INFECTION AND IMMUNITY, Apr. 1995, p. 1356–1361 Vol. 63, No. 4
0019-9567/95/$04.00+0

Identification of an Immunologically Important Hypervariable Domain of Major Outer Surface Protein A of *Borrelia burgdorferi*

BARBARA C. McGRATH,[1]* JOHN J. DUNN,[1] GINA GORGONE,[2]† BENJAMIN J. LUFT,[2] DAVID GUTTMAN,[3]‡ AND DANIEL DYKHUIZEN[3]

Biology Department, Brookhaven National Laboratory, Upton, New York 11973,[1] and Department of Medicine[2] and Department of Ecology and Evolution,[3] State University of New York at Stony Brook, Stony Brook, New York 11794

Received 30 September 1994/Returned for modification 15 November 1994/Accepted 11 January 1995

The gene for the major outer surface protein A (OspA) from several clinically obtained strains of *Borrelia burgdorferi*, the cause of Lyme disease, has been cloned, sequenced, and expressed in *Escherichia coli* by using a T7-based expression system (J. J. Dunn, B. N. Lade, and A. G. Barbour, Protein Expr. Purif. 1:159–168, 1990). All of the OspAs have a single conserved tryptophan at residue 216 or, in some cases, 217; however, the region of the protein flanking the tryptophan is hypervariable, as determined by a moving-window population analysis of *ospA* from 15 European and North American isolates of *B. burgdorferi*. Epitope-mapping studies using chemically cleaved OspA and a TrpE-OspA fusion have indicated that this hypervariable region is important for immune recognition. Biophysical analysis, including fluorescence and circular dichroism spectroscopy, have indicated that the conserved tryptophan is buried in a hydrophobic environment. Polar amino acid side chains flanking the tryptophan are likely to be exposed to the hydrophilic solvent. The hypervariability of these solvent-exposed amino acid residues may contribute to the antigenic variation in OspA. To test this, we have performed site-directed mutagenesis to replace some of the potentially exposed amino acid side chains in the B31 protein with the analogous residues of a *Borrelia garinii* strain, K48. The altered proteins were then analyzed by Western blot (immunoblot) with monoclonal antibodies which bind OspA on the surface of the intact B31 spirochete. Our results indicate that specific amino acid changes near the tryptophan can abolish the reactivity of OspA to these monoclonal antibodies, which is an important consideration in the design of vaccines based on recombinant OspA.

Lyme borreliosis is a multisystem infectious disease caused by the tick-borne spirochete, *Borrelia burgdorferi*. The major outer surface protein A (OspA) of *B. burgdorferi* is a basic lipoprotein with a size of approximately 31 kDa, which is encoded on a large linear plasmid along with the other major outer surface protein, OspB (21). Analyses of isolates of *B. burgdorferi* obtained from North America and Europe demonstrated that OspA has antigenic variability and that several distinct groups can be serologically and genotypically defined (24). The immune response to *B. burgdorferi* is characterized by an early, prominent and persistent humoral response to the endoflagellar protein p41 and to a protein constituent of the protoplasmic cylinder, p93 (6). Both of these proteins are physically cryptic antigens, protected from the immune system by the outer membrane whose major protein constituents are OspA and OspB. Paradoxically, the immune response to outer surface antigens has been demonstrated to occur late in infection, if at all (6, 7).

Mice immunized with a recombinant form of OspA are protected from challenge with the same strain of *B. burgdorferi* from which the protein was obtained (11). As a result, OspA has become the primary candidate for a genetically engineered Lyme disease vaccine. In mice, passively transferred anti-OspA monoclonal antibodies (MAbs) have been shown to be protective, and vaccination with a recombinant protein induced protective immunity against subsequent infection with the homologous strain of *B. burgdorferi* (19). However, immunization with the recombinant OspA does not necessarily confer resistance to a heterologous strain of *B. burgdorferi* (12). The overall efficacy of a recombinant OspA vaccine will therefore depend on the frequency of genetic variation in *B. burgdorferi*. Therefore, rational development of effective vaccines will require the determination of the extent of the overall variation in OspA, particularly in determinants responsible for antigenic variation.

Epitope mapping has been carried out with recombinant fusion proteins or a truncated form of OspA that lacks export and lipidation signals and is expressed to high levels in *Escherichia coli* (9). MAbs to OspA that recognize specific amino- and carboxy-terminal antigenic determinants have been reported (3, 17, 18, 24). However, only MAbs binding to the carboxy-terminal domains protect mice from challenge with spirochetes. In humans, anti-OspA antibodies produced during natural infection also recognize the carboxy-terminal portion of the OspA protein but do not recognize those epitopes which bind mouse MAbs (17).

Previous studies have shown that there are three to seven distinct types of *B. burgdorferi* that can be distinguished serotypically (24). Baranton et al. (2) distinguished three major groups, which they called genospecies. Recently, the *Borrelia* strains that cause Lyme disease have been classified into three species: *B. burgdorferi*, *B. garinii*, and *B. afzelii* (2, 4). Our recent phylogenetic analysis of 15 OspA variants from North

* Corresponding author. Phone: (516) 282-4960. Fax: (516) 282-3407.

† Present address: DNAX Research Institute, Palo Alto, CA 94304-1104.

‡ Present address: Department of Ecology and Evolution, University of Chicago, Chicago, IL 60637.

America and Europe also indicates that the protein falls into three phylogenetic groups that do not exchange genetic information and are, therefore, clonal (10). The amino termini of all 15 proteins are highly conserved; however, the immunologically important carboxy region varies considerably, with the exception of a single conserved tryptophan at residue 216 or, in a few cases, 217.

The *ospA* gene from the standard laboratory strain of *B. burgdorferi*, B31, has been cloned, sequenced, and expressed in *E. coli* in both the full-length lipidated form and a recombinant form that lacks the signals for export and lipidation (9). Unlike the lipidated form, the recombinant OspA is soluble and can be accumulated to high levels in cells expressing the cloned *ospA* gene. The recombinant B31 OspA has been purified and analyzed by circular dichroism (CD) and fluorescence emission spectroscopy (13–15). On the basis of the results of these studies, in conjunction with the epitope-mapping data (17, 24), a model that places the epitope around Trp-216 in an oriented alpha-helix has been proposed. Within this structure, Trp-216 is buried in a hydrophobic pocket, which thereby exposes more polar residues to the hydrophilic solvent. Sliding-window analysis demonstrates that these potentially solvent-exposed residues are included in one of the three hypervariable regions in OspA. Together, these data suggest that amino acid side chain differences at exposed sites around Trp-216 contribute to antigenic variation in OspA. To test this hypothesis, we have performed site-directed mutagenesis on residues flanking Trp-216 in recombinant B31 OspA in an effort to define the amino acid residues that are important for recognition by anti-OspA MAbs that bind the surface of intact spirochetes.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* BL21 (DE3)/pLysS (20) was grown at 37°C in phosphate-buffered tryptone broth supplemented with 25 μg of chloramphenicol per ml. BL21 (DE3)/pLysS cells carrying *ospA* clones in pET9c were grown in the same medium; however, 50 μg of kanamycin per ml was also added to maintain the pET9c plasmid. *E. coli* DH5α (Gibco/BRL, Gaithersburg, Md.) was grown at 37°C in phosphate-buffered tryptone broth which was supplemented with 50 μg of kanamycin when the strain carried the pET9c plasmid clones.

Sliding-window analysis of OspA polymorphism. OspAs from 15 clinically obtained European and North American variants of *B. burgdorferi* were cloned and sequenced as previously described (10). A moving window of fixed length was slid along the aligned sequences. A window size of 13 amino acids was chosen on the basis of the determination of the largest number of significantly deviating points (22). The average weighted polymorphism was calculated by adding the number of variant alleles for each site. Those sites that contained radical amino acid replacements on the basis of the PAM 250 amino-acid-weight matrix described by Dayhoff et al. (8) were given twice the weight as sites containing only conserved changes. The sum was normalized according to the window size and plotted.

An idea of the peaks that are likely to be biologically important was obtained by bootstrap resampling. With the sequences aligned, an amino acid position was sampled at random. This position formed the first position of a new protein and was replaced in the original sequence. A second position, which can be the same as the first, was chosen at random, and used to fill the second position of the new protein. The position was replaced in the original protein. This sampling with replacement was continued until a sequence with a length equal to that of the original sequence was generated. Then, a sliding window was run along the sequence, and the value of the weighted polymorphism was recorded for each position. This whole procedure was repeated 500 times, creating 500 new proteins, and many more sliding-window estimates. The value of the weighted polymorphism for which only 5% of the values are higher was determined. This should not be taken as a statistical test in the usual sense but as an indication of the regions which might contain biologically important polymorphism.

Site-directed mutagenesis. Three oligonucleotides were generated, each containing various changes to incorporate strain K48 amino acid residues at their analogous positions in the recombinant B31 *ospA* that had been cloned into the P-Alter vector (Promega, Madison, Wis.). Oligonucleotides used to create the three double mutants were as follows: OspA 613, CTTAATGACTCTGACAC TAGTGCTGCT (Thr-204-Ser and Ser-206-Thr); OspA 625, GCTACTAAA AAGGGAAATGGAATTCA (Ala-214-Gly and Ala-215-Lys); and OspA 640, GCAGCTTGGATTCCAAAAACATCCACTTTAACA (Asn-217-Asp and Gly-219-Lys). Mutagenesis was carried out per the instructions in the kit (Promega). The two quadruple mutants were generated by performing mutagenesis with pairs of the above-described oligonucleotides. This created OspA 613-625 (Thr-204-Ser, Ser-206-Thr, Ala-214-Gly, and Ala-215-Lys) and the OspA 613-640 protein (Thr-204-Ser, Ser-206-Thr, Asn-217-Asp, and Gly-219-Lys). Mutations at the appropriate positions were confirmed by standard DNA sequencing methods.

Cloning and expression in T7 system. The site-directed mutant OspA proteins in the P-Alter vector served as a template for PCR amplification and cloning into the pET9c T7 expression plasmid, which was carried out as described by Dunn et al. (9). The OspA DNAs were amplified in such a way that the first 17 codons, which include the signals for export and lipidation, were removed from the PCR product and restriction sites for ligation into the pET9c plasmid were introduced. The resulting PCR DNA was digested with *Nde*I (Gibco/BRL) and *Bgl*II (United States Biochemical Corp., Cleveland, Ohio) and then purified on a 0.8% low-melting-point agarose preparation gel. The isolated gel slice was melted and used for ligation into the pET9c vector that had been restricted with *Nde*I and *Bam*HI (United States Biochemical Corp.). Ligations were transformed into DH5α, and kanamycin-resistant colonies were then screened for the *ospA* insert by digestion of isolated plasmid DNA with *Bgl*II and *Eco*RI (New England BioLabs, Beverly, Mass.). Insert-bearing plasmids were then used to transform BL21 (DE3)/pLysS to kanamycin resistance.

Expression of the mutant *ospA* genes was carried out on a small (10-ml) scale by adding 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to cultures grown at 37°C to mid-log phase in phosphate-buffered tryptone broth supplemented with kanamycin and chloramphenicol (to maintain pLysS) (9, 20). After 4 h, a small aliquot (0.1 ml) was added to 0.2 ml of 3× sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS PAGE) loading dye and boiled for 2 to 3 min. A small amount of this material (10 to 15 μl) was analyzed by SDS-PAGE on a 12% gel (mini-Protean II; Bio-Rad, Richmond, Calif.), and the proteins were visualized by Coomassie blue staining. A prominent protein band migrating at 31 kDa was observed for clones carrying *ospA* inserts. Cells carrying the pET9c vector itself did not produce a major protein band of this size on induction with IPTG.

Western blot (immunoblot) analysis of site-directed mutant OspA proteins. *E. coli* cells carrying pET9c with the site-directed OspA mutant inserts were induced for expression, and the resulting cell lysates were subjected to SDS-PAGE as described above. The proteins were then transferred to an Immobilon-P membrane (Millipore) at 70 V for 2 h at 4°C. Western blot analysis was carried out as described by Schubach et al. (17). Primary antibodies were typically used at a 1:10 dilution, whereas the alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Bio-Rad) was diluted 1:1,000. MAb 105 was raised to B31 spirochetes and recognizes an epitope near the carboxy terminus of OspA (17). MAb 184 was also raised to B31 spirochetes; however, it binds a domain at the amino terminus of the protein and is highly cross-reactive among the OspA variants (17). MAb 336 was raised to whole spirochetes of the European OspA variant P/Gau. MAbs H5332 and H3TS were kindly provided by A. Barbour (University of Texas Health Sciences Center, San Antonio, Tex.). With the exception of MAb 184, all of the above MAbs will agglutinate spirochetes.

Chemical cleavage of B31 OspA with NCS. The reagent *N*-chloro-succinimide (NCS; Sigma Chemical Company, St. Louis, Mo.) cleaves proteins at tryptophan residues and therefore fragments OspA into two pieces with sizes of 23.2 and 6.2 kDa. To 150 μg of recombinant B31 OspA in 8 M urea, an equal volume of 1.3 mg of NCS per ml in 25% acetic acid was added. The reaction mixture was then incubated at room temperature for 30 min according to the method of Lischwe and Sung (16). The products were then separated by SDS-PAGE, transferred to Immobilon-P, and probed with MAbs 105 and 184 by using the Western blot procedure described above. The uncleaved OspA control was incubated under the same reaction conditions, without the addition of NCS.

RESULTS

Sliding-window analysis indicates that the helical domain around Trp-216 is hypervariable. Sliding-window analysis was carried out by plotting the moving, weighted-average polymorphism of a window (a fixed-length subsection of the total sequence) as it was moved along the sequence one amino acid at a time. The window size was 13 amino acids as established by the method of Tajima (22). Polymorphism calculations were weighted by the severity of the amino acid replacement (8). The purpose of this sliding-window analysis was to find regions of the protein in which there is an excess of variability. It is postulated that these highly variable regions are also immunologically important. The argument for this postulate is that a variant will be able to infect an animal that has already been infected with other types, without being eliminated by an immune response. This gives rare variants a selective advantage, since they are less likely to infect an organism previously ex-

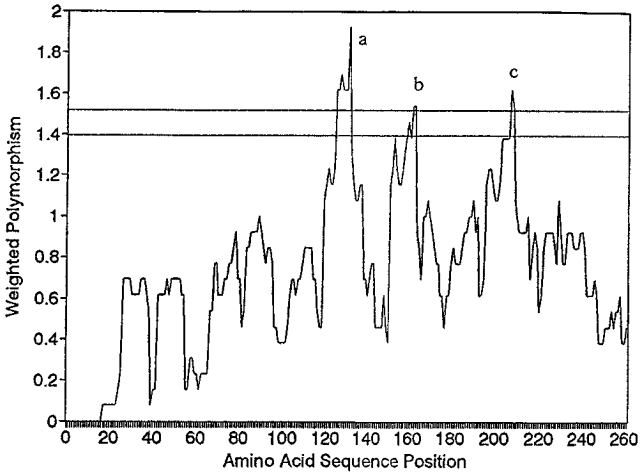

FIG. 1. Plot of weighted polymorphism versus amino acid position among 15 *B. burgdorferi* OspA variants. For this analysis a data set consisting of DNA sequences from 15 clinically obtained European and North American variants were used (10). A moving window of 13 amino acids was used on the basis of the largest number of deviating points (22). The average weighted polymorphism was calculated as the sum of the number of variant alleles at each position. Radical amino acid changes were given twice the weight of conservative ones (8). The sum was normalized to the window size and plotted. The amino acid sequence position corresponds to the first residue of any particular window (i.e., sequence position 1 corresponds to a window that spans amino acids 1 to 13). The marked peaks are amino acids 132 to 145, (a) 163 to 177 (b), and 208 to 221 (c). The lower horizontal line at polymorphism value 1.395 demarcates statistically significant excesses of polymorphism at P of 0.05. The upper horizontal line at 1,520 is the same, except that the first 29 amino acids at the monomorphic amino terminus have been removed for the original analysis. These values were obtained by bootstrapping (resampling with replacement) 500 times at each position, and the mean was calculated from the sum of all positions.

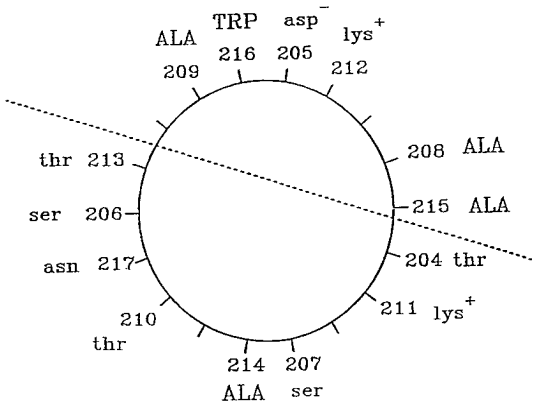

FIG. 2. Helical wheel projection of residues 204 to 217 of B31 OspA. Capital letters indicate hydrophobic residues; lowercase letters indicate hydrophilic residues; + and − indicate positively and negatively charges residues, respectively. The dashed line indicates division of the alpha-helix into a hydrophobic arc (above the line) and a polar arc (below the line) (14).

posed to the same type. This means that more variation will be maintained in a population than expected for regions of the protein that are not selectively important.

Fifteen OspA open reading frames were determined from isolates sampled from the United States and Europe. The sliding-window analysis demonstrated that the amino acid polymorphism is not randomly distributed throughout the protein but tends to be clustered in three regions of OspA. The peak values for these regions are for windows covering amino acid residues 132 to 145, 163 to 177, and 208 to 221 (Fig. 1). The region from amino acids 208 to 221 includes the region of OspA that has been modeled in the purified protein as an oriented alpha-helix in which the single conserved tryptophan is buried in a hydrophobic pocket, thereby exposing more polar residues to the solvent (Fig. 2) (15).

Chemical cleavage of OspA at Trp-216 abolishes binding of agglutinating MAbs. MAbs that agglutinate spirochetes, including several which are neutralizing in vitro, recognize epitopes that map to the hypervariable region around Trp-216 (3, 17). Western blot analysis (Fig. 3) demonstrates that chemical cleavage of OspA from the B31 strain at Trp-216 abolishes reactivity of the protein with agglutinating MAb 105, a MAb raised against B31 spirochetes. The reagent NCS cleaves OspA at its single Trp, forming a 23.2-kDa fragment and a 6.2-kDa peptide which is not retained on the Immobilon-P membrane after transfer. Uncleaved OspA incubated in reaction buffer without NCS binds MAb 105; however, the 23.2-kDa cleavage product is unreactive. Similar Western blots with a TrpE-OspA fusion protein containing the carboxy-terminal portion of the OspA protein demonstrated that the small 6.2-kDa piece (amino acids 217 to 273) fails to bind MAb 105 (17). This result demonstrates that the region of OspA flanking Trp-216 is a surface-exposed epitope, whose recognition requires continuity of the polypeptide chain.

Amino acid replacement of residues predicted to be solvent-exposed abolishes reactivity to agglutinating MAbs. To directly test the importance of the predicted solvent-exposed amino acid residues to MAb binding of this epitope, we performed site-directed mutagenesis to convert residues within the 204 to 219 domain of the recombinant B31 OspA to analogous residues of *B. garinii* K48, which originated in western Europe (10). We then expressed the mutant OspA proteins and subjected them to Western blot analysis using several independently derived agglutinating MAbs. An amino acid alignment between residues 200 and 220 for B31, K48, and the four site-directed mutants is shown in Fig. 4.

In the region of OspA between residues 204 and 219, which includes the helical domain (amino acids 204 to 217), there are seven amino acid differences between B31 OspA and K48 OspA. Because Trp-216 is known to be buried, it seemed likely that Trp-216 splits the 204 to 219 domain into two antigenically important subdomains. Rather than performing single replacements in these putative subdomains, we decided to make double replacements on either side of Trp-216, hoping that the resulting recombinant proteins would display clear changes in reactivity with different MAbs. We created three site-directed mutants, each with two of the seven changes (OspA 613, 625, and 640), as well as two proteins with four changes (OspA 613-625 and OspA 613-640). Both of these quadruple mutants contained the OspA 613 mutations (Thr-204-Ser and Ser-206-Thr); however, one was paired with the OspA 625 changes (Ala-214-Gly and Ala-215-Lys), while the other had the OspA 640 mutations (Asn-217-Asp and Gly-219-Lys).

MAbs H5332 and H3TS (3) have been shown by immunofluorescence to decorate the surface of fixed spirochetes (24). Furthermore, MAb H5332 has been shown to kill *B. burgdorferi* by antibody-dependent, complement-mediated killing in rhesus monkeys (1). Epitope mapping with fusion proteins has suggested that the epitopes which bind these MAbs, as well as MAb 105, are conformationally determined and reside in the carboxy half of the protein. MAb H5332 is cross-reactive among all known phylogenetic groups, whereas MAb H3TS and MAb 105 seem to be specific to the B31 strain against which they were raised. Like that of MAb 105, the reactivities of MAbs H5332 and H3TS to OspA can be abolished by

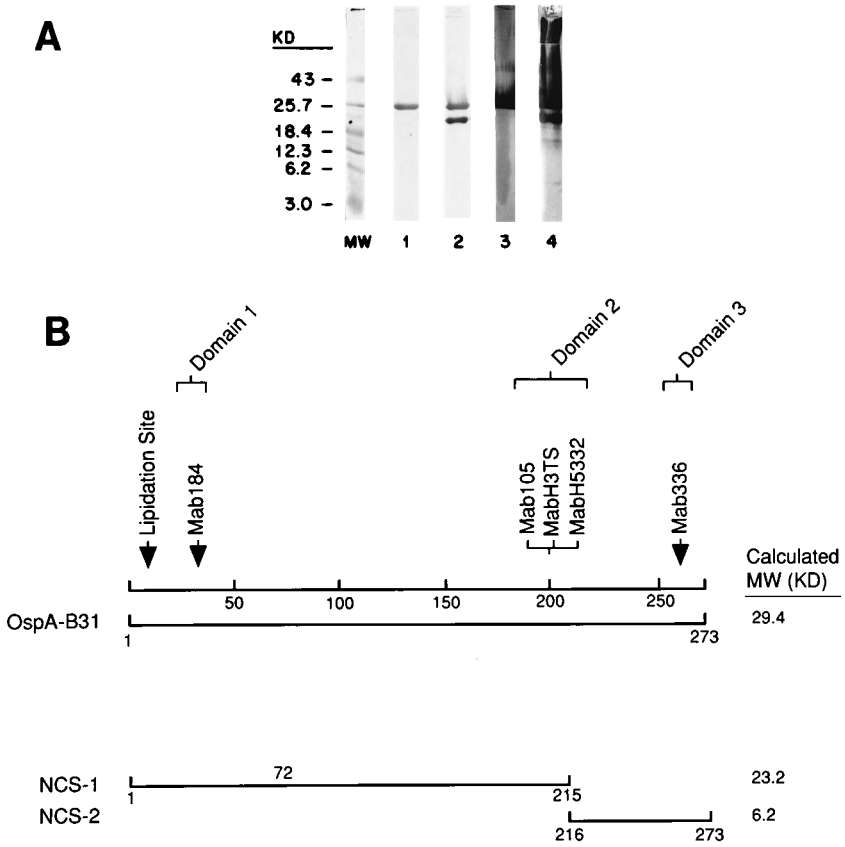

FIG. 3. Chemical cleavage of recombinant B31 OspA with NCS. Treatment of the recombinant, purified B31 OspA was carried out by the method of Lischwe and Sung (16) (see Materials and Methods). All samples, including the uncleaved OspA control, were incubated under standard NCS reaction conditions (8 M urea and 12.5% acetic acid). (A) Western blots. Lanes: MW, molecular size markers (with sizes in kilodaltons [KD] indicated on the left); 1, uncleaved OspA stained with Ponceau red (Sigma); 2, NCS-cleaved B31 OspA stained with Ponceau red; 3, NCS-cleaved B31 OspA probed with MAb 105; 4, NCS-cleaved B31 OspA probed with MAb 184. (B) Linear map of B31 OspA cleaved with NCS. MW, molecular sizes (in kilodaltons [KD]).

fragmentation of the protein at Trp-216 (data not shown). MAb 336 was raised against whole spirochetes of strain P/Gau, a *B. afzelii* strain (4, 10). It cross-reacts with OspA from spirochetes of the *B. burgdorferi* group but not with OspA of *B. garinii* strains. Previous studies using fusion proteins and chemical cleavage have indicated that this antibody recognizes a domain of OspA in the region between residues 217 and 273 (data not shown). All of these MAbs will agglutinate B31 spirochetes. These MAbs were used for Western blot analysis of the site-directed OspA mutants induced in *E. coli* by using the T7 expression system (Fig. 5; Table 1) (9).

```
                    ↓
B31:      ELNDTDSSAATKKTAAWNSGT
K48:      ALDDSDTTQATKKTGKWDSKT

613:      ELNDSDISAATKKTAAWNSGT
625:      ELNDTDSSAATKKTGKWNSGT
640:      ELNDTDSSAATKKTAAWDSKT
613/625:  ELNDSDISAATKKTGKWNSGT
613/640:  ELNDSDISAATKKTAAWDSKT
```

FIG. 4. Amino acid alignment of residues 200 through 220 for OspA proteins from B31 and K48, as well as for site-directed mutant OspA 613, 625, 640, 613-625, and 613-640. The arrow indicates Trp-216. Amino acid changes are underlined.

Western blot analysis indicates that only the OspA 625 double mutant (Ala-214-Gly and Ala-215-Lys) retains binding to the agglutinating MAb H3TS. However, the OspA 613-625 quadruple mutant, which has additional alterations on the amino-terminal side of Trp-216 (Thr-204-Ser and Ser-206-Thr), does not bind this MAb. Both OspA 640 and OspA 613-640, which have the Asn-217-Asp and Gly-219-Lys changes on the carboxy-terminal side of Trp-216, also fail to bind MAb H3TS. This suggests that the epitope of the B31 OspA which binds H3TS is comprised of amino acid side chains on both sides of Trp-216.

The OspA 613-625 quadruple mutant fails to bind MAbs 105 and H5332, while the other mutants retain their ability to bind these MAbs. This is interesting in light of the data obtained with fusion proteins that indicate that MAb 105 behaves more like MAb H3TS in terms of its serotype specificity and binding to OspA (23). The OspA 613-625 protein has, in addition to the differences at residues Thr-204 and Ser-206, changes immediately amino terminal to Trp-216 (Ala-214-Gly and Ala-215-Lys). The abrogation of the reactivities of MAbs 105 and H5332 to this protein indicates that the epitopes of OspA that bind these MAbs are comprised of residues on the amino-terminal side of Trp-216.

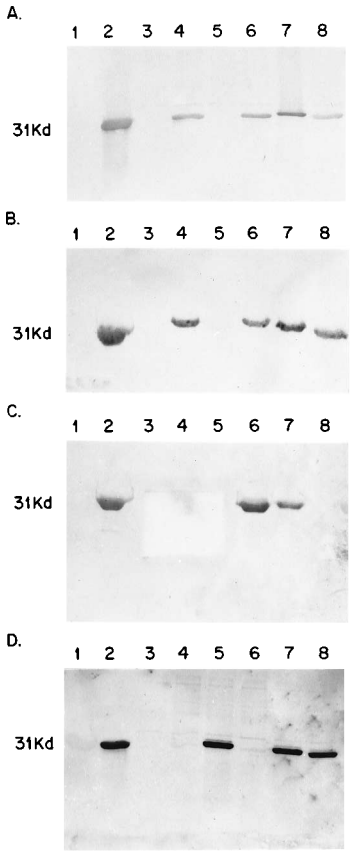

FIG. 5. Western analysis of site-directed mutant OspA proteins induced by using the T7 expression system (9). *E. coli* cells carrying the mutant OspA clones in pET9c were induced for expression and analyzed by Western blotting as described in Materials and Methods and in reference 17. Identical membranes were probed with MAb 105 (A), MAb H5332 (B), MAb H3TS (C), and MAb 336 (D). Lanes: 1, purified, recombinant K48 OspA; 2, purified, recombinant B31 OspA; 3, pET9c induced in *E. coli* without an *ospA* insert; 4, OspA 613-640 induced in *E. coli*; 5, OspA 613-625 induced in *E. coli*; 6, OspA 640 induced in *E. coli*; 7, OspA 625 induced in *E. coli*; 8, OspA 613 induced in *E. coli*. These data are summarized in Table 1. The position of the 31-kDa band (31 Kd) is indicated.

TABLE 1. Summary of Western blot data for OspA site-directed mutants

OspA	Reactivity to MAb:				
	184	105	336	H5332	H3TS
B31	+	+	+	+	+
K48	+	−	−	−	−
613	+	+	+	+	−
625	+	+	+	+	+
640	+	+	−	+	−
613-625	+	−	+	−	−
613-640	+	+	−	+	−

The two proteins carrying the Asn-217-Asp and Gly-219-Lys replacements on the carboxy-terminal side of Trp-216 (OspA 640 and OspA 613-640) retain binding to MAbs 105 and H5332; however, they fail to react with MAb 336, a MAb which has been mapped with TrpE-OspA fusion proteins and by chemical cleavage to a more carboxy-terminal domain. This may explain why MAb 336 fails to recognize the OspA proteins from *B. garinii* strains.

DISCUSSION

The efficacy of a recombinant vaccine for Lyme disease based on OspA will no doubt depend on its ability to protect against infection by a broad spectrum of *B. burgdorferi* variants. As new variant strains are identified both in Europe and North America and the phylogenetic tree expands, it becomes clear that the extent of variation in OspA must be an important consideration in vaccine development. Our results define three clusters of polymorphism. One includes the surface-exposed domain around Trp-216, which is the target of a number of MAbs that are agglutinating and, in some cases, neutralizing in vitro. The other hypervariable domains, which include residues 132 to 145 and 163 to 177, may also be antigenically important and may be involved in epitopes that are involved with cellular and/or humoral immune responses in humans. The biophysical data also suggest that the region from 163 to 177 may also have some helical character (15); however, the determination of the actual configuration of all of the domains of OspA is contingent on obtaining a well-resolved X-ray crystallographic structure.

For the agglutinating epitope around Trp-216, our current model suggests that OspA antigenic variability is the result of amino acid side chain differences at surface-exposed positions on the polar face of an alpha-helix, between residues 204 and 217. We have shown that changes in these solvent-exposed residues can alter the reactivity of OspA to agglutinating MAbs. It is clear that the amino acids Thr-204 and Ser-206 play an important part in the agglutinating epitopes in the region of the B31 OspA flanking Trp-216. Replacement of these two residues with the analogous residues present in the strain K48 protein alters the epitopes of the B31 OspA that bind MAbs 105, H5332, and H3TS. The ability of OspA 640 changes alone to abolish reactivity to MAb 336 indicates that Thr-204 and Ser-206 are not involved in direct interaction with MAb 336.

In these experiments, the amino acid residues altered by mutagenesis have been replaced with residues present in the analogous positions in K48 OspA. Recent circular dichroism analysis indicates that the structures of the B31 and K48 OspA proteins differ very little within this domain; therefore, it is unlikely that the changes made by mutation have radically altered the overall structure of the OspA protein (15). This hypothesis is supported by our finding that the recombinant, mutant OspA proteins exhibit the same high solubility and purification properties as the parental B31 OspA protein. During the course of mutagenesis experiments, we inadvertently produced an OspA that has a proline missense mutation a short distance upstream of Trp-216. This change perturbed the structure of the protein to the extent that it was misfolded during synthesis, producing an OspA that was insoluble (data not shown).

In summary, amino acid side chains at Thr-204 and Ser-206 seem to be part of a region encompassing the agglutinating epitope of OspA. However, a limited set of conservative changes at these sites are not sufficient to abolish binding to all of the agglutinating MAbs. These results suggest that while the agglutinating epitopes of OspA are distinct, they may have some overlap. This supports the hypothesis that the surface-exposed epitope around Trp-216, which is thought to be important for immune recognition and neutralization, is a complex domain of OspA. Previous work by Bockenstedt et al. (5) also demonstrated this complexity. They were able to show that fragments of OspA, including one that was capable of binding

a protective MAb in vitro, were not effective vaccines for mice in vivo. Conversely, vaccination of mice with full-length OspA was shown to provide effective, long-lived protection under the same conditions (5). These results do not suggest that an OspA-based vaccine for Lyme disease will be ineffective. However, they do call into question the usefulness of anti-OspA peptide vaccines and underscore the need to understand the specificity of protective OspA epitopes. To this end, we have established that amino acid side chains at solvent-exposed positions around Trp-216 make important contacts with agglutinating MAbs. Since these contacts tend to differ among OspA variants, it may be necessary to incorporate several variant proteins into an OspA-based vaccine in order to provide a broad spectrum of protection against Lyme disease.

ACKNOWLEDGMENTS

We gratefully acknowledge the gift of MAbs H5332 and H3TS from Alan Barbour (University of Texas Health Sciences Center, San Antonio). We also thank Cathy Lawson, Elizabeth Bosler, and Barbara Lade for careful review of the manuscript.

This work was supported by NIH grant RO1AI3254.

REFERENCES

1. **Aydintug, M. K., Y. Gu, and M. T. Phipipp.** 1994. *Borrelia burgdorferi* antigens that are targeted by antibody-dependent, complement-mediated killing in the rhesus monkey. Infect. Immun. **62:**4929–4937.
2. **Baranton, G., D. Postic, I. Saint Girons, P. Boerlin, J.-C. Piffatetto, M. Assous, and P. A. D. Grimont.** 1992. Delineation of *Borrelia burgdorferi* sensu stricto, *Borrelia garinii* sp. nov., and group VS461 associated with Lyme borreliosis. Int. J. Syst. Bacteriol. **42:**378–383.
3. **Barbour, A. G., S. L. Tessier, and W. J. Todd.** 1983. Lyme disease spirochetes and ixodid tick spirochetes share a common surface antigenic determinant defined by monoclonal antibodies. Infect. Immun. **41:**795–804.
4. **Belfaiza, J., D. Postic, E. Bellenger, G. Baranton, and I. Saint Girons.** 1993. Genomic fingerprinting of *Borrelia burgdorferi* sensu lato by pulse-field gel electrophoresis. J. Clin. Microbiol. **31:**2873–2877.
5. **Bockenstedt, L. K., E. Fikrig, S. W. Barthold, F. S. Kantor, and R. A. Flavell.** 1993. Inability of truncated recombinant OspA proteins to elicit protective immunity to *Borrelia burgdorferi* in mice. J. Immunol. **151:**900–906.
6. **Craft, J. E., D. K. Fischer, G. T. Shiamamoto, and A. R. Steere.** 1986. Antigens of *Borrelia burgdorferi* recognized during Lyme disease. J. Clin. Invest. **78:**934–939.
7. **Dattwyler, R. J., and B. J. Luft.** 1989. Immunodiagnosis of Lyme borreliosis. Rheum. Dis. Clin. North Am. **15:**727–734.
8. **Dayhoff, M. O., R. M. Schwartz, and B. C. Orcutt.** 1978. *In* M. O. Dayhoff, (ed.) Atlas of Protein Sequence and Structure, vol. 5, suppl. 3, p. 345. National Biomedical Research Foundation, Washington, D.C.
9. **Dunn, J. J., B. N. Lade, and A. G. Barbour.** 1990. Outer surface protein A (OspA) from the Lyme disease spirochete *Borrelia burgdorferi*: high level expression and purification of a soluble from of OspA. Protein Expr. Purif. **1:**159–168.
10. **Dykhuizen, D. E., D. S. Polin, J. J. Dunn, B. Wilske, V. Preac-Mursic, R. J. Dattwyler, and B. J. Luft.** 1993. *Borrelia burgdorferi* is clonal: implications for taxonomy and vaccine development. Proc. Natl. Acad. Sci. USA **90:**10163–10167.
11. **Fikrig, E., S. W. Bartold, F. S. Kantor, and R. A. Flavell.** 1990. Protection of mice against the Lyme disease agent by immunizing with recombinant OspA. Science **250:**553.
12. **Fikrig, E., S. R. Telford III, S. W. Batsford, F. S. Kantor, A. Spielman, and R. Flavell.** 1992. Elimination of Borrelia burgdorferi from vector ticks feeding on OspA-immunized mice. Proc. Natl. Acad. Sci. USA **89:**5418.
13. **France, L. L., J. Kieleczawa, J. J. Dunn, G. Hind, and J. C. Sutherland.** 1992. Structural analysis of an outer surface protein from the Lyme disease spirochete *Borrelia burgdorferi*, using circular dichroism and fluorescence spectroscopy. Biochim. Biophys. Acta **1120:**59–68.
14. **France, L. L., J. Kieleczawa, J. J. Dunn, B. J. Luft, G. Hind, and J. C. Sutherland.** 1993. Evidence for an alpha-helical epitope on outer surface protein A from the Lyme disease spirochete, *Borrelia burgdorferi*: an application of steady-state and time-resolved fluorescence quenching techniques. Biochim. Biophys. Acta **1202:**287–296.
15. **France, L. L., B. C. McGrath, J. J. Dunn, B. J. Luft, J. Kieleczawa, G. Hind, and J. C. Sutherland.** 1993. Structural analysis of antigenic variation in *Borrelia burgdorferi* using ultraviolet synchrotron radiation, p. 107–114. *In* Sima et al. (ed.), Frontiers in Photobiology. Elsevier Science Publishers, New York.
16. **Lischwe, M. A., and M. T. Sung.** 1977. Use of *N*-chloro-succinimide/urea for the selective cleavage of tryptophanyl peptide bonds in proteins. J. Biol. Chem. **252:**4976–4980.
17. **Schubach, W. H., S. Mudri, R. J. Dattwyler, and B. J. Luft.** 1991. Mapping antibody-binding domains of the major outer surface protein (OspA) of *Borrelia burgdorferi*. Infect. Immun. **59:**1911–1915.
18. **Sears, J. E., E. Fikrig, T. Y. Nakagaura, K. Deponte, N. Marcantonio, and R. A. Flavell.** 1991. Molecular mapping of OspA-mediated immunity to Lyme borreliosis. J. Immunol. **147:**1995–2000.
19. **Simon, M. M., U. E. Scnaible, M. D. Kramer, C. Eckerskorn, C. Museteanu, H. K. Muller-Hermelink, and R. Wallich.** 1991. Recombinant outer surface protein A from *Borrelia burgdorferi* induces antibodies protective against spirochetal infection. J. Infect. Dis. **164:**123–132.
20. **Studier, F. W., A. H. Rosenberg, J. J. Dunn, and J. W. Dubendorf.** 1990. Use of T7 RNA polymerase to direct expression of cloned genes. Methods Enzymol. **185:**60–89.
21. **Szezepanski, A., and J. L. Benach.** 1991. Lyme borreliosis: host responses to *Borrelia burgdorferi*. Microbiol. Rev. **55:**21–34.
22. **Tajima, F.** 1991. Determination of window size for analyzing DNA sequences. J. Mol. Evol. **33:**470–473.
23. **Wilske, B., B. J. Luft, W. H. Schubach, G. Zumstein, S. Jauris, V. Preac-Mursic, and M. D. Kramer.** 1992. Molecular analysis of the outer surface protein A (OspA) for conserved and variable antibody-binding domains. Med. Microbiol. Immunol. **181:**191–207.
24. **Wilske, B., V. Preac-Mursic, R. Fuchs, and E. Soutschek.** 1991. Immunodominant proteins of *Borrelia burgdorferi*, the etiologic agent of Lyme borreliosis. World J. Microbiol. **7:**130–136.