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A Monoclonal Antibody to OspA Inhibits Association of *Borrelia burgdorferi* with Human Endothelial Cells

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Previously, it has been shown that polyclonal antibodies to *Borrelia burgdorferi* and some monoclonal antibodies (MAbs) to borrelia major surface proteins caused inhibition of adherence of the bacteria to cultured human umbilical vein endothelial (HUVE) cells. In this study, fragment antigen binding (Fab) molecules generated from the immunoglobulin G fraction of rabbit anti-recombinant OspA serum were found to inhibit the adherence of *B. burgdorferi* to HUVE cells by 73%. Subsequently, MAbs were generated for use in determining whether or how *B. burgdorferi* outer surface proteins (Osps) A and/or B are involved in mediating attachment to, and/or invasion of, HUVE cells by *B. burgdorferi*. Twenty-two MAbs were generated to borrelial proteins with apparent molecular masses (determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) of 19, 31 (OspA), 34 (OspB), and 35 kDa. Fab molecules from one anti-OspA MAb, 9B3D, demonstrated an inhibitory effect on bacterial association with HUVE cells. None of the other MAbs, including the other anti-OspA MAbs, showed an inhibitory effect on cell association of greater than 5%. This effect of Fab 9B3D was concentration dependent and plateaued at approximately 6 μg of Fab per ml (nearly 80% inhibition of the bacterial association with the monolayer). Penetration assays and cell association experiments performed by using immunofluorescence also suggested that the inhibitory action of 9B3D occurs at the level of adherence. MAb 9B3D recognized the OspA of every North American strain tested (n = 19) but only 4 of 20 strains from western Europe, Russia, and Japan, suggesting that the North American strains and strains from other parts of the world may use different molecules and/or different OspA epitopes to interact with endothelial cells. Immunoblots of *Escherichia coli* expressing different OspA fusion peptides suggested that the 9B3D epitope resides in the carboxy-terminal half of OspA. MAb 9B3D promises to be a valuable tool for elucidating the domain or domains of OspA involved in the endothelial cell cytadherence of North American strains of *B. burgdorferi*.

Lyme borreliosis is a multisystem disorder which can affect the myocardium, central nervous system, and joints. The spirochetal agent of this infection, *Borrelia burgdorferi*, possesses an outer surface which contains relatively few species of major proteins. The genes for two abundant outer surface proteins, OspA and OspB, which migrate by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 31 and 34 kDa, respectively (5, 7), have been cloned and sequenced (10, 19, 20) and are encoded on a 49-kbp linear plasmid (2).

OspB has been correlated with virulence since Schwan et al. (28) demonstrated that an American strain passaged in culture ceased to express the 34-kDa OspB and also exhibited decreased infectivity for white-footed mice (*Peromyscus leucopus*) between passages 11 and 15. Among North American isolates of *B. burgdorferi*, OspB varies both in its migration on SDS-PAGE (5) and in its reactivity with monoclonal antibodies (MAbs) (5). Additionally, this protein has been shown to be polymorphic in a clonal population (11). The variability of OspB may contribute to the virulence of this organism by immune evasion, as occurs with *Borrelia hermsii* major surface proteins (6). However, a defined functional role for OspB in virulence has yet to be established.

OspA has been shown to be more conserved among North American strains than OspB (4). This feature suggests that OspA may be a good target for a vaccine. An immune response to OspA has been shown to be protective in two different animal models (15, 27). Passive immunization of severe combined immunodeficient (scid) mice with either MAbs or polyclonal antibodies to OspA prevented the development of a chronic infection (27), suggesting that an anti-OspA response is protective. Additionally, C3H/HeJ mice that had been actively immunized with a recombinant OspA were protected from a subsequent challenge with live organisms (15). Benach et al. have also reported that OspA may affect the chemotactic response of human neutrophils in vitro (9).

To date, no other biological functions of this or any other outer surface proteins of *B. burgdorferi* have been proposed or defined. Since these bacteria are not known to produce toxins, interaction and association of the bacteria with host cells is presumed to be important in the development of disease. Such bacterial-host cell interactions would also be, presumably, mediated by bacterial outer surface molecules.

Borrelias have been shown to adhere to cultured human umbilical vein endothelial (HUVE) cells along the lengths of the bacteria (32). Adherence to host cells can be inhibited by

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65% by pretreating the bacteria with immune serum and by 36 to 39% by pretreating them with either of two different MAbs to OspB (32). After attachment, the borrelias are able to enter the cells and translocate across the cytoplasm (13). This translocation process, as well as intercellular penetration, requires the viability of both the eucaryotic cell and the bacterium (14). Treatment of the bacteria with proteinase K (which preferentially cleaved OspA and OspB but did not affect bacterial viability or motility) decreased spirochetal adherence by 70% and decreased penetration of endothelial cells by 95% (14).

A major goal of this laboratory is to identify borrelial molecules involved in attachment to and penetration of cultured host cells. On the basis of the findings listed above, the present study was initiated to determine what portions of OspA and/or OspB are involved in mediating attachment to, and/or penetration of, endothelial cells by *B. burgdorferi*.

Because site-specific, in vitro mutagenesis procedures have yet to be developed for spirochetes, we chose to approach this aim by generating MAbs to OspA and OspB and testing these antibodies for the ability to inhibit bacterial association with endothelial cells. Previous studies using whole immunoglobulin molecules have described partial inhibition of cell attachment with various Osp MAbs (9, 32). We have observed that whole antibodies, which frequently cause aggregation of the bacteria, can yield unpredictable and unreliable results in quantitative cell association or invasion assays. To avoid this problem, we chose to prepare fragment antigen binding (Fab) molecules from MAbs by proteolytic cleavage and physical separation of digestion products. These fragments not only eliminated the aggregation problem but, presumably, also produced less steric hindrance.

We describe here in detail one MAb, reactive with OspA, which inhibited borrelia-HUVE cell association by 69%. The results demonstrated the involvement of OspA in borrelia host cell adherence. Furthermore, data from an experiment using polyclonal anti-OspA Fabs suggested that strains from other parts of the world may use different OspA epitopes to interact with endothelial cells. The demonstration of a proposed functional activity for OspA implies an important role for OspA in the virulence of *B. burgdorferi*.

TABLE 1. *B. burgdorferi* strains used

Strain	Passage	Geographic locale	Animal source[a]
HB19	Low	United States (Conn.)	Human blood
Lenz	High	United States (N.Y.)	Human skin
ECM-87	Low	United States (N.Y.)	Human skin
ECM-NY86	Low	United States (N.Y.)	Human skin
MAC 13	High	United States (N.Y.)	Human skin
26816	Low	United States (R.I.)	*Microtus* sp. (vole)
BB Mouse	Low	United States (Wis.)	*Peromyscus leucopus*
21343	Low	United States (Wis.)	*P. leucopus*
Veery	Low	United States (Conn.)	*Catharus* sp. (Veery)
B31	High	United States (N.Y.)	*Ixodes dammini*
SH-2-82	Low	United States (N.Y.)	*I. dammini*
NJ114	Low	United States (N.J.)	*I. dammini*
27985	Low	United States (Conn.)	*I. dammini*
N40	Low	United States (N.Y.)	*I. dammini*
DN127	Low	United States (Calif.)	*Ixodes pacificus*
CA-2-87	High	United States (Calif.)	*I. pacificus*
CA-8	Low	United States (Calif.)	*I. pacificus*
CA-25	Low	United States (Calif.)	*I. pacificus*
CA-27	Low	United States (Calif.)	*I. pacificus*
20004	Low	France	*Ixodes ricinus*
VS219	High	Switzerland	*I. ricinus*
VS461	Low	Switzerland	*I. ricinus*
HV-1	High	Austria	Human heart
LV-4 (K59)	High	Austria	Human CSF
G1	Low	Germany	Human CSF
G2	High	Germany	Human CSF
PKa1	Low	Germany	Human CSF
P/Gau	Low	Germany	Human skin
ACAI	High	Sweden	Human skin
ECMI	Low	Sweden	Human skin
FI	High	Sweden	*I. ricinus*
G25	Low	Sweden	*I. ricinus*
NBS16	Low	Sweden	*I. ricinus*
NBS23a	Low	Sweden	*I. ricinus*
NBS23b	Low	Sweden	*I. ricinus*
UOI	Low	Sweden	*I. ricinus*
Ip21	High	Russia	*Ixodes persulcatus*
Ip90	High	Russia	*I. persulcatus*
J-1	Low	Japan	*I. persulcatus*

[a] CSF, cerebrospinal fluid.

MATERIALS AND METHODS

Bacteria. The *B. burgdorferi* strains used are listed in Table 1. Borrelias were propagated at 34°C in BSK II (1) medium containing 6% normal rabbit serum and were harvested at the exponential phase of growth. Cloned populations of North American strains HB19, a human blood isolate (30), and N40, a tick isolate (8), were used.

Borrelial outer membrane-enriched material (OME) was prepared by resuspending washed bacteria at approximately 10^{10} bacteria per ml in phosphate-buffered saline (PBS), pH 7.2, and then adding *n*-octyl-β-D-glucopyranoside (Sigma Chemical Co., St. Louis, Mo.) to 0.8%. After incubation at 25°C for 15 min, the protoplasmic cylinders were pelleted at 10,000 × *g* for 5 min, and the supernatant was collected. These preparations contained relatively little 41-kDa flagellin protein as determined both by Coomassie blue staining of SDS-polyacrylamide gels and by Western blotting (immunoblotting) with H9724, an anti-flagellin MAb (3) (data not shown). Protein concentrations were estimated by using a protein assay kit (Bio-Rad, Richmond, Calif.) as described by the supplier.

Cultured endothelial cells. HUVE cells were regularly isolated from freshly delivered human umbilical cords by the method of Jaffe et al. (21). Cells were maintained in 5% CO_2 at 37°C in medium 199 (M199; GIBCO Laboratories, Grand Island, N.Y.) supplemented with 20% fetal calf serum (FCS; GIBCO), 100 μg of heparin (Sigma) per ml, and 50 ng of endothelial cell growth supplement (GIBCO) per ml. Experiments were performed with cells from passage 15 or lower. The endothelial character of the cultures was assured by demonstrating that cells (i) stained positively with fluorescein isothiocyanate-labeled rabbit antihuman factor VIII (von Willebrand factor), (ii) gave high transendothelial electrical resistance, and (iii) contained Weibel-Palade bodies and tight junctions when examined by transmission electron microscopy (33).

MAb production. BALB/c mice were immunized intraperitoneally with 50 μg of OME from *B. burgdorferi* HB19 in Freund's complete adjuvant (Sigma) on day 1 and with 50 μg of OME in Freund's incomplete adjuvant on day 14. On day 35, mice received 25 μg of OME intraperitoneally and 25 μg intravenously, each in PBS. Mice were euthanized on day 39, their spleens were removed, and the homogenized spleen cells were fused with NS-1 myeloma cells as described previously (17).

Ten to 14 days after the fusion, hybridomas were tested

for the production of MAbs against OME by an enzyme-linked immunosorbent assay (ELISA). Briefly, microtiter plates were coated overnight at room temperature (RT) with 2.5 μg of OME in 50 μl of carbonate buffer. Coated wells were washed with PBS containing 0.05% Tween 20 (PBST; Fisher Scientific, Pittsburgh, Pa.) and blocked with 100 μl of 3% nonfat milk in PBS (PBS-milk) for 20 min at RT. Twenty microliters of hybridoma supernatant was then added, and the plates were incubated for 1 h at 37°C and then washed with PBST. Wells were then incubated for 1 h at RT with 50 μl of horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G (IgG) (1:1,000 in PBST; Boehringer Mannheim Biochemicals, Indianapolis, Ind.). Finally, the plates were washed and incubated with 50 μl of substrate for 5 min at RT. The colorimetric substrate mixture consisted of 2.5 ml of 0.12% tetramethylbenzidine dihydrochloride (Sigma) in ethanol and 10 ml of 0.2 M Tris-citrate (pH 5.2) plus 12 μl of 30% hydrogen peroxide. Positive wells developed a blue color detected by visual inspection, and the corresponding antibody-positive hybridomas were cloned at least twice by limiting dilution (17).

Isotype and competition grouping of MAbs. The isotypes of MAbs were determined by ELISA with a commercial kit (Boehringer Mannheim; see Table 3). Antibodies from hybridoma supernatants were purified by using a protein A/G agarose affinity column (Pierce, Rockford, Ill.), dialyzed extensively against distilled water, and lyophilized. To segregate the MAbs into competition groups on the basis of epitopic recognition, 0.1-mg aliquots of purified MAbs were conjugated to ImmunoPure-activated peroxidase (Pierce) as described in the manufacturer's directions.

Competition experiments were performed by using an ELISA-based assay. Microtiter plates coated with OME were blocked with 100 μl of PBS-milk for 20 min at 37°C. After the blocking solution was removed, 50 μl of hybridoma supernatant was added and incubation was continued for 2 h at 37°C. A different HRP-conjugated MAb was then added to wells at a concentration predetermined to give a positive reaction when no primary antibody was present. Plates were reincubated at 37°C for 1 h and then washed and developed as described in the previous section. If no color developed, the primary and secondary MAbs were placed in the same competition group (CG; i.e., competition for the binding site had occurred; see Table 3). Conversely, if no color developed (i.e., competition for a single binding site had not occurred), the primary and secondary antibodies were placed in different CGs.

An additional competition experiment was performed to compare the epitopal specificities of MAbs 9B3D and CIII.78 (15). An octyl glucoside-solubilized extract of *B. burgdorferi* HB19 was spotted (10 μg) onto nitrocellulose membranes. These spot blots were blocked in PBS-milk and then preincubated with either MAb 9B3D Fabs, whole CIII.78 (IgG), or PBS. One of the blots preincubated with MAb 9B3D Fabs was washed with PBS and subsequently incubated with MAb CIII.78, while the remaining blots were treated with PBS. Finally, all blots were washed in PBS and then probed with a solution containing 1% HRP-conjugated protein A (Sigma). After the blots were washed in PBS, substrate solution (described in a previous section) was added to them for detection.

Ascites production. Ascitic fluid was generated in and recovered from BALB/c mice as described previously (17). The fluid was clarified by centrifugation at 3,000 × *g* for 30 min prior to antibody precipitation by ammonium sulfate at a final saturation of 50% for 6 h at 4°C. The precipitate was resuspended in PBS, and the suspension was dialyzed against 12 liters of PBS at 4°C.

Preparation of rabbit anti-OspA and anti-*Chlamydia trachomatis* IgG. Sera from a rabbit immunized with recombinant OspA (from *B. burgdorferi* B31) and from a control rabbit were prepared as previously described (24). An anti-*C. trachomatis* L1 serum was prepared in rabbits as described by Kaul and Wenman (22). The IgG fractions from these sera were purified on protein A affinity columns (Pierce) prior to production of polyclonal Fabs as described below.

Fab generation. Immunoglobulins purified from the ascitic fluid of the 22 MAbs, from the supernatant of MAb CIII.78 (15), from rabbit anti-recombinant OspA serum (24), from rabbit anti-*C. trachomatis* serum, and from control rabbit serum were cleaved to Fab molecules with papain, by using a method previously described (25). Digestion was continued at 37°C for 1 to 8 h, depending on the isotype of the McAb, or for 12 h for polyclonal anti-OspA immunoglobulins. Iodoacetamide was used to alkylate and inactivate the residual papain. The presence of Fab molecules was confirmed by SDS-PAGE (data not shown), and, subsequently, Fab molecules were separated from intact immunoglobulins and F_c molecules by affinity chromatography over protein A-Sepharose columns (Pierce) before being dialyzed extensively against PBS and quantitated for protein concentration.

SDS-PAGE and Western blots. Discontinuous SDS–10 or 12.5% PAGE was performed as described previously (14). Molecular weight standards were obtained from Pharmacia (Piscataway, N.J.). Proteins were electrotransferred (34) onto Immobilon or nitrocellulose matrix filters (Millipore Corp., Bedford, Mass.) for antibody binding studies. After blocking with PBS-milk, MAbs were incubated with the blotted proteins for 1 h in PBS-milk. After three washes with PBS, the blots were incubated for 1 h at RT with HRP-labeled goat anti-mouse IgG (Boehringer Mannheim) diluted 1:750 in PBS. The blots were washed three times in PBS before being developed by incubating for 15 min at RT in a substrate solution consisting of 2 ml of 0.006 M 4-chloro-1-naphthol (Sigma) in methanol, 10 ml of 0.01 M imidazole (Sigma) in PBS, and 10 μl of 30% hydrogen peroxide. In some experiments, HRP-labeled protein A (Sigma) was used in place of a secondary antibody (i.e., anti-IgG-HRP) to differentiate between Fab and/or whole antibody binding to immobilized antigens.

Agglutination assay. Ascitic fluid was added to bacteria in BSK II medium (10^8 bacteria per ml) to a final concentration of 5% ascitic fluid, and the mixture was incubated for 30 min at 34°C. The extent of clumping was assessed by dark-field microscopy. The clumping of 5 to 15% of the bacteria observed by dark-field microscopy was deemed significant and was graded 1+; 15 to 25% clumping was graded 2+; 25 to 35% clumping was graded 3+; and greater than 35% clumping was considered a 4+ reaction. The aggregation of bacteria by normal mouse serum was compared as a control and judged to be less than 5%, or a <1+ reaction.

Cell association assay. The ability of *B. burgdorferi* to adhere to HUVE cells was assessed as previously described (32). Briefly, borrelias were intrinsically radiolabeled in BSK II medium with [^{35}S]methionine (Expresslabel; New England Nuclear, Boston, Mass.; 10 μCi/ml), washed with PBS, and resuspended to a density of 10^8 bacteria per ml in M199 containing 15% FCS (M199-FCS). Aliquots (0.5 ml) were added to confluent HUVE cell monolayers grown in 24-well plates (Corning Glass Works, Corning, N.Y.). After

incubation for 3 h in 5% CO_2 at 37°C, the monolayers with associated organisms were washed with PBS at RT and solubilized with 0.5% SDS. Lysates were emulsified in scintillation cocktail (Universol ES; ICN Pharmaceuticals, Inc., Irvine, Calif.) and analyzed by liquid scintillation spectrophotometry.

In studies to test Fab inhibition of adherence, borrelias (10^8/ml) were preincubated at 34°C with various concentrations of Fab solutions for 30 min prior to addition to HUVE cell monolayers. Succeeding assay steps were as described above, with Fab fragments present for the duration of the attachment assay.

Penetration of HUVE cells. Penetration assays were performed essentially as described previously (13, 33). Briefly, 5×10^4 HUVE cells were seeded onto sterile polycarbonate filters (pore size, 3 μm; diameter, 13 mm; Nuclepore Corp., Pleasanton, Calif.) mounted on plastic chemotaxis chambers (PC-2; ADAPS Inc., Dedham, Mass.). The chambers were placed in 24-well plates containing 1.5 ml of M199-FCS per well and incubated in 5% CO_2 for 48 h, at which time the monolayers were confluent and possessed high transendothelial electrical resistance (33). Bacteria were resuspended in M199-FCS, and 0.2-ml samples containing 5×10^8 radiolabeled bacteria were added to the upper portions of the chambers. After incubation for various intervals at 37°C, aliquots from beneath the filters were removed and spirochetes were quantitated by dark-field microscopy and/or liquid scintillation spectrophotometry. For some experiments, Fab molecules were mixed with the bacteria in M199-FCS for 30 min at 34°C prior to addition to the endothelial monolayers, and these Fabs were present for the duration of the assay.

Fluorescence assays. The reactivity of each MAb with various *B. burgdorferi* strains was determined by immunofluorescence. Washed bacteria (10 μl at 2×10^8 bacteria per ml) were air dried at RT onto microscope slides. Ascitic fluid (30 μl) of one MAb chosen from each CG (diluted 1:20 in PBS-milk) was added to the dried bacteria, and the mixture was incubated at 37°C for 30 min in a humidified chamber. Unbound antibody was washed away with PBS, and a fluorescein isothiocyanate-labeled goat anti-mouse IgG (diluted 1:100 in PBS; Boehringer Mannheim) was added for an additional 30 min at 37°C. Fluorescence was assessed with a Leitz fluorescence microscope.

To qualitatively assess the number of bacteria associated with the monolayer after the cell association assay, a second fluorescence procedure similar to one described previously was used (12). After the cell association assay, monolayers grown on round coverslips were fixed with 3% paraformaldehyde for 20 min at RT. The remaining paraformaldehyde was quenched by incubation with 50 mM ammonium chloride for 10 min at RT. To observe attached bacteria, monolayers were first incubated with rabbit anti-OME serum (diluted 1:50 in PBS) for 30 min at 37°C and then washed with PBS at RT and subsequently incubated with rhodamine-labeled goat anti-rabbit IgG (1:100 in PBS, Boehringer Mannheim) for 30 min at 37°C. Surface-bound bacteria were therefore rhodamine labeled. After the monolayers were washed with PBS, the fluorescence of the attached bacteria and the differential interference contrast images of the monolayers were observed with an Olympus BH-2 microscope.

Mapping of MAb 9B3D and other OspA MAbs to cloned OspA fragments. The generation of cloned OspA fragments has been described previously (29). Briefly, OspA (*B. burgdorferi* N40) gene fragments were amplified by using the polymerase chain reaction with primers based on the OspA-N40 gene sequence (GenBank accession number M38375). Sixteen nanograms of pDC-197-12-OspA-N40 (a plasmid containing the OspA-N40 gene) was used as the template. Gene fragments were cloned into pMX (a modified pGEX-2T expression vector; Pharmacia) which expressed the recombinant antigen as a translational fusion protein with glutathione transferase. The following fragments (with the corresponding OspA amino acid sequences in parentheses) were used: F1 (1 to 36), F2 (1 to 76), F3 (1 to 143), F5 (1 to 219), F6 (1 to 273), F7 (66 to 273), F8 (133 to 273), F9 (183 to 273), F11 (216 to 273), F12 (243 to 273), F13 (66 to 143), F15 (90 to 167), F16 (133 to 240). *E. coli* containing pMX (expressing glutathione transferase) served as a negative control.

Lysates of OspA protein fragments expressed in *E. coli* were separated on SDS-12.5% polyacrylamide gels and electrotransferred to nitrocellulose (34). The filters were stained with Ponceau S (Sigma) and then blocked for 2 h with a mixture of 5% dried milk, 0.01% antifoam A (Sigma), and 0.02% sodium azide in PBS. The blocked filters were incubated with MAb 9B3D or with MAbs representative of the five OspA CGs (see Table 3) at a 1:100 dilution in the same solution. Filters were washed three times for 10 min each in PBS, PBS containing 0.05% Nonidet P-40, and PBS, respectively. Secondary probing with ^{125}I-labeled sheep anti-mouse IgG for 1 h was followed by three washes as described above. The filters were dried in air and exposed to X-ray film (Kodak XAR) for 1 day at 4°C.

TABLE 2. Inhibition of cell association of various *B. burgdorferi* strains with polyclonal anti-recombinant OspA Fabs[a]

Strain	% Associated		% Inhibition
	No OspA Fabs[b]	With OspA Fabs[c]	
HB19	11.3 ± 0.4	2.9 ± 0.3	74.3
VS219	7.2 ± 0.3	2.8 ± 0.4	61.1
Ip90	8.6 ± 0.6	3.4 ± 0.4	60.5
ACAI	8.2 ± 0.6	3.1 ± 0.3	62.2

[a] The experiment was conducted for 3 h at 37°C.
[b] Bacteria were incubated with HUVE cell monolayers in the absence of polyclonal OspA Fabs. Similar results were obtained when bacteria were preincubated with Fabs prepared from (i) normal rabbit serum or (ii) rabbit anti-*C. trachomatis* serum.
[c] Bacteria were preincubated at 34°C with 25 μg of polyclonal OspA Fabs per ml for 30 min prior to the assay. Fabs were also present during the HUVE cell association assay.

RESULTS

Inhibition of cell association with polyclonal OspA Fab molecules. Fab molecules were prepared from IgG purified from rabbit antiserum to recombinant OspA. When borrelias were preincubated with these polyclonal Fabs prior to the addition of the bacteria to HUVE cell monolayers, the adherence of the borrelias to the host cells was inhibited (Table 2). Cytadherence of *B. burgdorferi* HB19 was inhibited by 74% at a Fab concentration of 25 μg/ml. Greater than 60% inhibition of adherence to the HUVE cell monolayers was observed when three *B. burgdorferi* strains from Western Europe and Russia were incubated with the OspA Fabs (Table 2).

MAbs generated. Six fusions which yielded 22 MAbs were performed. These MAbs were generated to four different borrelial proteins with apparent molecular masses by SDS-PAGE of 19, 31 (OspA), 34 (OspB), and 35 kDa (Fig. 1). The

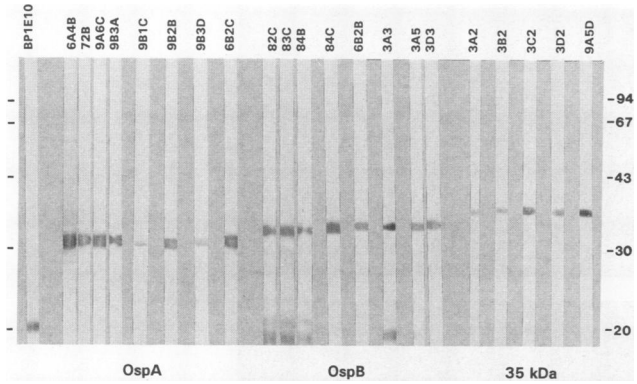

FIG. 1. Immunoblot analysis of *B. burgdorferi* HB19 reactive MAbs. SDS-12.5% PAGE gels were loaded with 40 μg of HB19 OME per lane. Blotted proteins were incubated with the indicated hybridoma supernatants diluted 1:25. Apparent molecular masses of protein standards (Pharmacia; in kilodaltons) are shown on the right.

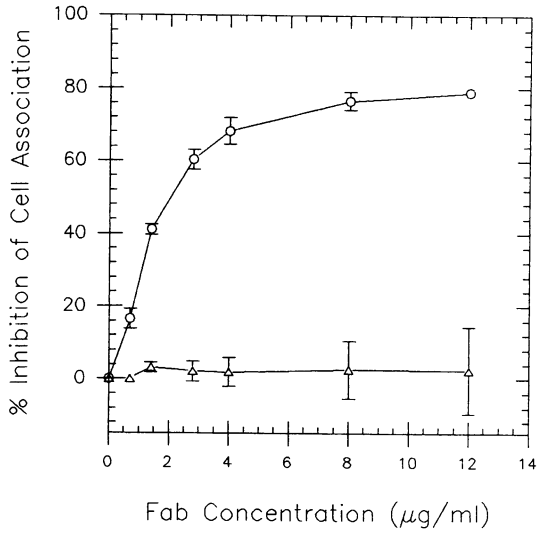

FIG. 2. Inhibition of HB19 association with HUVE cells by Fab 9B3D. Bacteria were mixed with the indicated concentrations of Fab 9B3D (○) or Fab 9B2B (△) for 30 min prior to and for the 3-h duration of the assay. Error bars represent standard deviations of three determinations for each concentration.

isotype and specificity of each MAb are shown in Table 3. Of the 22 MAbs generated, one recognized a 19-kDa protein, eight reacted with OspA, eight reacted with OspB, and five reacted with a 35-kDa protein. Both the 34-kDa band and the band that appears at ~20 kDa with MAbs 82C, 83C, 84B, and 3A3 were also recognized by MAb 4610, an antibody known to bind an epitope in the N-terminal region of OspB (26). Therefore, the two-band reactivity of these MAbs is, in fact, due to OspB specificity. MAb 9B3D, which was studied further, also bound to Western and dot immunoblotted lysates of *E. coli* containing plasmid pTRH44, which codes for the OspA protein of *B. burgdorferi* B31 (19).

The eight OspA antibodies were placed into five different CGs (Table 3). One OspA CG (designated OspA 1) predominated, with four of the eight MAbs belonging to this CG. The eight OspB MAbs also segregated into five OspB CGs (OspB 1-5), and each of the MAbs to the 35-kDa protein constituted separate CGs.

TABLE 3. Isotype and competition grouping of *B. burgdorferi* HB19 MAbs

MAb	Isotype	CG
BP1E10	IgG1	19 kDa
6A4B[a]	IgG2b	OspA 1
72B	IgG1	OspA 1
9A6C	IgG1	OspA 1
9B3A	IgG1	OspA 1
9B1C	IgG1	OspA 2
9B2B	IgG1	OspA 3
9B3D	IgG1	OspA 4
6B2C	IgG2b	OspA 5
82C	IgG1	OspB 1
83C[a]	IgG2b	OspB 1
84B	IgG1	OspB 1
84C	IgG2b	OspB 2
6B2B	IgG3	OspB 3
3A3	IgG1	OspB 4
3A5[a]	IgG1	OspB 5
3D3	IgG1	OspB 5
3A2	IgG1	35 kDa 1
3B2	IgG1	35 kDa 2
3C2	IgG2b	35 kDa 3
3D2	IgG1	35 kDa 4
9A5D	IgG2b	35 kDa 5

[a] Hybridoma selected from each CG to generate ascites fluid.

Surface reactivities. The ability of the MAbs to bind surface-exposed epitopes was determined by the ability of the MAbs to aggregate live HB19 cells. All MAbs aggregated the bacteria at a level of 4+ compared with normal mouse serum, except for BP1E10 (CG 19 kDa) and 3A2 (CG 35 kDa 1), which both gave a <1+ aggregation score, similar to that of control normal mouse serum.

Inhibition by Fabs of HB19 association with HUVE cells. Of the 16 CGs from which Fab molecules were made, only the OspA 4 CG (composed of one member, MAb 9B3D) demonstrated an inhibitory effect on bacterial association with HUVE cells. None of the other Fab molecules showed a change in cell association of greater than 5%. The inhibition by Fab 9B3D was concentration dependent (Fig. 2) and leveled off at approximately 6 μg of Fab per ml (nearly 80% inhibition of the bacterial association with the monolayer). At 4 μg of Fab per ml, Fab 9B3D inhibited cell association by 69%. Fab 9B3D did not affect bacterial motility or viability nor did 9B3D adversely affect HUVE cells, as determined by trypan blue exclusion. When any of the other Fab molecules was used individually or in conjunction with 9B3D, no further or additive change in the inhibition was observed. None of the Fab preparations at the concentrations used in the assays caused bacterial clumping.

Fab molecules of MAb CIII.78 (shown to provide passive protection in in vivo studies [15]) were subsequently found also to inhibit host cell interaction of *B. burgdorferi*. However, when tested at the same concentration (4 μg/ml) at which Fab 9B3D inhibited host association by about 70%, MAb CIII.78 resulted in only 21% inhibition. When a threefold increase in CIII.78 Fabs (12 μg/ml) was used in the host association assay, only 26% inhibition was obtained.

In a dot blot competition experiment performed to compare the epitopal specificity of MAbs 9B3D and CIII.78, some faint signal developed on a spot incubated sequentially with an excess of MAb 9B3D Fabs and then with whole CIII.78 IgG. After detection with protein A-HRP, some faint

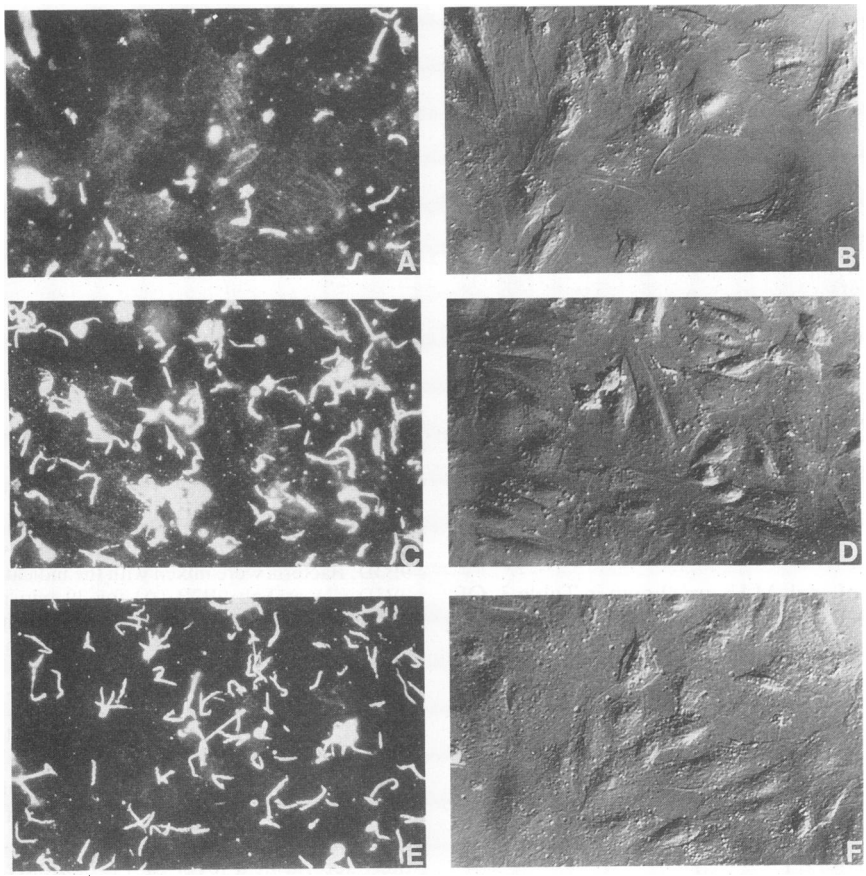

FIG. 3. Rhodamine-labeled borrelias attached to HUVE cell monolayers. (A) Attached bacteria after incubation with 4 μg of Fab 9B3D per ml; (B) differential interference contrast (DIC) image of the monolayer of the field shown in panel A; (C) attached bacteria with no Fab added; (D) DIC image of the monolayer shown in panel C; (E) attachment after incubation with 4 μg of Fab 9B2B per ml; (F) DIC image of the monolayer shown in panel E. Magnification, ×250.

signal developed in the Fab control spot, indicating that a small amount of intact IgG might have been present in this 9B3D Fab preparation. Because the color development on this 9B3D-CIII.78 competition spot was less intense than that of the CIII.78 control, the results indicated that MAb CIII.78 competed with MAb 9B3D for binding to immobilized OspA and may therefore bind the same or at least overlapping epitopes.

Strain reactivity of MAb 9B3D. MAb 9B3D reacted with the OspA molecules of all 19 North American strains tested but did not react with 16 of 20 strains from humans or ticks from Western Europe or Japan. The 9B3D-reactive exceptions were a human cerebrospinal fluid isolate from Germany (PKa1) and three *Ixodes ricinus* isolates, including two from Switzerland (VS219 and VS461) and one from France (20004) (data not shown).

Characterization of the inhibition of cell association by Fab 9B3D. Studies were then conducted to determine if Fab 9B3D inhibited the initial attachment of the bacteria, the invasion into cells, or both processes. In a 2-h qualitative cell association assay, the bacterial attachment to host cells (as assessed by immunofluorescence) was demonstrated to be greatly reduced when borrelias were preincubated with Fab 9B3D (Fig. 3A and B) than when borrelias were preincubated with no Fab (Fig. 3C and D) or with Fabs prepared from another anti-OspA MAb, Fab 9B2B (Fig. 3E and F). Fabs from MAb 9B2B (the same isotype as 9B3D and also reactive with OspA) did not inhibit cell association and served as a negative control in these experiments. A very diffuse background fluorescence was observed on HUVE cells, but this was easily distinguished from fluorescent bacteria.

To show more conclusively that Fab 9B3D was inhibiting the initial adherence process of the bacteria to the HUVE cell monolayer, cell association experiments were performed at both 37 and 4°C. At 4°C, invasion into cells and penetration of the monolayer are inhibited (13). At 37°C, 8.6% ± 0.5% of untreated bacteria associated with the monolayer. Borrelia-host cell association at 4°C was inhibited by 84% with Fab 9B3D pretreatment when compared with bacteria without Fab 9B3D pretreatment at the same temperature (Fig. 4), suggesting that Fab 9B3D blocked the adherence stage of the cell association process.

Inhibition of cell association of other *B. burgdorferi* strains with Fab 9B3D. Experiments were performed to examine whether Fab 9B3D could inhibit the ability of different strains to associate with HUVE cell monolayers. These results are presented in Table 4. The other 9B3D-reactive strain tested, VS219, was also inhibited in its ability to associate with the monolayer (56.5%) when preincubated with 4 μg of Fab 9B3D per ml. However, strains Ip90 and ACAI (neither of which reacted with 9B3D on Western blots) were able to associate with the monolayer to the same extent both in the presence and absence of Fab 9B3D (i.e.,

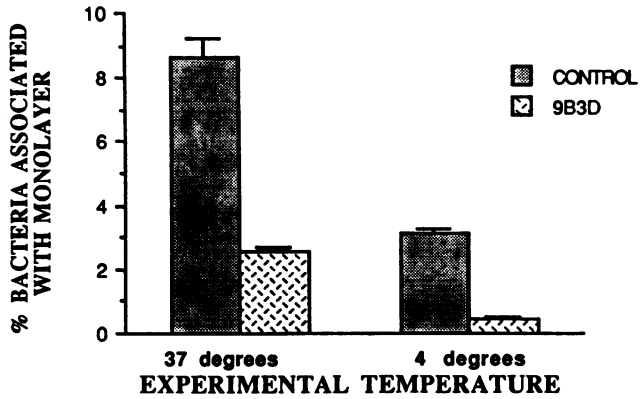

FIG. 4. Bacterial association with HUVE cells at 4 and 37°C with Fab 9B3D inhibition. Assays using 4 μg of Fab 9B3D per ml were conducted for 3 h. Bars represent averages from triplicate experiments and error bars indicate standard deviations.

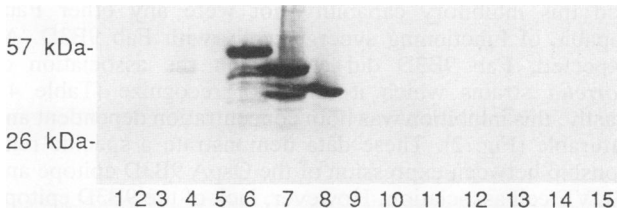

FIG. 5. Immunoblot analysis of *E. coli* expressing OspA fusion proteins probed with MAB 9B3D. Lanes 1 to 15 contain lysates of *E. coli* expressing the various OspA fragments. The fragment numbers for the lanes are indicated below, and the corresponding OspA amino acids are listed in parentheses. Lanes: 1, low-molecular-weight markers (not visualized); 2, F1 (1 to 36); 3, F2 (1 to 76); 4, F3 (1 to 143); 5, F5 (1 to 219); 6, F6 (1 to 273); 7, F7 (66 to 273); 8, F8 (133 to 273); 9, F9 (183 to 273); 10, F11 (216 to 273); 11, F12 (243 to 273); 12, F13 (66 to 143); 13, F15 (90 to 167); 14, F16 (133 to 240); 15, control (*E. coli* transformed with pMX, expressing glutathione transferase).

Fab 9B3D was noninhibitory for their HUVE cell adherence).

Mapping of MAb 9B3D to cloned OspA fragments. To determine the physical locations of the OspA epitopes recognized by MAb 9B3D and representative MAbs of the other four OspA CGs, these OspA MAbs were used to probe immunoblots of *E. coli* lysates expressing OspA fusion proteins. MAb 9B3D (CG OspA 4) recognized the previously described fragments 6, 7, and 8 (Fig. 5). These data indicate that MAb 9B3D binds to an epitope in the C-terminal half of OspA, as does MAb CIII.78, an antibody previously shown to have in vivo protective effects (15). MAbs 9B3A and 6A4B (CG OspA 1) also recognized epitopes in the C-terminal half of OspA. MAbs 9B1C (CG OspA 2) and 9B2B (CG OspA 3) bound epitopes within amino acids 90 to 167, and MAb 6B2C (CG OspA 5) recognized a region within amino acids 66 to 143 (i.e., the N-terminal half of the OspA sequence).

DISCUSSION

In previous reports, we have characterized the interactions of *B. burgdorferi* with cultured human endothelial cell monolayers (13, 14, 33). In the present study, we present a partial characterization of a set of 22 MAbs generated to molecules present in outer-membrane-enriched fractions of *B. burgdorferi* HB19. Sixteen of these MAbs recognized either OspA or OspB, while the six other MAbs recognized *B. burgdorferi* proteins of 19 or 35 kDa. Furthermore, we have demonstrated the ability of one anti-OspA MAb to inhibit the in vitro association of *B. burgdorferi* HB19 with human endothelial cells.

Several studies have reported on the adherence of *B. burgdorferi* to various types of host cells (9, 16, 18, 32) and on the penetration of host cell monolayers (13, 14, 23, 31, 32), but only a few have addressed the inhibition of cell association by antibodies directed against borrelias. Benach et al. (9) reported that two MAbs to OspA inhibited the adherence of *B. burgdorferi* B31 to HEp-2 epithelial cells in culture by 45%. Another report from the same laboratory stated that a sole OspA MAb tested did not inhibit the adherence of borrelias to cells of primary rat brain cultures (16). In addition, immune rat serum had no inhibitory effect on the adherence of the bacteria to these host cells, while normal rat serum effected a 27% inhibition of attachment. However, the anti-OspA status of the tested immune serum was neither described nor shown (16). Our own previous observations indicated that two OspB MAbs caused modest inhibition, while the single OspA MAb tested did not (32).

The differing results and ensuing confusion may be attributable, in whole or in part, to the use in these studies of whole immunoglobulin molecules. We now know that aggregation of the borrelias frequently leads to trapping of clumped organisms on the monolayers and, therefore, to unpredictable and spurious results. Indeed, the number of recovered counts per minute sometimes was greater than that of the non-antibody-treated controls when these clumps stuck to HUVE cell monolayers. The choice to use MAbs and Fabs prepared from these monospecific antibodies was made to more clearly show which molecules are important to the host cell interaction of the bacteria and to begin to describe important regions or epitopes of those proteins.

This study, along with previous data from this lab (14), implicates OspA as a borrelial surface molecule which is involved in attachment. Specifically, these results demonstrated that an epitope of OspA recognized by MAb 9B3D acts as an adhesin in *B. burgdorferi* strains which possess this epitope. These two hypotheses are supported by several findings. First, the Fab fragment of an MAb generated to OspA inhibited association of the bacteria with HUVE cells by 80%. Furthermore, no other Fab fragments from MAbs (OspA specific or otherwise) generated in this study exhib-

TABLE 4. Inhibition of cell association of various *B. burgdorferi* strains with Fab 9B3D[a]

Strain	% Associated		% Inhibition
	No Fab 9B3D[b]	With Fab 9B3D[c]	
HB19	9.2 ± 0.7	3.1 ± 0.3	66.3 ± 4.3
VS219	8.5 ± 0.7	3.7 ± 0.2	56.5 ± 2.8
Ip90	8.7 ± 0.4	9.1 ± 0.4	
ACAI	8.0 ± 0.5	7.8 ± 0.4	

[a] The experiment was conducted for 3 h at 37°C.

[b] Bacteria were incubated with HUVE monolayers in the absence of Fab 9B3D. Similar results were obtained when bacteria were preincubated with Fab molecules prepared from MAb 9B1C (cg OspA 2).

[c] Bacteria were preincubated at 34°C with 4 μg of Fab 9B3D per ml for 30 min prior to the assay. Fabs of 9B3D were also present during the assay at 37°C.

ited this inhibitory capability nor were any other Fabs capable of functioning synergistically with Fab 9B3D. As expected, Fab 9B3D did not inhibit the association of *Borrelia* strains which it does not recognize (Table 4). Lastly, this inhibition was both concentration dependent and saturable (Fig. 2). These data demonstrate a specific relationship between expression of the OspA 9B3D epitope and HUVE cell association. However, lack of the 9B3D epitope does not preclude the ability of a strain to associate with HUVE cells in an OspA-related manner (Tables 2 and 4).

We present the first results showing that anti-OspA polyclonal antibodies inhibit *B. burgdorferi* cytadherence. In retrospect, we propose that inhibition of cytadherence was not observed in earlier studies (9, 32) employing anti-OspA MAbs because the antibodies used were not directed against the appropriate domain.

The inhibition by Fab 9B3D was shown to occur at the level of attachment. Relatively few bacteria pretreated with 9B3D were observed attached to the surface of HUVE cell monolayers (Fig. 3). In addition, when the cell association assay was conducted with Fab 9B3D at 4°C (a temperature at which borrelial attachment, but no penetration, occurs), cell association was inhibited by 84% compared with the results when untreated bacteria were used (Fig. 4), demonstrating that Fab 9B3D inhibits the adherence event.

Because the data reported herein indicate that the inhibition of bacterial association with cells by Fab 9B3D plateaued at 80%, it is possible that another molecule(s) or other domains of OspA are also involved in mediating adherence. Additionally, it is possible that the 9B3D domain may be an adhesion domain among strains reactive with this antibody, yet other accessory molecules or domains may also participate in attachment. We propose that the proteins or epitopes which function in host cytadherence of *B. burgdorferi* are heterogeneous among *B. burgdorferi* isolates from different parts of the world. Also, we propose that OspA is involved in the cytadherence of these non-9B3D-expressing strains, since polyclonal anti-OspA Fabs inhibited HUVE cell association by approximately 60% (Table 2). However, demonstration of a putative function of the 9B3D epitope, and its degree of conservation among North American isolates, argues for the importance of an OspA function in the pathogenesis of Lyme borreliosis.

In this study, a function of OspA in mediating borrelial association with host cells has been described. This function also implies that OspA may be important as a virulence factor. Adhesin molecules can be good candidates for vaccines because the resultant antibodies produced are often able to prevent the initial establishment of infection by inhibiting adherence to host cells.

OspA has previously been shown to have potential as a subunit vaccine, since C3H/HeJ mice immunized with OspA were protected from infection by subsequent challenge with *B. burgdorferi* (15). In addition, scid mice and C3H/HeJ mice passively immunized with a specific MAb to OspA (CIII.78) were resistant to infection upon subsequent challenge with *B. burgdorferi* (15, 27). However, in both the C3H and scid mouse models, MAbs to OspA other than CIII.78 did not mitigate infection (15, 27). These data demonstrate specific protective capabilities of only certain OspA MAbs and support our findings that only MAbs directed against a particular domain(s) of OspA affect cytadherence.

Taken together, results from these in vivo studies and the data from this report suggest that an antibody or antibodies generated by the OspA vaccine and the passively protective anti-OspA MAb may have inhibited the initial association of the bacteria with the host cells and, therefore, prevented the establishment of infection. The prevention of adherence would influence the efficacy of a potential OspA vaccine in a positive fashion.

MAb 9B3D mapped to the same cloned fragments of OspA as did CIII.78, the MAb observed to be protective in animal studies (29). However, mapping with the available clones is sufficient only to locate MAbs 9B3D and CIII.78 to the C-terminal half of the OspA molecule. It is interesting that the inhibition observed when the same concentration of MAb CIII.78 was used (21%) was lower than that obtained when 9B3D was used (65%). It is possible that these two antibodies (i) may recognize the same epitope, (ii) may bind overlapping epitopes, or (iii) could possess different affinities for the same epitope. Additionally, while the cloned OspA fragment binding patterns of MAbs 9B3D, 6A4B, and 9B3A were similar on immunoblot (all bound the C-terminal one-half of the OspA protein; Fig. 5), these three MAbs bound to three different, and possibly distant, epitopes in the C terminus, as suggested by the competition grouping (Table 3), and is a potential explanation for why only 9B3D inhibited adherence.

Future studies should determine if MAb 9B3D, when passively transferred, will protect mice from subsequent challenge. The results from these studies may indicate that this portion of OspA is protective because it contains the adhesin domain. Finally, MAb 9B3D will be a valuable tool for elucidating the exact region of OspA which is involved in adherence.

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