

Molecular Analysis of Neutralizing Epitopes on Outer Surface Proteins A and B of *Borrelia burgdorferi*

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The neutralizing epitopes of the major outer surface proteins A and B (OspA and OspB) of *Borrelia burgdorferi* B31 were investigated by epitope mapping using overlapping synthetic peptides, encompassing full-length OspA and OspB, and antiborrelial monoclonal antibodies (MAbs). OspA MAb N4B12 and OspB MAbs N5G5, W7C2, and P4D1 displayed a complement-independent antiborrelial activity, and complement failed to enhance the antiborrelial activity, as measured by a sensitive colorimetric assay. A combination of N4B12 with N5G5 displayed a higher antiborrelial activity than did the MAbs individually. OspA MAbs B3G11 and L3B5, however, exhibited a significant antiborrelial activity only in the presence of complement. Epitope mapping showed that B3G11 bound to one OspA synthetic peptide with the sequence of amino acids 247 to 256 (QYDSNGTKLE) and produced more than sixfold-higher reactivity than with other sequences, as measured by an enzyme-linked immunosorbent assay. OspB MAb N5G5 bound to an OspB peptide with the sequence of amino acids 211 to 220 (TLKREIEKDG), yielding at least threefold-higher reactivity than with other sequences. These two peptide sequences were found to contain neutralizing epitopes. Other MAbs had weak binding activities with the synthetic peptides, and their specific epitopes remain to be further analyzed. Thus, this study demonstrated both complement-independent and complement-dependent antiborrelial MAbs and identified the linear epitopes on OspA and OspB capable of inducing neutralizing antibody responses.

Borrelia burgdorferi is the etiological agent of Lyme disease, a tick-borne zoonosis (7). The early signs of the disease include the flu-like symptoms and characteristic skin rashes termed erythema migrans. Untreated patients may experience systemic infection resulting in multisystem disorder, characterized by arthritis, cardiac, and neurological manifestations (39). Antibiotic treatment of early Lyme disease is generally effective (5, 11, 39). However, failure of antibiotic therapy can occur, as evidenced by persistent signs and symptoms of the illness and survival of the spirochete in patients after treatment (4, 34). The rising incidence and geographic spread of this zoonosis have made Lyme disease an important public health problem worldwide (1, 3, 12, 24, 41). Thus, development of a vaccine against Lyme disease has been one of the recent efforts in Lyme disease research.

Studies on the development of vaccines against Lyme disease have mainly focused on the major outer surface proteins A and B (OspA and OspB) as vaccine candidates. Immunization with recombinant OspA and/or OspB confers protection against experimental challenge (13–15, 26). Transfer immunoprotection with immune sera and monoclonal antibodies (MAbs) reveals that serum antiborrelial activity is a major protective mechanism against spirochetal infection (16, 33). Characterization of immune response domains on OspA by using truncated OspA fusion proteins, murine MAbs, clinical serum samples, and T lymphocytes from Lyme disease patients has recently been reported (36–38, 43). Approximately half of the OspA sequence at the C terminus contains antibody binding domains recognized by neutralizing MAbs and sera from Lyme disease patients (37). Furthermore, 60 C-terminal amino acids of OspA have been suggested to contain B- and T-cell

epitopes (38). Recently, a linear epitope near residue 253 on OspB, capable of inducing bactericidal antibody, has been identified (31).

Our previous studies have indicated that the conserved neutralizing epitopes exist among the different genospecies of *B. burgdorferi* (26). The goal of our study was to analyze these epitopes by using neutralizing MAbs raised against OspA and OspB of strain B31 as a basis for future Lyme disease vaccine-related research. We describe here both complement-independent and complement-dependent antiborrelial murine MAbs and the identification of the precise locations of neutralizing epitopes on OspA and OspB by epitope mapping. Amino acids 247 to 256 on OspA and 211 to 220 on OspB have been found to contain functional epitopes capable of inducing antiborrelial antibody responses.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The sources of the low-passage (<10 passages in vitro) *B. burgdorferi* sensu stricto strain B31 and *Borrelia garinii* sp. nov. strain G25 (2) have been described previously (27). *B. burgdorferi* CA-2-87 (low passage) and *Borrelia afzelii* sp. nov. strain R-IP-3 (8) (unknown passage) were generously provided by Tom G. Schwan (Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Mont.) and David Dennis (Centers for Disease Control and Prevention, Fort Collins, Colo.), respectively. All strains used were uncloned, and their infectivities, except that for strain R-IP-3, have been demonstrated in C3H/HeJ mice. Spirochetes were grown in modified Barbour-Stoenner-Kelly (mBSK) medium at 32°C as previously described (26).

Molecular cloning and expression of OspA and OspB. The genes encoding OspA and OspB of *B. burgdorferi* B31 were cloned and expressed in *Escherichia coli* MZ-1 as described previously (26). Plasmid pLCBC1, containing the heat-inducible p_L promoter, the Shine-Dalgarno sequence, and the first 16 amino acid codons derived from the lambda cII gene, was used as an expression vector. Expression of OspA and OspB was induced by growing bacteria to logarithmic phase at 32°C and then incubating them at 42°C for 1 h. The bacteria were lysed, and proteins were purified by anion-exchange chromatography as previously described (26). Protein concentration was determined by the bicinchoninic acid assay (Pierce, Rockford, Ill.).

Bacterial lysates. Borrelial strains B31, CA-2-87, G25, and R-IP-3 were grown to the late logarithmic growth phase. Borrelial lysates were prepared by washing

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spirochetes three times and resuspending the organisms in 0.5% sodium dodecyl sulfate (SDS) as described previously (26).

MABs. MABs to OspA and OspB of *B. burgdorferi* B31 were produced as described previously (18, 23). Briefly, BALB/c mice were immunized at least twice with recombinant OspA, OspB, and borrelial lysate formulated with QS-21 (19) or monophosphoryl lipid A plus trehalose dicorynomycolate emulsion (RIBI ImmunoChem Research, Inc. Hamilton, Mont.). One week after the last immunization, spleen cells were fused with SP2/0 myeloma cells at a 4:1 ratio in the presence of 50% polyethylene glycol 1500 (Boehringer Mannheim, Indianapolis, Ind.). Hybridoma cultures producing antibodies of interest were cloned at least twice by limiting dilution. Specific MABs and their isotypes were identified by enzyme-linked immunosorbent assay (ELISA) and immunoblotting. MABs were purified by using a protein A affinity column.

ELISA. ELISA was performed by using plates coated with *B. burgdorferi* antigens (Cambridge Biotech Corporation, Worcester, Mass.) as described previously (25). Reactivities of MABs with OspA and OspB in borrelial antigens were measured by using serially diluted MABs at an initial concentration of 2 µg/ml and goat anti-mouse immunoglobulin G (IgG) conjugated to horseradish peroxidase (Fisher Scientific, Pittsburgh, Pa.). Antibody isotypes were examined by using an isotype-specific goat anti-mouse IgG conjugate as described above.

Immunoblotting. Immunoblotting was conducted by a modification of the procedure of Towbin et al. (42). Ten micrograms of bacterial lysates was subjected to SDS-polyacrylamide gel electrophoresis using an 11% separating gel (22). Separated antigens were transferred to a nitrocellulose membrane and probed with 10 µg of purified MABs per ml. Goat anti-mouse IgG conjugated to horseradish peroxidase (Fisher Scientific) and 4-chloro-1-naphthol substrate were used to detect specific antibody and antigen reaction.

Microagglutination. Specific MAB-mediated agglutination of spirochetes was performed as previously described (26). A *B. burgdorferi* culture was adjusted to a density producing approximately 60 organisms per microscopic field. One hundred microliters of the borrelial suspension was incubated in 96-well plates with an equal volume of heat-inactivated, serially diluted MABs at an initial concentration of 100 µg/ml in mBSK medium at 32°C for 2 h. Normal mouse serum and mouse antiserum to OspA formulated with QS-21 (26) were used for the agglutination negative and positive controls, respectively. Microagglutination was determined by dark-field microscopy, and the titer was defined as the highest dilution that resulted in fewer than 15 individual spirochetes per microscopic field.

Colorimetric antiborrelial assay. A colorimetric assay was performed to determine the antiborrelial activity of MABs as described previously (27). Briefly, heat-inactivated MABs at a concentration of 100 µg/ml were serially diluted in mBSK containing 120 µg of phenol red per ml in 96-well plates (Costar, Cambridge, Mass.). An equal volume of spirochetal suspension of strain B31 or CA-2-87 containing approximately 6×10^6 spirochetes in fresh mBSK was added to each well. Assays with and without guinea pig complement (Gibco/BRL) were tested simultaneously. Normal mouse serum and mouse antiserum to OspA formulated with QS-21 (26) were used as negative and positive controls, respectively. Each sample was tested in triplicate. After 72 h of incubation at 32°C, absorbance was measured at wavelengths of 562 and 630 nm by an ELISA microplate reader. Antiborrelial activity was evaluated by measuring borrelial growth as indicated by a change in the color of the medium from red to yellow because growing spirochetes release acids into the medium. High absorbance at 562 and 630 nm indicates a high antiborrelial activity; low absorbance indicates a lack of antiborrelial activity. The antiborrelial activity was expressed as the MAB minimal neutralizing concentration and defined as the lowest antibody concentration resulting in a mean absorbance 0.10 higher than that of a nonantiborrelial MAB at the same antibody concentration tested.

Epitope mapping. Epitope mapping was performed by ELISA for reactivity of MABs with synthetic peptides. Pin-bound peptides of 10 amino acids with overlapping 7 amino acid residues were constructed on the basis of the deduced amino acid sequences of OspA and OspB of strain B31 (6), using an epitope scanning kit (Cambridge Research Biochemicals, Cheshire, England). ELISA was conducted as instructed by the manufacturer. MABs were used at 300 ng/ml for binding to the specific peptides, and the isotype-specific goat anti-mouse IgG conjugated to horseradish peroxidase (Fisher Scientific) was used for the colorimetric reaction.

RESULTS

OspA- and OspB-specific MABs. The properties of a panel of MABs reactive with OspA and OspB of *B. burgdorferi* B31 are summarized in Table 1. Reactivities and specificities of these MABs with denatured and native OspA and OspB were determined by ELISA, immunoblotting, and microagglutination. Nine MABs are OspA specific, and three are OspB specific.

Immunoblotting of borrelial lysates of strains B31, CA-2-87, G25, and R-IP-3 with these MABs revealed that only T2H12 reacted with OspA of all four borrelial strains tested (Fig. 1,

TABLE 1. Characterization of MABs to OspA and OspB of *B. burgdorferi* B31

MAB	Antigen specificity	Isotype	Microagglutination titer ^a	MNC (µg/ml) ^b	Complement dependency	Epitope (amino acids)
H3G4	OspA	IgG1	<2	ND		
L3B5	OspA	IgG1	128	6.25	Yes	
T1F6	OspA	IgG1	<2	ND		
B3G11	OspA	IgG1	128	1.56	Yes	247–256
T2H12	OspA	IgG1	<2	ND		
N6F4	OspA	IgG1	16	ND		
N4B12	OspA	IgG2a	256	1.56	No	
Q6H12	OspA	IgG2a	<2	ND		
Q7H6	OspA	IgG2a	2	ND		
W7C2	OspA/B	IgG1	128	25	No	
P4D1	OspB	IgG1	8	25	No	
N5G5	OspB	IgG2a	512	0.31	No	211–220

^a Determined by incubating 100 µl of serially diluted, heat-inactivated MAB with an equal volume of live spirochetal suspension, with a density producing 60 organisms per microscopic field, at 32°C for 2 h. The microagglutination titer is defined as the highest dilution of MAB which caused $\geq 50\%$ of the spirochetes to agglutinate.

^b MNC (minimal neutralizing concentration) is defined as the lowest antibody concentration resulting in a mean absorbance 0.10 higher than that of a nonantiborrelial MAB at the same antibody concentration tested. ND, not determined.

lanes 5). Another seven OspA MABs tested bound to the OspA protein band of strains B31 and CA-2-87 but not to that of strains G-25 and R-IP-3 (Fig. 1, lanes 1 to 4 and 6 to 8). OspB MAB W7C2 recognized OspB of all four strains and also OspA of strains B31 and CA-2-87 (Fig. 1, lanes 9). P4D1 and N5G5 bound only to OspB of strains B31 and CA-2-87, not to that of strains G25 and R-IP-3 (Fig. 1, lanes 10 and 11).

Reactivities of the MABs with the accessible epitopes of strain B31 were determined by a microagglutination assay. OspA MABs L3B5, B3G11, and N4B12 strongly agglutinated the live spirochetes of strain B31 with titers of 128 to 256. N6F4

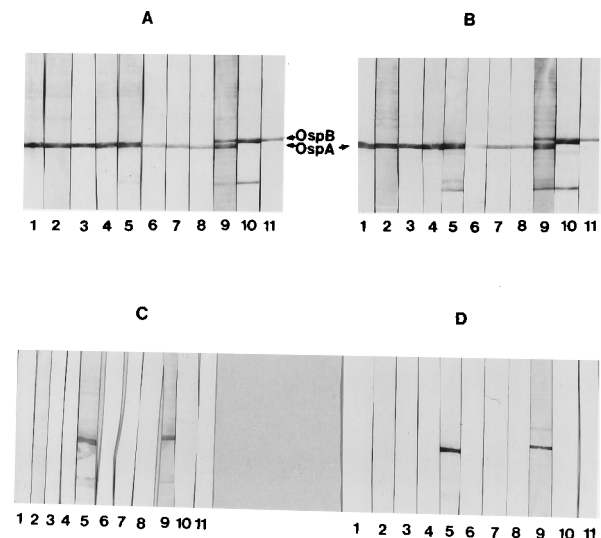


FIG. 1. Immunoblot of borrelial lysates with OspA and OspB MABs. Ten micrograms each of lysates of strains B31 (A), CA-2-87 (B), G25 (C), and R-IP-3 (D) was subjected to SDS-polyacrylamide gel electrophoresis using an 11% separating gel (22). Separated antigens were transferred to a nitrocellulose membrane and probed with 10 µg of purified MABs per ml. Goat anti-mouse IgG conjugated to horseradish peroxidase and 4-chloro-1-naphthol substrate were used for developing colorimetric reaction. Lanes: 1, H3G4; 2, L3B5; 3, T1F6; 4, B3G11; 5, T2H12; 6, N6F4; 7, N4B12; 8, Q6H12; 9, W7C2; 10, P4D1; 11, N5G5.

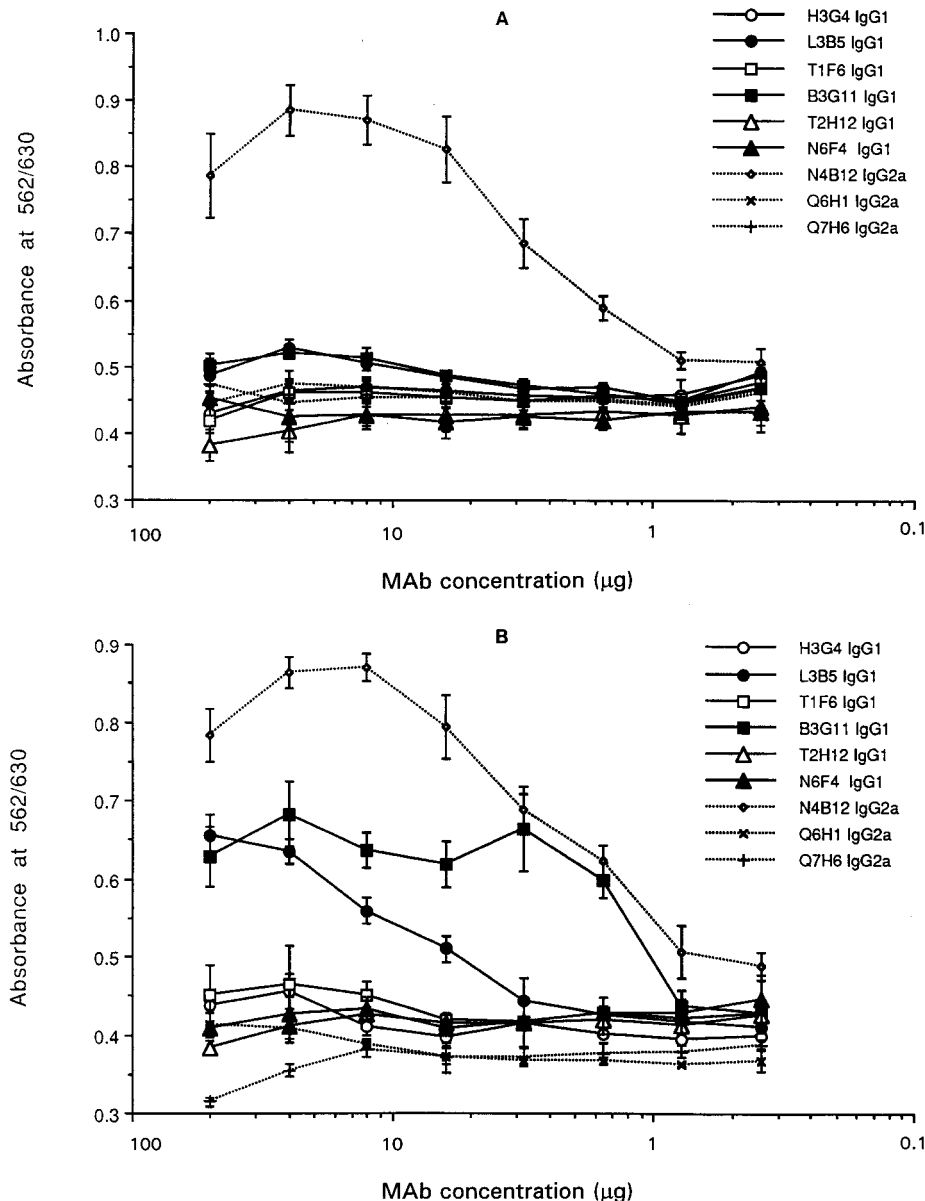


FIG. 2. Antiborrelial activities of murine MAbs to OspA of *B. burgdorferi* B31. Antiborrelial activity of the purified MAbs in the absence (A) and presence (B) of complement was determined by a colorimetric assay (27). High absorbance indicated a high antiborrelial activity; low absorbance represented a lack of antiborrelial activity. Each error bar represents the standard error of three measurements.

and Q7H6 had low microagglutination titers of 16 and 2, respectively. Four other OspA MAbs lacked microagglutination activities at the antibody concentrations tested (Table 1). OspB MAbs W7C2 and N5G5 were also able to agglutinate strain B31 spirochetes with titers of 128 and 512, respectively. P4D1 had a microagglutination titer of 8.

Antiborrelial activities of MAbs. All MAbs were examined in the same experiment for antiborrelial activity against different geographic borrelial isolates. N4B12, in the absence of complement, exhibited a strong antiborrelial activity against strain B31, with an antiborrelial concentration of approximately 1.56 $\mu\text{g/ml}$ (Fig. 2A). Addition of complement failed to enhance the antiborrelial activity (Fig. 2B). In contrast, B3G11 and L3B5 displayed clear antiborrelial activities only in the presence of complement (Fig. 2). The antiborrelial concentra-

tions of B3G11 and L3B5 were 1.56 and 6.25 $\mu\text{g/ml}$, respectively. These antiborrelial MAbs were also antiborrelial to *B. burgdorferi* CA-2-87 but not to the different genospecies of strains G25 and R-IP-3 (28). None of the other six OspA MAbs with complement possessed a detectable antiborrelial activity at the MAb concentration tested. OspB MAb N5G5, in the absence of complement, was strongly antiborrelial at a MAb concentration of as low as 0.31 $\mu\text{g/ml}$ (Fig. 3A). P4D1 and W7C2, however, had antiborrelial activities detectable only at a MAb concentration of at least 25 $\mu\text{g/ml}$ (Fig. 3A). Addition of complement failed to enhance the antiborrelial activity of these OspB MAbs (Fig. 3B). The minimal neutralizing concentrations of MAbs with their dependency on complement against strain B31 are summarized in Table 1.

Interestingly, a combination of OspA MAb N4B12 with

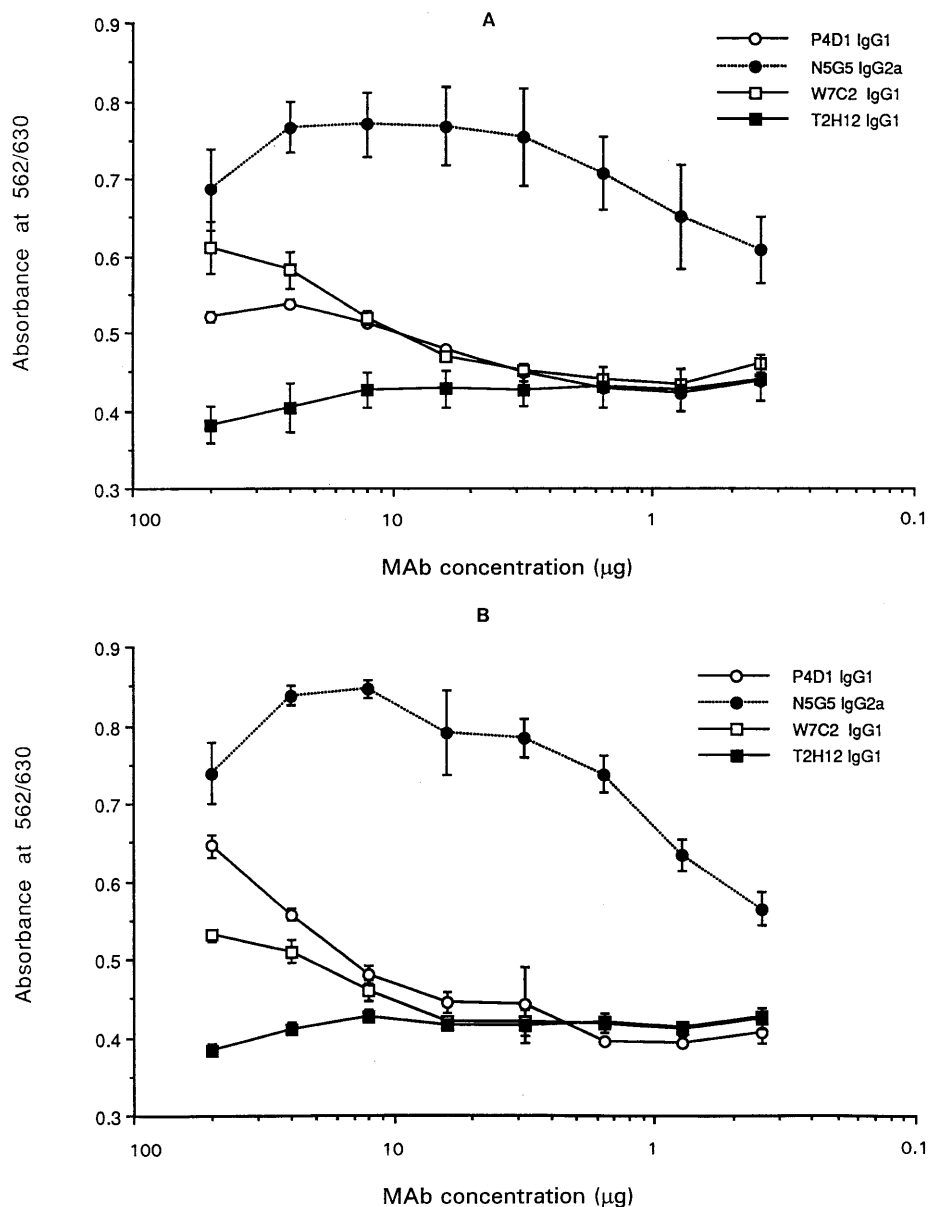


FIG. 3. Antiborrelial activity of murine MAbs to OspB of *B. burgdorferi* B31. Antiborrelial activity of the purified MAbs in the absence (A) and presence (B) of complement was evaluated as described for Fig. 2. Each error bar represents the standard error of three measurements.

OspB MAb N5G5 exhibited higher antiborrelial activity than did the individual MAbs against strain B31 (Fig. 4A) and heterologous strain CA-2-87 (Fig. 4B). Nevertheless, the combination of these MAbs did not neutralize *B. garinii* G25 and *B. afzelii* R-IP-3 (28).

Functional epitopes on OspA and OspB. A series of overlapping peptides of 10 amino acid residues encompassing the full-length OspA and OspB of strain B31 were synthesized and used for epitope mapping with the antiborrelial MAbs. B3G11 bound to an OspA peptide with an absorbance of 1.01, similar to that (1.03) of a positive control peptide with its specific MAb provided by the manufacturer (28). Absorbances of the reactivities of other peptides with B3G11 were below 0.16. This more highly reactive peptide has the sequence of amino acids 247 to 256 (QYDSNGTKLE) on OspA (Fig. 5). N5G5 bound to an OspB peptide with the sequence of amino acids 211 to

220 (TLKREIEKDG), producing an absorbance of 1.14. Its reactivities with other peptides yielded absorbances of less than 0.36 (Fig. 6). Repeat tests showed the same reaction profiles. Other MAbs bound to multiple peptides with weak reactivities (28). Their specific epitopes remain to be further elucidated.

DISCUSSION

In this study, we generated a number of OspA and OspB MAbs which recognized different epitopes. Four OspA MAbs, H3G4, T1F6, T2H12, and Q6H1, failed to agglutinate the spirochetes; their specific epitopes on OspA could have been sterically hindered by the membrane from antibody binding. Five other OspA MAbs and three OspB MAbs were obviously raised against the accessible epitopes, as indicated by their

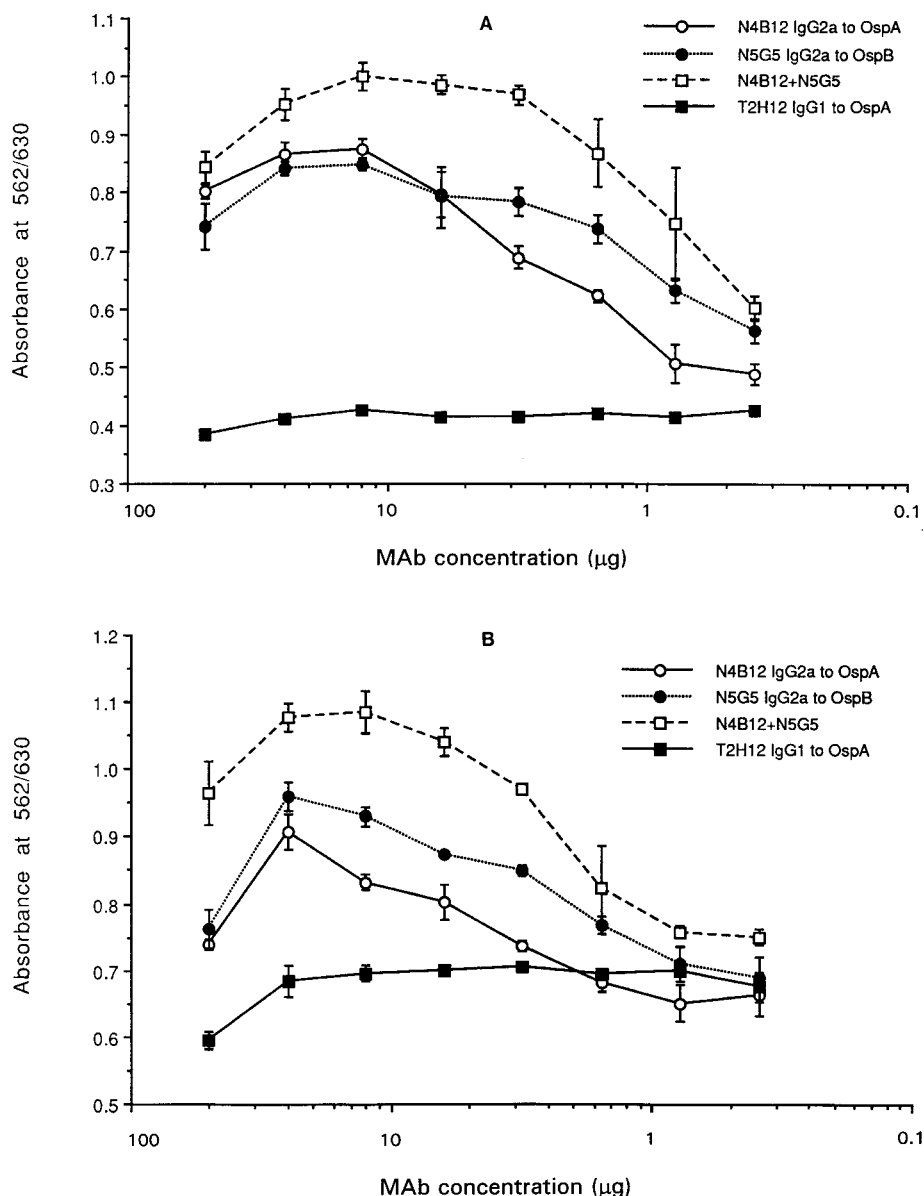


FIG. 4. Antiborrelial activity of OspA MAb N4B12 and OspB MAb N5G5, alone and in combination, against *B. burgdorferi* B31 (A) and CA-2-87 (B). Antiborrelial activities of MAbs were evaluated as described for Fig. 2. Each error bar represents the standard error of three measurements.

agglutinating activities. Their antiborrelial activities, however, were displayed in different fashions. N5G5 and N4B12 are IgG2a antibodies which exhibited a strong complement-independent antiborrelial activity; complement did not significantly enhance the antiborrelial activity. In contrast, both B3G11 and L3B5 are IgG1 antibodies which displayed antiborrelial activity only in the presence of complement. The mechanism through which these IgG1 isotype MAbs display complement-dependent antiborrelial activity remains to be investigated because it has been shown that murine IgG1 antibody does not fix complement as efficiently as IgG2 antibody (9).

It has been documented by *in vitro* studies that killing of *B. burgdorferi* by convalescent human serum and rabbit immune IgG Fab fragment is complement dependent (20, 21). The antiborrelial activity, however, was also observed in heat-inactivated human and rat immune sera to *B. burgdorferi* (30, 32).

Fab fragments of mouse OspA and OspB MAbs in the absence of complement also possess antiborrelial activity (10, 32). *In vivo* passive protection tests have shown that complement-dependent, and possibly complement-independent, antibodies to *B. burgdorferi* are involved in protection against hamster Lyme arthritis (35). Our present study extended the previous observation and demonstrated both complement-independent and complement-dependent neutralizing antibodies to Osp proteins of *B. burgdorferi*.

Antiborrelial MAbs used in the present investigation were also capable of neutralizing *B. burgdorferi* California isolate CA-2-87. None of these MAbs, however, neutralized *B. garinii* G25 and *B. afzelii* R-IP-3 even in the presence of complement (28). These MAb-specific epitopes, thus, may not be conserved among the genospecies of Lyme disease spirochete tested. Among 12 MAbs, only T2H12 and W7C2 recognized the spe-

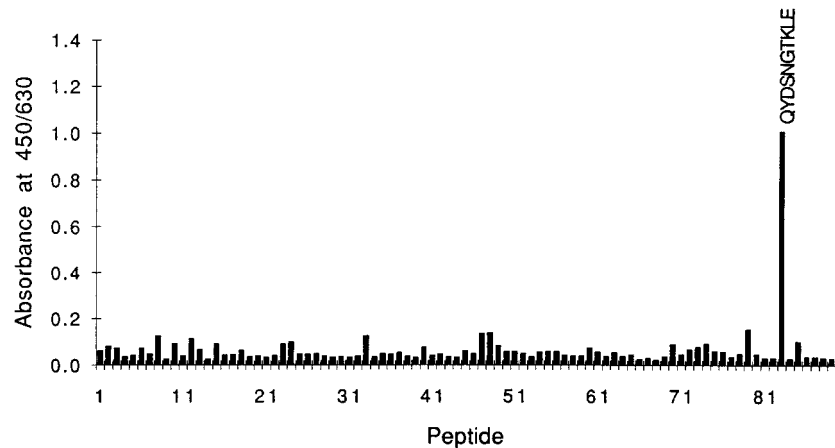


FIG. 5. Identification of an epitope on OspA by peptide scanning analysis. Epitope mapping was conducted by ELISA for reactivity of MAb B3G11 with the pin-bound OspA peptides. Those synthetic peptides of 10 amino acid residues with overlapping 7 amino acid residues were constructed on the basis of the deduced amino acid sequence of OspA (6), using an epitope scanning kit (Cambridge Research Biochemicals). MAbs were used at 300 ng/ml in the ELISA, and the isotype-specific goat anti-mouse IgG conjugated to horseradish peroxidase was used for detection of the specific binding of MAb to a peptide. The OspA-specific MAb B3G11 reacted with a single peptide with the sequence of amino acids 247 to 256 (QYDSNGTKLE).

cific epitopes conserved on OspA and OspB, respectively, of the three genospecies of strains tested. W7C2 also recognized an epitope on OspA apparently conserved among strains B31 and CA-2-87. Unfortunately, T2H12 was not antiborrelial, and W7C2 was weakly antiborrelial only to homologous strains (28). Therefore, these conserved epitopes are unlikely to induce protective immunity against different genospecies of borrelial infection. Nevertheless, these conserved epitopes may prove useful for developing testing methods for serodiagnosis of Lyme disease. MAbs N6F4 and Q7H6 had low microagglutination activities, and neither of them displayed detectable antiborrelial activity. It appears that not all outer surface-exposed epitopes are capable of inducing antiborrelial antibodies. This difference may be related to the epitope constituents and their locations on the surface of the borrelial cell membrane (31).

The combination of antiborrelial OspA and OspB MAbs significantly enhanced antiborrelial activity against homologous strain B31 and heterologous strain CA-2-87 but did not expand the activity endpoint. This phenomenon apparently

reflected a cooperative action of OspA and OspB antibodies on spirochetes, resulting in an enhanced neutralizing activity. Thus, a Lyme disease vaccine containing both OspA and OspB may be more efficacious than a single-protein-based vaccine in prevention and control of Lyme disease spirochetal infection.

Epitope mapping, applying antiborrelial MAbs and overlapping synthetic peptides of OspA and OspB, has identified a number of immunogenic epitopes. B3G11 bound to an OspA peptide with the sequence of amino acids 247 to 256 (QYDSNGTKLE). N5G5 reacted with an OspB peptide with the sequence of amino acids 211 to 220 (TLKREIEKDG). Antiborrelial N4B12 weakly bound to an OspA peptide with the sequence of amino acids 208 to 217 (AATKKTAAWN) (28). Schubach et al. described an OspA MAb, 105.5, binding to a region centered around amino acids 214 to 217 (AAWN) (36). It is possible that both N4B12 and 105.5 are raised against the same epitope or overlapping epitopes. Therefore, amino acids 208 to 217 (AATKKTAAWN) and 247 to 256 (QYDSNGTKLE) on OspA and amino acids 211 to 220 (TLKREIEKDG) on OspB are identified as containing neu-

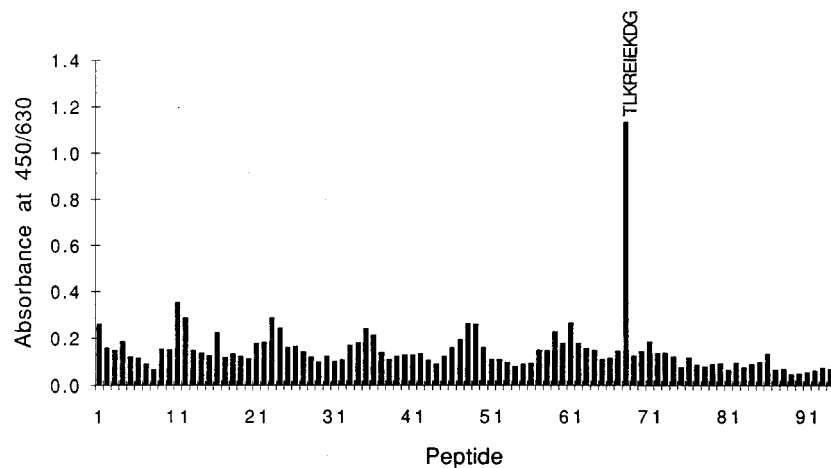


FIG. 6. Identification of an epitope on OspB by peptide scanning analysis. Epitope mapping was performed for reactivity of OspB MAb N5G5 with the pin-bound OspB peptides, using 300 ng of MAb per ml as described for Fig. 5. N5G5 bound to an OspB peptide with the sequence of amino acids 211 to 220 (TLKREIEKDG).

tralizing epitopes able to elicit functional antibody responses. Both OspA and OspB epitopes identified in the present study are apparently located at the variable regions of their respective proteins (31, 43). Epitope mapping with other MAbs has met with some difficulty because of multiple binding and weak reactivities (28). It is possible, however, that some MAbs recognize conformational epitopes.

Clinical observations have revealed indirect evidence for the possible association of Osp protein-induced immune responses and development of chronic Lyme disease (17, 39). OspA and OspB have been shown to stimulate the production of inflammatory cytokines by murine and human mononuclear cells (29, 40). Therefore, a safe and effective Lyme disease vaccine may require the use of synthetic peptides containing protective domains or an immunogen excluding the autoimmunity inducing epitope(s). Molecular mapping of neutralizing epitopes on immunogens of the Lyme disease spirochete will be of significance for Lyme disease vaccine development.

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