis. We have expressed the OspA open reading frame in *Escherichia coli* and generated a series of deletion constructs of the gene and expressed them as *trpE* fusion proteins in *E. coli*. These constructs were used to identify antibody-binding sites of both rabbit antiserum and mouse monoclonal antibodies (MAbs) directed against OspA. All antibodies tested failed to bind to a fusion protein containing the first 61 amino acids of OspA, suggesting that the amino-terminal domain of OspA is unexposed to the cell surface. The binding site for one MAb, 184.1, was identified in a region centered around amino acid 61, while the binding site for MAb 105.5 was identified in a region centered around amino acids 214 to 217. Sera from two patients which were reactive to OspA identified distinct epitopes that lie between those recognized by our MAbs.

Lyme borreliosis is the most common vector-borne disease in the United States. The earliest sign of infection is erythema migrans, a distinctive skin lesion noted in two-thirds of patients infected with *Borrelia burgdorferi*. Early dissemination may give rise to multiple systemic complications, including myocarditis, meningitis, radiculopolyneuritis, skin rash, and arthritis. If untreated, the infection may become latent and subsequently recur as a chronic form of the disease that may involve joints, skin, the heart, or the nervous system (15).

Infection by *B. burgdorferi* is characterized by a gradually evolving, delayed humoral response. An early-appearing immunoglobulin M (IgM) response is directed primarily against the 41-kDa periflagellar antigen. This is followed by a specific anti-41-kDa-antigen IgG response. Subsequently, antibodies appear that are directed against larger (60, 66, 73, and 93 kDa) antigens. The chronic phase is often associated with the development of a humoral response directed against several smaller antigens, including the most prominent outer surface proteins OspA and OspB (1, 2, 4, 5, 7, 8). Interestingly, passive transfer experiments have shown that monoclonal antibodies (MAbs) directed against OspA protect mice with severe combined immunodeficiency acutely infected with *B. burgdorferi* from clinical infection (18).

In an effort to identify important immunogenic and structural domains of OspA, we have isolated mouse MAbs against the protein and have generated a molecular clone encoding its open reading frame. We have used this clone in order to express OspA in *Escherichia coli* and have constructed a family of deletions of OspA encoding various regions of the protein. By using these constructs to identify antibody-binding domains of the protein, we have identified important structural features of the molecule. We have also used these constructs to identify a region of the molecule that may be preferentially recognized by immunoreactive human sera.

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MATERIALS AND METHODS

Antibodies. The isolation of MAbs 184.1 and 105.5 has been described previously (11). MAb 184.1 is IgG2b and MAb 105.5 is IgG1. Both were used as hybridoma supernatants. The polyclonal rabbit antiserum used in these experiments was obtained following immunization with the B31 strain of *B. burgdorferi*.

Construction of OspA expression vectors. The OspA open reading frame of the B31 strain of *B. burgdorferi* was retrieved from a genomic expression library in λgt11 by screening with a rabbit polyclonal antiserum (9). This clone was modified for the purposes of expression cloning by attaching a *Bam*HI site at the 5′ end of the coding sequence and a *Sac*I site at the 3′ end. This was accomplished by removing the open reading frame following 25 cycles of polymerase chain reaction (PCR) amplification (19) with the following primers: 5′GGATCCGGAGAATATATTATGA AA3′ for modification of the 5′ end and 5′GAGCTCTA TTTTAAAGCGTTTTTA3′ for modification of the 3′ end. The resultant OspA fragment was cleaved with *Bam*HI and *Sac*I and ligated to the pATH3 (13) vector which had been cleaved with *Bam*HI and *Hin*dIII. Following this ligation, blunt ends were created by incubation with Klenow DNA polymerase at 37°C for 30 min, after which the reaction was made 2.5 mM in all four deoxynucleoside triphosphates and incubated for another 30 min. This mixture was religated to generate the p3/A clone.

Deletion constructs of p3/A were generated by restriction digestion, blunt-end formation, and religation of the p3/A parent plasmid. The enzymes used to generate the indicated constructs were the following: *Hin*dIII and *Cla*I for p3/AΔBH; *Spe*I partial and *Cla*I for p3/AΔBS; *Pst*I partial and *Cla*I for p3/AΔBP; and *Bam*HI and *Hin*dIII for p3/AΔHC. p3/AΔHS was made by cleaving p3/AΔBS with *Bam*HI and *Hin*dIII, blunt-end formation, and religation. p3/AΔHP was made by cleaving p3/AΔBP with *Bam*HI and *Hin*dIII, blunt-end formation, and religation. p3/AΔEC was made by *Eco*RI digestion followed by religation. p3/AΔSE was made from p3/AΔSC by *Eco*RI cleavage and religation. p3/AΔSC was isolated from the p3/A clone by PCR amplification with oligonucleotide primers flanking the region. These primers were 5′CGCGGGATCCACACTAGTATCAAAAAAAGT

A3′ for the 5′ end and 5′TCGTCTTCAAGAATTAAT3′ for the 3′ end. The PCR-amplified product was cleaved with *Bam*HI and *Cla*I and ligated into the *Bam*HI and *Cla*I sites of pATH3.

Internal deletions of OspA were generated by PCR amplification of sequences flanking the deleted segment and ligation of these sequences back into pATH3. For example, p3/AΔ108-143 was obtained by PCR amplification with primers 5′CCGAATTCGGGGGGATCC3′, from the polylinker region of pATH3, and 5′AGCTCTAGATGTTTTGCCATCT TCTTT3′, from OspA, as primers for the 5′ and 3′ ends, respectively, of the amino-terminal segment of the fusion construct. The primer sequences 5′AGCTCTAGAACCAG ACTTGAATACACA3′ and 5′GTTTGACAGCTTATCATC GAT3′ were used as 5′ and 3′ primers, respectively, to amplify the carboxy end. This strategy generates an amino-terminal template encompassing amino acids 1 to 108 which is flanked by *Eco*RI and *Xba*I restriction sites and a carboxy-terminal template extending from residues 143 to 273 flanked by *Xba*I and *Cla*I sites. Cleavage with these enzymes and religation into *Eco*RI-*Cla*I-cleaved pATH3 results in the production of a fusion protein in which amino acids 108 to 143 are deleted and replaced by Ser-Arg.

Induction of OspA fusion proteins. Recombinant constructs in *E. coli* C600 were grown overnight in M9 (17) complete medium supplemented with L-tryptophan (20 μg/ml). The cells were then removed from this medium and grown in M9 without tryptophan at 37°C for 1 h. Following this, cells were induced by the addition of β-indole acrylic acid to a final concentration of 5 μg/ml and growth for 2 h at 37°C. The cells were then harvested by centrifugation and suspended in sample buffer for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (14).

Immunoblot analyses. Following induction, 20 μg of bacterial cell lysate protein was separated by 10% SDS–PAGE, transferred electrophoretically to Immobilon P membranes (Millipore) (16), and incubated for 18 h in 50 mM Tris (pH 8)–150 mM NaCl (TBS) with 5% nonfat dry milk (Carnation). Blots were then incubated with the primary antibody in TBS with 0.05% Tween 20 (TBST) and 0.5% bovine serum albumin (BSA) for 90 min at room temperature. MAbs were used at a protein concentration of 1 μg/ml, rabbit antiserum was used at 30 μg/ml, and human sera were used at 300 μg/ml. Blots were then washed in TBST plus 0.5% BSA and incubated with 1 μCi of ^{125}I-labeled *Staphylococcus aureus* protein A (specific activity, 65 mCi/mg; ICN), followed by washing in TBST. Blots were exposed to autoradiographic film (Kodak XAR) at −70°C with Cronex Lightning-Plus enhancement screens.

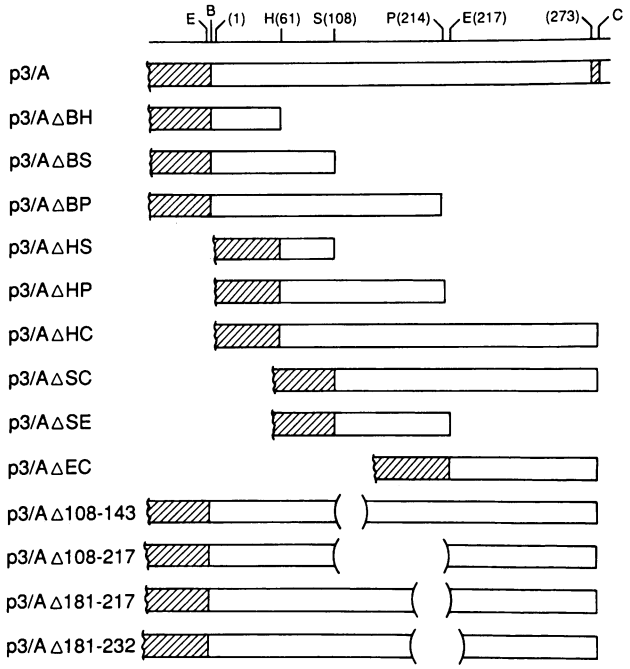

FIG. 1. Deletions of the OspA coding region. The OspA open reading frame was fused to the *trpE* gene of the expression vector pATH3 (13) at the polylinker site in the vector to create p3/A. Deletion constructs were then made by fusing various fragments of the OspA gene to the vector by using the restriction sites indicated at the top of the figure or else by PCR-mediated deletion of internal sequences. The designation of each construct corresponds to the restriction sites used in the cloning or the boundaries of amino acid deletions. *trpE* sequences are indicated by the cross-hatched regions; open boxes designate OspA sequences. Restriction sites and the coordinates of the amino acids encoded at those sites are indicated at the top of the figure. E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; S, *Spe*I; P, *Pst*I; C, *Cla*I.

RESULTS AND DISCUSSION

Construction of deletion mutants of OspA. The OspA open reading frame was isolated from a λgt11 expression clone following screening of a library with a rabbit polyclonal serum directed against the whole *B. burgdorferi* organism. Sequence analysis of the first 250 nucleotides of this clone confirmed that it encoded OspA since it was identical over this interval to the published sequence of OspA (3). The 819-bp open reading frame was cloned into the pATH3 expression vector (13). This plasmid vector contains a polylinker sequence near the 3′ end of the *E. coli trpE* gene. Subcloning of specific coding sequences within the polylinker creates inducible fusion proteins between the *trpE* gene and the expressed protein. The fusion construct for the OspA gene is designated p3/A in Fig. 1. Deletions of various regions of the protein were constructed by restriction digestion and religation or by PCR-mediated deletion, as indicated in Fig. 1, to create a family of fusion proteins that contain various amounts of OspA coding sequence.

Each of these expression clones was induced by the addition of indole acrylic acid in order to visualize the fusion protein in each clone. Figure 2 shows an SDS-PAGE analysis of several of the constructs illustrated in Fig. 1 following the induced overexpression of these fusion proteins. With the exception of p3/AΔHC, each of the constructs encoded a fusion product which was clearly distinguishable on the Coomassie-stained gel depicted in Fig. 2. While no induced band was visualized on the Coomassie-stained gel for p3/AΔHC, an antigenic band was clearly induced that was detectable on an immunoblot (Fig. 3A). This band was apparently of low abundance and was not visible above the background bands.

Mapping antibody-binding domains of anti-OspA MAbs. Four independently derived MAbs were generated against OspA. MAbs 184.1 and 105.5 have been described previously (11). Two other MAbs, 40.1 and 12.2, were prepared in the same fashion and were found to be functionally identical to 184.1 and 105.5, respectively, in all of the studies reported here. For this reason, no data are shown for these antibodies. Our previous studies had shown that MAbs 184.1 and 105.5 showed a pattern of reactivity suggesting that each

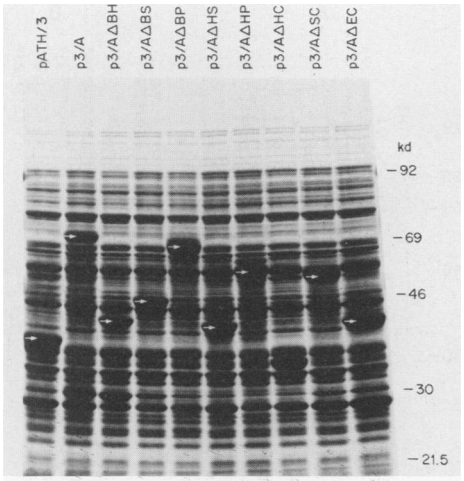

FIG. 2. Induction of OspA fusion constructs. Nine of the constructs illustrated in Fig. 1 were induced to high-level production of fusion proteins by the addition of indole acrylic acid; cell lysates were prepared, products were separated by 10% SDS–PAGE, and the gel was stained with Coomassie blue. The locations of molecular mass standards are indicated to the right of the gel. Individual induced bands are indicated by arrows. No induced band was visible above background for p3/AΔHC.

recognized a different domain (11). In these studies we found that MAb 184.1 reacted strongly with OspA and in addition reacted with a family of low-molecular-weight proteins. MAb 105.5, which also reacts strongly with OspA, cross-reacts with a group of higher-molecular-weight proteins. The present study was undertaken to identify the domains of OspA that determine antibody binding by these two MAbs.

In order to map the binding sites in OspA for each of these MAbs, immunoblot analysis was performed with the p3/A

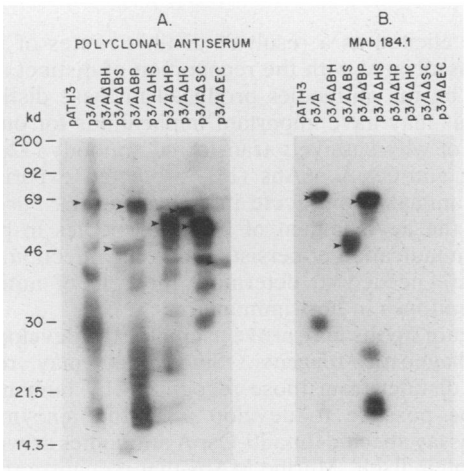

FIG. 3. Immunoblots of OspA fusion constructs with rabbit polyclonal serum and MAb 184.1. Induced fusion constructs of pA/3 and the various deletion constructs of OspA were lysed, electrophoresed, transferred to Immobilon P, reacted with the indicated MAb, developed with ^{125}I-protein A, and exposed as autoradiographs. The locations of molecular mass standards are indicated at the left of panel A. Panel A shows the result for rabbit polyclonal serum raised against the whole organism. Panel B shows the result for MAb 184.1. Reacting bands are indicated by arrowheads.

expression clone and the various deletion constructs depicted in Fig. 1. Figure 3 shows the results of this analysis with MAb 184.1 (Fig. 3B) and a polyclonal rabbit antiserum (Fig. 3A). The rabbit polyclonal serum directed against the whole organism detected the induced fusion proteins for all constructs except p3/AΔBH and p3/AΔHS. None of the antibodies or antisera used in this set of analyses bound to p3/AΔBH, which encodes the amino-terminal region of the protein (see Fig. 3, 4, and 5), suggesting that the antibody-binding site of OspA encoded in this construct is not exposed to the cell surface. The amino-terminal sequence of OspA contains a signal sequence which contains the tetrapeptide L-X-X-C, which confers the specificity needed for posttranslational lipidation at the cysteine (22). In *B. burgdorferi*, this signal sequence may be cleaved and the protein may be anchored to the outer membrane by the lipid modification. The failure of rabbit antiserum to bind to p3/AΔHS is somewhat surprising in view of the fact that the antiserum recognizes p3/AΔBS. The lack of binding to p3/AΔHS suggests that sequences amino terminal to amino acid 61 are important for the establishment of a recognizable conformation of the antibody-binding site in this region. An alternative explanation would be that peptides in the adjacent *trpE* domain of p3/AΔHS mask the antibody-binding site of this region of OspA.

When the same constructs were probed with MAb 184.1 (Fig. 3B), only the p3/A clone and the p3/AΔBS and p3/AΔBP deletion constructs were recognized. This places the recognition site in the 108-amino-acid domain between the amino terminus and amino acid 108. Since none of the constructs in which *trpE* was fused with OspA at the *Hin*dIII site lying at codon 61 were recognized by this MAb (p3/AΔHS, p3/AΔHP, and p3/AΔHC), the determinant must lie in the region that includes the *Hin*dIII site around amino acid 61. A computer analysis (10) of antigenic domains of OspA identified a segment predicted to be highly immunogenic lying between amino acids 37 and 61. The autoradiograms also revealed several lower-molecular-weight bands that were detected by our antibody preparations. These may result from internal transcription initiation in some of the constructs, from premature termination of translation resulting from the codon preference bias distinguishing *Borrelia* spp. and *E. coli*, or from proteolytic degradation of antigens during sample processing.

A similar analysis was performed with MAb 105.5, and the results of this analysis are shown in Fig. 4A and B. In Fig. 4A, the strongest pattern of binding was found with mutant p3/AΔSC, placing the recognition site between amino acid 108 and the carboxy terminus of OspA. Much weaker but detectable binding was found with p3/AΔHC, which contains all of the sequences contained in p3/AΔSC. This apparent paradox can be explained by the finding that the p3/AΔHC fusion protein is induced to very low levels following indole acrylic acid treatment (Fig. 2) and results in relatively weak binding to polyclonal rabbit antiserum as well (Fig. 3B). In addition, variable and weak binding was seen with p3/AΔBP, which terminates at amino acid 214. MAb 105.5 did not react with fusion proteins of the internal deletion constructs p3/AΔ181-217, p3/AΔ108-217, and p3/AΔ181-232 but did react with those from p3/AΔ108-143. This places the determinant in the segment from residues 143 to 217. The finding that MAb 105.5 bound to no constructs other than p3/AΔBP that contain boundaries at amino acid 214 (p3/AΔHP) or 217 (p3/AΔSE and p3/AΔEC) suggests that the region important for antibody binding by MAb 105.5 lies very near amino acids 214 to 217 within the 181 to 217 region.

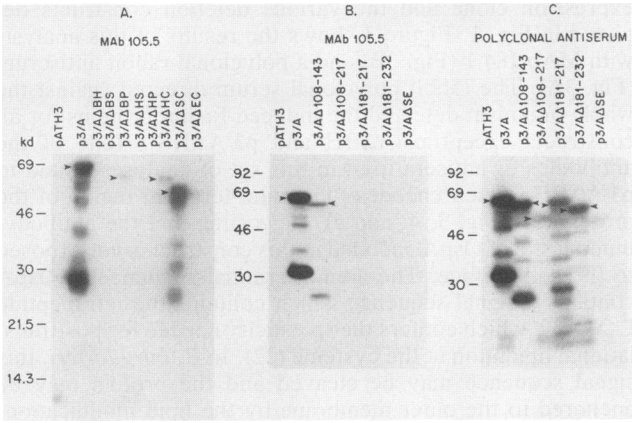

FIG. 4. Immunoblots of OspA fusion constructs with MAb 105.5. The protocol was essentially the same as for Fig. 3. Molecular mass standards are indicated at the left of each panel. Panel A shows the result for the set of constructs whose induced bands are shown in Fig. 2, and panel B shows the results obtained with an additional set of deletions. Panel C shows the same constructs as panel B probed with a polyclonal rabbit antiserum against *B. burgdorferi*. Arrowheads indicate fusion protein bands.

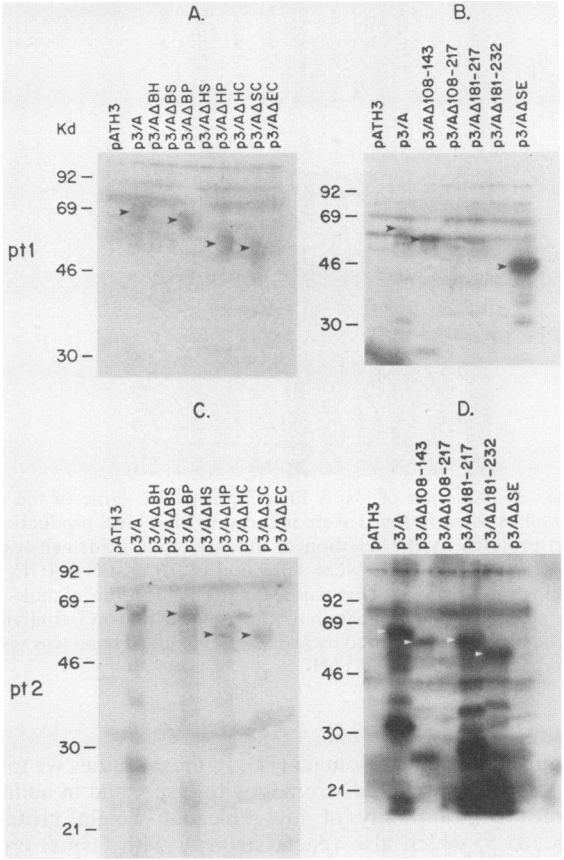

FIG. 5. Immunoblots of OspA fusion constructs with sera from patients with Lyme borreliosis. Sera from two patients with Lyme disease were reacted with the various OspA constructs shown in Fig. 4 following the same protocol as for Fig. 3 and 4. Reacting bands are indicated by arrowheads. (A and B) Patient 1; (C and D) patient 2.

Mapping binding sites of human sera from patients with Lyme borreliosis. Serum was obtained from seven patients with Lyme disease and used to perform immunoblot analysis on the same set of OspA expression constructs used in the mapping of binding sites for MAbs. Two of the seven sera showed reactivity to the p3/A clone, representing the complete OspA open reading frame. The immunoblots from the analyses of two of these sera are shown in Fig. 5. By a process of reasoning analogous to that applied to the previous studies, we can conclude that the antibody recognition region common to these human serum samples falls in the region between amino acids 108 to 214. Serum from patient 1 reacted strongly with p3/AΔSE and p3/AΔ108-143 but not with p3/AΔ181-217. This places the determinant between amino acids 181 and 214. The failure to react with p3/AΔHC can again be explained by the low abundance of the antigen and the relatively low affinity of the human antibody compared with rabbit antiserum. Using a similar line of reasoning, we conclude that serum from patient 2 recognized a region of OspA between amino acids 143 and 181.

Although only two of seven human serum samples reacted with our OspA recombinant fusion proteins, all seven human antisera detected a protein of 29 to 30 kDa that migrated close to OspA in SDS-PAGE and which we have found to be a more dominant antigen from *B. burgdorferi* (data not shown).

OspA and OspB are the most abundant surface proteins of *B. burgdorferi*. Interestingly, the immune response in humans to this surface protein does not reflect its abundance on the cell surface. Mice inoculated with a crude preparation of antigen or following laboratory infection develop a prominent immune response to OspA, as illustrated in this work. Similarly, laboratory infection of dogs elicits a prominent immune response to both OspA and OspB. By contrast, dogs undergoing natural infection following a tick bite do not develop a prominent antibody response to these antigens (7). This result may partially explain the infrequent immune response to OspA in this small sample, since following natural infection of humans, an antibody response to OspA develops late in infection and is seen in only a minority of cases. In our studies we show that the variable immune response elicited as a result of distinct routes of infection may be associated with the recognition of distinct antigenic domains by the antibodies produced in these distinct contexts. This may have important implications for our understanding of why passively transferred antibody (12, 18, 20), including anti-OspA MAbs (18), protected experimentally infected animals from acute infection with *B. burgdorferi*, whereas the development of these antibodies in people is usually an indication of persistent chronic infection. Further studies are needed to determine the role of antibody to specific epitopes in host immunity.

This set of MAbs may prove useful for the development of immunodiagnostic reagents. Since they may recognize epitopes distinct from those recognized by human sera, it should be possible to develop a capture enzyme-linked immunoassay specific for anti-OspA antibodies as well as the OspA protein itself. Murine MAbs that recognize regions of the OspA protein unreactive with human sera may be particularly useful in this regard if antigen-antibody complexes (21) are confirmed to explain our inability to detect the response to this antigen. The antigen complex could be captured by MAbs, and complexed antibody or antigen could be determined by an enzyme-linked immunoassay with these reagents, which do not compete with the human antibody.

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