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Borrelia burgdorferi Mutant Lacking Osp: Biological and Immunological Characterization

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All *Borrelia burgdorferi* sensu lato isolates characterized to date have one or a combination of several major outer surface proteins (Osps). Mutants of *B. burgdorferi* lacking Osps were selected with polyclonal or monoclonal antibodies at a frequency of 10^{-6} to 10^{-5}. One mutant that lacked OspA, -B, -C, and -D was further characterized. It was distinguished from the $OspA^{+}B^{+}$ cells by its (i) autoaggregation and slower growth rate, (ii) decreased plating efficiency on solid medium, (iii) serum and complement sensitivity, and (iv) diminished capacity to adhere to human umbilical vein endothelial cells. The Osp-less mutant was unable to evoke a detectable immune response after intradermal live cell immunization even though mutant survived in mouse skin for the same duration as wild-type cells. Polyclonal mouse serum raised against Osp-less cells inhibited growth of the mutant but not of wild-type cells, an indication that other antigens are present on the surface of the Osp-less mutant. Two types of monoclonal antibodies (MAbs) with growth-inhibiting properties for mutant cells were identified. The first type bound to a 13-kDa surface protein of *B. burgdorferi* sensu stricto and of *B. afzelii*. The MIC of the Fab fragment of one MAb of this type was 0.2 μg/ml. The second type of MAb to the Osp-less mutant did not bind to *B. burgdorferi* components by Western blotting (immunoblotting) but did not bind to unfixed, viable cells in immunofluorescence and growth inhibition assays. These studies revealed possible functions Osp proteins in borrelias, specifically serum resistance, and indicated that in the absence of Osp proteins, other antigens are expressed or become accessible at the cell surface.

Lyme disease is a complex, multisystemic illness caused by at least three genomic species of the spirochete *Borrelia burgdorferi* sensu lato (reviewed in reference 6). Virtually all North American isolates have been classified as *B. burgdorferi* sensu stricto (1, 17, 58). European and Asian isolates also include at least two other genomic species, *B. garinii* and *B. afzelii* (1, 23). The clinical features and epidemiology of Lyme disease have been well characterized (reviewed in reference 6). Comparatively less, however, is known about the pathogenic features of Lyme disease borrelias and immunopathological responses of the host. Ignorance of precise mechanisms of Lyme disease pathogenesis is partly attributable to the paucity of basic information about all spirochetes, which are unique in several aspects (31).

One of the known features of spirochetes of the genus *Borrelia* is an abundance of one or more lipoproteins in the outer cell membrane (4, 16, 19, 33, 35, 37, 42, 43, 59). The lipoproteins OspA and OspB of *B. burgdorferi* are major contributors to antigenic distinctness of Lyme disease borrelias (6). Many European and some North American *B. burgdorferi* sensu lato strains express a third immunodominant major protein, OspC (59). Another protein of this group, OspD, has been also reported (43). Proteins designated OspE and OspF have been described, but their surface exposure in live cells has not yet been established (39).

OspA and OspB may contribute to the spirochete's ability to adhere to or invade host cells (15, 25, 49). It has been suggested that OspA may affect the chemotactic response of human neutrophils in vitro (15). Mitogenic and cytokine-stimulatory properties of OspA and OspB have been also shown (41). In a previous study, we found that reduced size and amounts of OspB were associated with lowered infectivity (49). The findings of Cadavid et al. indicated that differences in invasive properties and tissues tropism between serotypes of the related spirochete *B. turicatae*, a relapsing fever agent, may be determined by the expression of a single surface protein that is analogous to Osp proteins of *B. burgdorferi* (22).

Studies of Osp protein function are still limited in number, though. One approach to obtain further insight into Osp function and contributions to pathogenesis is to select and then characterize mutants with altered surface lipoproteins. One such mutant lacks OspA, -B, -C, and -D (51, 54). In the present study, we further characterized this Osp-less mutant. We examined whether borrelias lacking these Osp proteins would be altered in such functional properties as generation time, growth on solid medium, association with mammalian cells, serum sensitivity, potential to evoke an immune response, and survival in the skin. Another aspect was the immunological characterization of the Osp-less mutant. A question was: In the absence of Osp proteins, against what surface-exposed antigens are antibodies directed?

MATERIALS AND METHODS

Strains and culture conditions. *B. burgdorferi* sensu stricto mutants were of the B31 (ATCC 35210) lineage (Table 1). The Osp phenotypes and plasmid contents of clonal, high-passage, noninfectious derivatives B311, B312, B313, and B314 were described previously under these or other designations (3, 7, 30, 54). Low-passage isolates had not been passaged in medium more than 10 times. The low-passage, infectious progenitor for this lineage retained the original strain designation, B31 (21). In some experiments, we also used *B. burgdorferi* sensu strictro HB19 (12) and Sh.2 (49), *B. afzelii* ACAI (17), and *B. garinii* IP90 (1) (Table 1). *B. hermsii* HS1 serotype 33 (ATCC 35209 [11]) is abbreviated Bh33. Borrelias were grown in BSK II medium and harvested by methods described previously (3, 5). For culturing of tissues from animals, rifampin (50 μg/ml), phosphomycin (100 μg/ml), and, for skin samples, amphotericin (25 μg/ml) were added to the medium. Spirochetes were counted in a Petroff-Hauser chamber by phase-contrast microscopy. In some experiments, borrelias were also grown on solid BSK II medium as described previously (30, 51). To estimate growth rate,

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TABLE 1. Isolates of *B. burgdorferi* sensu lato used in the study and their Osp profiles

Genomic species	Isolate	Osp profile[a]				Reference(s)
		OspA	OspB	OspC	OspD	
B. burgdorferi	B31	+	+	−	+	21, 54
	B311	+	+	−	−	3, 7, 54
	B312	+	+	+	−	30, 54
	B313	−	−	−	−	51, 54
	B314	−	−	+	−	54
	HB19	+	+	+	+	12
	Sh.2	+	+	+	−	49
B. afzelii	ACAI	+	+	+	−	17
B. garinii	IP90	+	+	+	−	1, 17

[a] Determined by Western blot analysis.

borrelias at an initial concentration of 2×10^6 cells per ml were grown in tightly capped polystyrene culture tubes (13 by 100 mm; Falcon Labware, Lincoln Park, N.J.) containing 6 ml of medium. Growth at 34°C in a 1% CO_2 atmosphere was monitored visually and by cell counts every 12 h for 3 days. The amount of total cellular protein in the final cell pellet was determined with the Bradford reagent (Bio-Rad Laboratories, Richmond, Calif.) (12).

ELISA. The enzyme-linked immunosorbent assay (ELISA) was performed essentially as described previously (52). For a "dry" ELISA, B311 cells at a total protein concentration of 1.4 μg/ml in phosphate-buffered saline (PBS) were dried onto polystyrene 96-well microtiter plates at 37°C for 18 h. For a "wet" ELISA, B311 cells at a total protein concentration of 3 μg/ml in 15 mM Na_2CO_3–35 mM $NaHCO_3$ buffer (pH 9.6) were coated onto plates at 4°C for 24 h. Adherence of *B. burgdorferi* cells to the wells in the wet ELISA was confirmed with anti-*B. burgdorferi* monoclonal antibody (MAb) H5332 (12). Absorbance values were recorded at 490 nm on a model 580 ELISA reader (Dynatech Laboratories, Chantilly, Va.); wells with values of ≥0.2 were considered positive.

Immunofluorescence assays. Indirect immunofluorescence assay (IFA) of fixed, dried cells was performed as described previously (11, 12). Harvested, fresh borrelias were washed with RPMI 1640 medium and mixed with a suspension of washed rat erythrocytes in 50% RPMI 1640–50% fetal calf serum, and a thin smear of the suspension was coated on the slides. Slides were fixed in methanol, air dried, and kept in a desiccator at −20°C until use.

Binding of MAbs to unfixed live spirochetes was assessed by a modification of the procedure of Barbour et al. (12). Borrelias (10^7) were washed with 2% (wt/vol) bovine serum albumin (BSA) in PBS with 5 mM $MgCl_2$ (PBS-Mg-BSA) and then resuspended in 0.5 ml of undiluted hybridoma culture supernatant or 0.5 ml of PBS-Mg-BSA containing the MAb of interest. The cell mixture was incubated at room temperature with gentle rotation for 60 min. The cells were centrifuged, washed twice with PBS-Mg-BSA, resuspended in 30 μl of PBS-Mg-BSA with 20 μg of anti-mouse immunoglobulin (Ig)-fluorescein $F(ab')_2$ fragment (Boehringer Mannheim, Indianapolis, Ind.) per ml, and incubated for 30 min under the same conditions. Before microscopic evaluation, the volume of the cell suspension was adjusted to 300 μl with PBS-Mg-BSA.

For the direct immunofluorescence assay (DFA), purified MAbs and their Fab fragments were conjugated with fluorescein isothiocyanate (QuickTag FITC conjugation kit; Boehringer Mannheim). Fractions containing the antibody-fluorescein conjugate were mixed together, dialyzed in the dark against PBS for 24 h, and concentrated with an Amicon Centriprep-10 (Beverly, Mass.). Borrelias (10^7) in log-phase growth were resuspended in RPMI 1640 medium with 10 to 100 μg of antibody-fluorescein conjugate per ml and examined for fluorescence at 3, 15, 30, 60, and 360 min.

IA and serum sensitivity assay. The growth inhibition assay (GIA) was described previously (53). Briefly, to a 100-μl volume of BSK II containing 2×10^6 borrelias was added an equal volume of heat-inactivated (56°C for 30 min) MAb or polyclonal antiserum serially diluted twofold in BSK II. To evaluate the susceptibility of borrelias to fresh, nonimmune serum, we applied the same growth inhibition technique using pooled unheated serum from C3H/HeN mice (Taconic, Germantown, N.Y.). Blood was drawn on ice, separated from the erythrocyte clot, and immediately frozen at −135°C. Heat-inactivated serum from the same mice served as a control. To determine the susceptibility of borrelias to complement, unheated or heated (56°C for 30 min) guinea pig complement (Diamedix, Miami, Fla.) was added to each well at an activity ranging from 1 to 6 hemolytic units (HU; 50% hemolytic complement units) per well. In some experiments, 2 HU of unheated guinea pig complement was added to each well for a final concentration of 10 HU/ml of medium after addition of antibody. Growth in flat-bottom, 96-well polystyrene microtiter plates was monitored visually for changes in the color of the phenol red indicator and by phase-contrast microscopy of wet mounts. The MIC was the lowest concentration of MAb that produced pink instead of yellow wells and represented at least 20-fold fewer cells (53). All growth inhibition studies were performed at least twice.

PAGE and Western blotting (immunoblotting). Whole-cell lysates were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) as described previously (2, 11). In some experiments, cleavage of surface-exposed proteins of intact borrelias with proteinase K (Boehringer Mannheim) was carried out (51). Western blot analysis was essentially as described before (44). The dilutions of ascitic and hybridoma supernatant fluids were 1:100 and 1:10 in milk-TS, respectively.

Antisera and MAbs. The origins of the OspA-specific MAb H5332 (12), OspB-specific MAb H6831 (10), and Vmp33-specific MAb H4825 (10) have been given. MAb H9724 binds to native and denatured flagellins of different *Borrelia* species (9). These antibodies are IgG2a.

Additional polyclonal and monoclonal antibodies were produced for this study. Female 6- to 8-week-old BALB/c mice (Jackson Laboratory, Bar Harbor, Maine) were used. Freshly harvested borrelias were washed with and resuspended in PBS (pH 7.0). The total cellular protein in the suspension was estimated with the Bradford reagent and adjusted with PBS for a total protein concentration of 200 μg/ml. One-half milliliter of antigen suspension was emulsified in 0.5 ml of complete Freund's adjuvant, and 200 μl of emulsion (20 μg of total protein) was administered as six subcutaneous injections on day 0. Control mice received a 200-μl emulsion of equal parts of complete Freund's adjuvant and PBS alone. After 4 weeks, mice were boosted with the same dose. Mice were bled by eye sinus puncture 10 days after the boost. After collection, these polyclonal sera were evaluated by ELISA and GIA. On day 52, the mice received intravenously 2×10^8 viable borrelias in 100 μl of PBS. Fusion of mouse splenocytes with NS1 myeloma cells was performed on day 56 by a modification of the method of Oi and Herzenberg (44). Undiluted hybridoma supernatant fluids without antibiotics were screened by wet ELISA, unfixed-cell IFA, and Western blotting. Those fluids that were positive by one of the three assays were then evaluated by GIA. For GIA, hybridoma supernatant fluids were dialyzed against PBS (pH 7.0) and concentrated with Centriprep-10 (Amicon) cartridges. The isotypes of antibodies were determined by using a commercial kit (Immunotype; Sigma). Ascitic fluids from hybridomas, purified MAbs, and univalent Fab fragments were produced as described previously (51, 53). Reactivities of purified MAbs and Fab fragments were confirmed by DFA and IFA, Western blotting, and GIA.

Cell association assay. An assay for association of intrinsically labeled borrelias to human umbilical vein endothelium (HUVE) cells was carried out essentially as described previously (49). Briefly, spirochetes were intrinsically radiolabeled with [^{35}S]methionine, washed with PBS, and resuspended to a density of 1.7×10^8 per ml of medium 199 with 20% fetal calf serum. Aliquots (300 μl) of radiolabeled spirochetes were added to confluent HUVE cell monolayers grown in 24-well plates. After a 4-h incubation at 4°C, monolayers with associated spirochetes were washed, solubilized, and mixed with Universol ES scintillation cocktail (ICN Pharmaceuticals, Irvine, Calif.), and the radioactivity was measured. The assay was done with triplicate samples and performed twice. Differences in numbers of cell-associated spirochetes were analyzed by Student's *t* test.

Experiments in mice. Six- to eight-week-old female C3H/HeN mice (Taconic) were used. Spirochete cells were counted and diluted in BSK. For intradermal infections with live cells (48), 100 μl of cells in BSK II medium was mixed with 900 μl of sterile PBS. A 100-μl volume of this suspension or 0.1× BSK II in PBS, as a control, was inoculated intradermally in shaved skin. Some mice were sacrificed at different times after injection, and full-depth skin clippings from the injection sites were cultured. A second group of mice were bled from the tail vein on day 24 after inoculation, and their sera were examined by ELISA and GIA. The second group of mice were challenged on day 28 at the base of the tail with 10^4 cells of strain Sh.2 and euthanized on day 42, and plasma, the whole bladder, macerated heart, and cross-cuttings of both tibiotarsal joints were cultured as described previously (51).

RESULTS

Growth rate, culture yield, and plating efficiency. Isolate B313 lacked OspA, -B, -C, and -D (Table 1). By preliminary characterization, Osp-less mutant B313 was distinguishable from B311 in culture by the microscopic and macroscopic autoaggregation of B313 cells and the delayed color change of the phenol red indicator of B313 cultures. For this study, the growth rates of cultures of B311 and B313 were determined. Cell counts in triplicate were determined every 12 h, and the $\log_{10}$ of means of cell counts were plotted against time. The mean generation times (± standard error of the mean) was 6.6 ± 0.1 h for B311 and 9.5 ± 0.2 h, 50% slower, for B313. Stationary-phase cultures had densities in cells per milliliter of 2.0×10^8 for B311 and 0.5×10^8, fourfold lower, for B313. Total cell protein levels of the cell pellets were 0.65 mg for B311 and 0.16 mg for B313. The Osp-less cells grew more

TABLE 2. Association of B311 and B313 spirochetes with HUVE cells

Expt[a]	Cell population	Cell association[b]	
		Mean cpm adhered ± SEM[c]	Mean % of inoculum cell associated
I	B311	14,719 ± 134	5.0
	B313	7,360 ± 36	2.5
II	B311	13,447 ± 92	5.7
	B313	6,801 ± 83	2.9

[a] The specific activities of inocula for association assays in experiments I and II were 2.9×10^5 and 2.3×10^5 cpm, respectively.
[b] Measured following incubation for 4 h at 4°C.
[c] Radioactivity bound to host cells following incubation and washing, expressed as the mean of three samples.
[d] $P < 0.001$, Student's t test.

slowly and achieved a lower final cell mass than did their Osp-bearing counterparts.

Another biological characteristic is growth as colonies on solid medium (30). Wild-type *B. burgdorferi* strains B31 and Sh.2 have efficiencies of plating on solid medium of between 50 and 100% (30, 49, 51). The experiment was performed twice, each time plating in triplicate 10^1 to 10^6 cells per plate. B311 cells had an expected plating efficiency of 50%. The efficiency of plating under the same conditions of the B313 population was 0.01%, 2,000-fold lower. Three arbitrarily colonies of B313 that grew on the solid medium retained the Osp-less phenotype by PAGE (data not shown).

Adherence to endothelial cells. Adherence of radiolabeled *B. burgdorferi* B311 and B313 cells to HUVE cell monolayers was measured after 4 h at 4°C. At this temperature, borrelias do not detectably enter endothelial cells and adherence of cells becomes maximal by 4 h (26). Results of the two experiments are given in Table 2. The ability of Osp-less cells to adhere to the HUVE monolayer both times was half that of wild-type borrelias ($P < 0.001$).

Serum and complement sensitivity. Wild-type *B. burgdorferi* is resistant to the bactericidal activity of nonimmune serum, in spite of classical and alternative complement pathway activation (38). To assess whether Osp proteins contributed to serum resistance, we exposed B311 and B313 cells to twofold serially diluted fresh, naive mouse serum in a GIA. Heat-inactivated serum was applied in parallel. As expected, B311 cells were resistant to the nonimmune serum; no growth-inhibitory effect on the cells was observed at the lowest serum dilution of 1:8. In contrast, the minimum inhibitory titer of nonimmune serum against Osp-less borrelias was 1:64. Heat-inactivated serum at a 1:8 dilution did not inhibit the growth either B311 or B313 cells.

To determine whether the serum susceptibility of the Osp-less mutant was wholly or in part attributable to complement susceptibility, we exposed B313 and B311 cells to doses of 1 to 6 HU of guinea pig complement in the GIA. As a control, the same complement doses were heat inactivated. The study was performed twice. Whereas heat-inactivated complement had no growth-inhibitory effect on either isolate at doses of 6 HU or less per well, B313 and B311 differed in their growth in the presence of unheated complement. As little as 1 HU of complement inhibited growth of B313 in both experiments. In contrast, B311 grew without detectable inhibition in the presence of 6 HU.

We also estimated the frequency of B313 cells surviving in the presence of complement. Because of B313's poor growth on solid medium, the experiment was performed in 96-well microtiter plates (51). B311 or B313 cells (5×10^6) were exposed to 3 HU of guinea pig complement per tube for 6 h. After this time, cell suspensions were diluted to a complement concentration of less than 1 HU per tube and aliquoted in 200-μl volumes to individual microtiter plate wells at estimated inocula ranging between 10^0 and 10^5 cells per well. Cells that were exposed to heat-inactivated complement or no complement at all served as controls. The frequency in the B313 population of cells that could grow in the presence of complement was 3×10^{-5} to 6×10^{-5} by the Poisson distribution (51).

Survival in the skin. The preceding experiment suggested that Osp proteins have a role in providing serum resistance to these blood-borne pathogens. The first step in invasion, though, occurs in the host's skin (6). Accordingly, we next evaluated whether Osp proteins also might protect borrelias from nonspecific resistance factors in the skin of the mouse (34). We determined how long 10^7 B311 or B313 cells would survive in the skin after intradermal inoculation. Mice were sacrificed at 0.25, 0.5, 2, 6, 9, 12, 18, and 24 h following inoculation; at each time point, 8 to 12 separate skin biopsies were cultured. All cultures up to 9 h were positive with both B311 and B313. In 12 h, four of eight and five of eight skin cultures were positive with B311 and B313 cells, respectively. Cultures at 18 and 24 h after inoculation did not have growth of spirochetes. Six randomly chosen cultures each of B311 and B313 from the 12-h time points were subjected to PAGE; the protein profiles were unchanged (data not shown).

Although the Osp-less mutant cells survived in the skin for approximately as long as wild-type cells, inoculation with 10^6 live cells did not elicit a detectable immune response by the criteria of ELISA, GIA, and infectious challenge with strain Sh.2 (48, 49). Live-cell immunization of five mice with B311 yielded the following results: reciprocal ELISA titers of 128 to 256, reciprocal GIA titers of 128 to 1,024, and protection of all mice against challenge with strain Sh.2. In contrast, for the live-cell immunization with B313 cells, the reciprocal ELISA and GIA titers of five mice were 2 to 8, which were indistinguishable from value for five mice immunized with 0.1× BSK-PBS alone. None of the five mice immunized with B313 cells or the five control mice were protected against challenge with Sh.2.

Polyclonal antisera to Osp-less cells. A possible explanation for the lack of a detectable antibody response to B311 in mice immunized with live B313 cells was an anti-B313 immune response that was directed against antigens not presented by or accessible in B311 cells. There are reports of other surface proteinaceous antigens besides Osps (19, 36, 40, 55, 56). It was possible that the immune response in B313-immunized mice was against one or more of these antigens.

Mice were immunized with B311 or B313 whole cells with adjuvant and boosted once. The results are presented in Table 3. By dry ELISA, reciprocal titers for a homologous reaction were as high as 32,768. When heterologous sera were evaluated, the reciprocal titers were up to 16,384 for anti-B311 serum against B313 cells and up to 4,096 for anti-B313 serum against B311 cells. Sera from mice immunized with complete Freund's adjuvant alone were negative at a dilution of 1:2 by ELISA.

Pooled antisera were also evaluated by GIA for functional activity; the experiment was performed twice with the same results (Table 3). To prevent complement effects on the more susceptible Osp-less cells, the serum was heat-inactivated. There was no growth inhibition of either B311 or B313 cells by sera of mice immunized with adjuvant and PBS alone. The reciprocal growth-inhibitory titer of anti-B311 against B311

TABLE 3. Analysis of polyclonal mouse antisera to B311 and B313 cells by ELISA and GIA[a]

Polyclonal serum	Mouse no.	ELISA[b]		IA[c]	
		B311	B313	B311	B313
Anti-B311	1	16,384	16,384	8,192	32
	2	16,384	16,384		
	3	32,768	16,384		
	4	32,768	16,384		
Anti-B313	1	4,096	16,384	<8	4,096
	2	4,096	16,384		
	3	4,096	32,768		
	4	2,048	32,768		
Control[d]	1	<4	<4	<8	<8
	2	<4	<4		

[a] Mice were immunized with B311 and B313 whole-cell emulsion in adjuvant and were boosted once with the same immunogen.
[b] Reciprocal ELISA titers from individual mouse sera.
[c] Reciprocal growth-inhibitory titers of heat-inactivated pooled mouse sera; the experiment was repeated twice with the same results.
[d] Control mice were immunized with adjuvant emulsion in PBS.

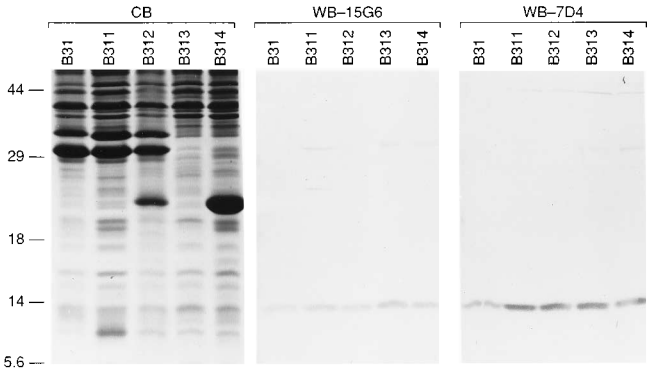

FIG. 1. Coomassie blue-stained polyacrylamide gel (CB) and Western blot analysis (WB) of whole-cell lysates of *B. burgdorferi* isolates B31, B311, B312, B313, and B314 with either MAb 15G6 or MAb 7D4. The acrylamide concentration was 17%. Molecular size standards in kilodaltons (shown to the left) were ovalbumin (44 kDa), carbonic anhydrase (29 kDa), β-lactoglobulin (18 kDa), lysozyme (14 kDa), and bovine trypsin inhibitor (5.6 kDa).

was high at 8,192. Anti-B313 serum did not detectably effect the growth B311 cells at any of the dilutions examined, and Osp-less mutant cells were inhibited by anti-B311 polyclonal serum only at a dilution of 1:32. The latter result, while indicating the specificity of the response, nevertheless suggested that growth-inhibitory antibodies to non-Osp components were produced. This was more evident in the anti-B313 versus B313 cell reaction, in which a reciprocal growth-inhibitory titer of 4,096 was found.

MAbs to the Osp-less mutant. To further characterize the surface antigens of the Osp-less mutant, MAbs to B313 were produced. Procedures used for production and screening of hybridoma supernatant fluids were designed to select for and identify those MAbs that were directed against surface proteins. To screen the hybridoma supernatants, we first used an ELISA in which whole borrelias remained wet in the wells and then used the unfixed-cell immunofluorescence assay. MAbs identified by these assays were further characterized by Western blotting.

Two types of MAbs were distinguished by Western blotting. One class of MAbs did not bind to any protein in the blots, a result suggesting that these MAbs were directed against conformational epitopes or nonproteinaceous antigens. The Western blot reactions of two MAbs, 15G6 and 7D4, of the other type are shown (Fig. 1); both MAbs were IgG2b. The antigens in this assay were B31 lineage isolates with different Osp protein phenotypes (Table 1). Both MAbs bound to a protein with an apparent size of 13 kDa present in each isolate and in approximately the same amounts. An identically sized protein bound by 15G6 and 7D4 was also present in HB19 and Sh2 strains of *B. burgdorferi* sensu stricto (data not shown). This protein was designated p13. Both MAbs produced minor bands with proteins with apparent sizes of 26, 32, and 44 kDa.

In a second Western blot study, we determined whether antibodies 15G6 and 7D4 recognized similar or identical proteins in other genomic species of Lyme disease borrelias. The results with 15G6 are shown in Fig. 2A; the same results were obtained with 7D4. Representatives of *B. afzelii* and *B. garinii* were evaluated at the same time as B311, B313, and *B. hermsii* cells by Western blot analysis. The MAb recognized a protein of slightly higher apparent molecular weight in *B. afzelii* ACAI. Neither 15G6 nor 7D4 recognized any protein in *B. garinii* IP90 or *B. hermsii*.

Figure 2 also shows the effect of proteinase K treatment on whole cells of B313 (20). After proteinase K digestion of wild-type and Osp-less mutant cells, no band was observed by Western blotting with either anti-p13 MAb, an indication that p13 is surface exposed. The results with MAb 15G6 and B313 cells are shown in Fig. 4B.

Immunofluorescence studies of p13. To further assess the topography of p13 in the cell, and in particular to determine if p13 is exposed over B313's entire surface, we used fixed and unfixed cells in IFA and DFA. In this series of experiments, we used purified MAb 15G6; for unfixed-cell DFA, purified MAb 15G6 was conjugated with fluorescein.

In the fixed-cell IFA B311 and B313 cells were individually mixed with a suspension of washed rat erythrocytes, coated as a thin smear over the the slides, and fixed with methanol. No fluorescein-labeled spirochetes were seen with either wild-type or mutant cells when cells were exposed to MAb 15G6 (data not shown). In contrast, antiflagellin MAb H9724, used as a control, showed uniform fluorescein labeling of fixed to the glass spirochetes as demonstrated previously (9). This study suggested that the epitope for MAb 15G6 was susceptible to the experimental conditions and treatment required for the sample preparation. Although this epitope was accessible to

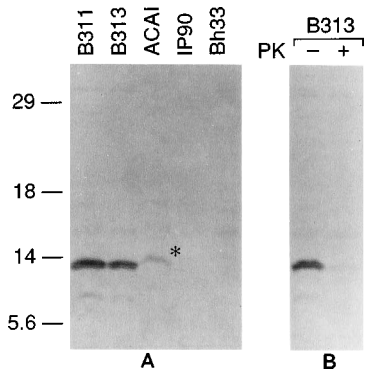

FIG. 2. Western blot analysis with MAb 15G6. (A) *B. burgdorferi* B311 and B313, *B. afzelii* ACAI, *B. garinii* IP90, and *B. hermsii* Bh33 were probed with MAb 15G6. (B) B313 cells treated (+) or untreated (−) with proteinase K (PK) were probed with MAb 15G6. Molecular size standards in kilodaltons (shown to the left) were carbonic anhydrase (29 kDa), β-lactoglobulin (18 kDa), lysozyme (14 kDa), and bovine trypsin inhibitor (5.6 kDa).

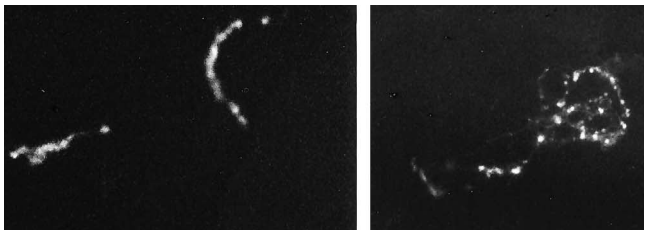

FIG. 3. Photomicrograph under UV illumination of binding of fluorescein-conjugated MAb 15G6 to unfixed, suspended cells of *B. burgdorferi* B313 for 3 min (left) and 15 min (right). Magnification, ×504.

MAb 15G6 by the Western blot assay in the whole-cell lysates and by unfixed-cell immunofluorescence assay, it was not recognized in the dried and fixed borrelias.

We then assessed the binding of fluorescein-labeled antibodies to unfixed borrelias by DFA. B313 cells were examined at 3, 15, 30, 60, and 360 min after addition of the 15G6 conjugate. The antibody was uniformly distributed over the lengths of 90 to 100% of the cells by 15 min (Fig. 3). In contrast to B313, less than 1% of B311 cells at any time point were detectably bound by the 15G6 conjugate by DFA with unfixed cells (data not shown).

Functional characterization of anti-p13 MAb. For further functional characterization of MAb 15G6, we used the whole purified IgG molecules and univalent Fab fragments of 15G6 MAb. Two other growth-inhibiting antibodies, anti-OspB MAb H6831 and anti-Bh33 MAb H4825, which were prepared in the same way, served as controls in a GIA with B311 and B313 (Table 4). The experiment was repeated three times with the same results. Anti-OspB MAb H6831 as whole IgG molecules inhibited B311 cells but did not inhibit the growth of Osp-less B313 cells even at 25 μg/ml, more than 100-fold higher than the MIC for B311 cells. The opposite was found with MAb 15G6. The MIC of this antibody as whole IgG was 0.02 μg/ml for B313 cells but 12.5 μg/ml for the Osp-bearing B311 cells. Antibody H4825, which is specific for *B. hermsii*, had no discernible effect on either B311 or B313. Univalent Fab fragments had a lower inhibitory activity against B311 or B313 than whole IgG; this phenomenon had been found in previous studies (53). Even then, Fab fragments of MAb 15G6 were 10-fold more active against B313 than were Fab fragments of H6831 against B311.

DISCUSSION

A mutant of *B. burgdorferi* that did not have OspA, -B, -C, and -D was further characterized with respect to selected biological functions and surface antigens. The basis for the mutant's Osp-less phenotype was the absence of *ospA*, *ospB*, and *ospD* genes and an unexplained lack of expression of the *ospC* gene in the cells (43, 51, 54). In this study, the Osp-less mutant was compared with the isolate from which it was derived, which expressed OspA and OspB but neither OspC nor OspD. The study showed that the Osp-less mutant differed in several ways from the OspAB-bearing parent with which it was compared. The most apparent genetic difference between the $OspA^+B^+$ B311 and $OspA^-B^-$ B313 was the presence or absence of the entire 49-kb linear plasmid. Although differences between B311 and B313 in other proteins besides OspA and OspB were not detected, we cannot exclude the possibility that the observed distinctions between B311 and B313 in biological functions were wholly or in part attributable to other genetic loci on the 49-kb plasmid. Definitive understanding of Osp function will require more specific mutagenesis of the Osp genes. Nevertheless, the present study provides some insights on Osp function and identified a non-Osp protein exposed the cell surface in the absence of Osp proteins.

Biological characteristics distinguishing Osp-less and $OspA^+B^+$ cells were growth rate and the population density at which stationary phase occurred. Isolate B313 grew more slowly than did B311 and stopped dividing at a lower cell density than did B311. This may be attributable wholly or in part to the greater autoagglutination displayed by the mutant cells. The triad of self-aggregation, slower growth rate, and lower cell density at stationary phase has also been noted with low-passage, infectious isolates of *B. burgdorferi* (3, 53). Some of these low-passage isolates of *B. burgdorferi* sensu lato also have a poor plating efficiency on solid medium (47). The diminished ability of aggregrated Osp-less borrelias to move about the broth medium may explain their slower growth under that condition, but why B313 cells could not grow on solid medium when singly dispersed is unknown. In previous studies, antibody-resistant variants of the B31, HB19, and Sh.2 lineages plated with the same high efficiency as B31, HB19 and Sh.2 themselves (49, 51). An exception was the very low plating efficiency of mutant B314 (54). Inasmuch as B314 cells still express OspC protein, the lower plating efficiency cannot be attributed to lack of Osp proteins per se.

Curiously, while $OspA^-B^-$ cells were inherently sticky for one another, they were less disposed than $OspA^+B^+$ cells to adhere to human endothelial cells, an indication that self-aggregation is not equivalent to the association of the borrelias with mammalian cells. Prior studies had revealed a possible role for OspA in endothelial cell adherence and for OspB in cell penetration (25, 27, 49). The findings of the present study are thus consistent with these functions for OspA and/or OspB in mammalian cell association.

We also examined another possible function of Osp proteins, namely, resistance to nonimmune effects of serum. For a blood-borne pathogen, this would seem to be a requirement for successful transmission between hosts and for proliferation within a mammalian host. Much is known about what confers serum resistance to gram-negative and gram-positive bacteria; less is known about this aspect of spirochetes. Although borrelias have two membranes sandwiching a peptidoglycan layer, as do gram-negative bacteria, the outer membrane of borrelias appears to be more fluid than that of gram-negative bacteria (8) and lacks lipid A-containing glycolipids (57). Thus, it was

TABLE 4. Growth inhibition by purified whole IgG and Fab fragments of MAbs 15G6, H6831, and H4825

Cell population	MIC (μg/ml)					
	15G6		H6831		H4825	
	Whole IgG	Fab fragment	Whole IgG	Fab fragment	Whole IgG	Fab fragment
B311	12.5	25	0.15	2	>25	>25
B313	0.02	0.2	>25	>25	>25	>25

likely a priori that spirochetes would have a different mechanism for avoiding the alternative complement pathway and other nonimmune defenses against bacteria. Indeed, the results suggest that OspA and/or OspB protect the cells from complement attack. When OspA, -B, -C, and -D are lacking, the borrelias were more susceptible than $OspA^{+}B^{+}$ cells to either unheated, nonimmune serum or to guinea pig complement. Brade et al. also suggested that differences in surface properties between *Borrelia* strains might be responsible for differences in complement activation and serum resistance (18). The B31 lineage isolates with different Osp phenotypes provide tools for further investigations of the interaction of complement and possibly other defense factors in the serum with *B. burgdorferi*.

Whatever protection OspA and OspB appeared to confer to the borrelias in serum did not seem to provide an advantage to cells in skin. In this experiment, we used two isolates that were not infectious by the criterion of detectable dissemination to the blood or other tissues. Still, we expected that the Osp-bearing cells would survive for a longer period in the skin than would their Osp-less counterparts. This did not occur in the two experiments in which this was examined. By 18 h after inoculation, both B311 and B313 could not be recovered from skin samples placed in culture medium. This finding suggests that OspA and/or OspB provide no additional protection against host noncomplement antimicrobial defense mechanisms in the skin (34). Complement components are also found in skin but at levels only 15% of that in serum (46).

Given the indistinguishability of B311 and B313 with respect to skin survival, one might expect that the immune responses to intradermal inoculation of viable borrelias would be comparable. Although the Osp-less mutant lacked two proteins, OspA and OspB, that are immunodominant when syringe inocula of 10^5 or greater are used (13, 28), other antigens, such as flagellin, commonly recognized by antibodies in immune sera were still present. Instead, we found that there was little detectable immune response to *B. burgdorferi* by ELISA, GIA, and protection against infection when B313 was the immunogen. Under the same conditions and with the same dose, mice given B311 had high titers to *B. burgdorferi* by immunoassays and were protected against challenge with strain Sh.2. The experiments results suggest that OspA and/or OspB are immunogens not only in their own right but also perhaps through their mitogenic properties (41), immunostimulatory with regard to other antigens. B313's equivalent survivability but reduced immunogenicity in comparison with B313 suggests that nonexpression of Osp proteins may confer a selective advantage to the borrelias once in the mammalian host.

Another possible conclusion from this experiment was that there were no antigens on the cell surface in B313 cells. The surface of *B. burgdorferi* cells without Osp proteins conceivably could be like the *Treponema pallidum* outer membrane, which is notably inert to the immune system (45). To further assess this possibility, we immunized mice with B313 by using a protocol that included an adjuvant. The resultant antiserum inhibited the growth of B313 cells in the GIA. The GIA results also showed that whatever was expressed on the surface of B313 was either not expressed or otherwise cloaked in B311 cells. The minimal effect polyclonal anti-B311 sera against growing B313 cells indicated that it was antibodies to OspA and/or OspB that inhibited the growth of B311 cells.

Surface-exposed antigens of the Osp-less mutant were further investigated with MAbs. The screening procedures were designed to identify antibodies that had the functional activity of growth inhibition. The antibodies that inhibited growth but did not bind to a borrelia component by Western blotting were identified but not further characterized for this study. The target or targets for this class of MAbs remains to be determined. It is also possible that they also bind to p13 but that their epitopes are sufficiently conformation dependent that Western blots would be negative. Alternatively, there may be other proteins or other nonproteinaceous components in the outer membrane against which these functional antibodies act.

Antibodies that bound to borrelia components in the Western blot were directed against p13, a 13-kDa protein. The evidence that p13 was surface exposed in the Osp-less mutant was the following: (i) growth inhibition by whole Ig and Fab fragment; (ii) direct immunofluorescence staining of live cells by an antibody conjugate; and (iii) cleavage of the antibody's epitope from the cell surface by in situ treatment with protease. p13 was present in all members of the B31 lineage and in approximately equal amounts. The expression of the protein did not vary according the amount of one or another of the Osp proteins. A slightly larger protein recognized by the MAb was present in a *B. afzelii* strain. If IP90, a representative of *B. garinii* strain, has a homologous protein, it does not share the MAb's epitope.

We considered whether p13 was identical to one of the other low-molecular-weight *B. burgdorferi* proteins to which antibodies have been developed. Like antibody to p13, antibody to a 10-kDa protein, as reported by Katona et al. (36), bound to only a small proportion of Osp-bearing cells in immunofluorescence assays. However, the molecular size of 10 kDa protein did not vary between strains, and uniform fluorescein labeling was seen in fixed-cell preparation when probed with a MAb to the 10-kDa protein (36). Furthermore, 15G6 does not bind to the 10-kDa protein in Western blots (29). Sambri et al. reported the presence of a 14-kDa protein of *B. burgdorferi* (55). This was identified with a MAb and by immunofluorescence of live borrelias. In contrast with what was observed by us with MAbs to p13 and by Katona et al. with an antibody to the 10-kDa protein (29), an antibody to the 14-kDa protein of Sambri et al. bound to the majority of cells (49). These differences suggest that p13 is neither the 10-kDa nor the 14-kDa protein of *B. burgdorferi*. A 14-kDa mitogenic lipoprotein of *B. burgdorferi* was recently reported by Honavar et al. (32). It has yet to be determined if this surface protein is similar or identical to p13 or the 14-kDa protein reported by Sambri et al.

The effect of MAb 15G6 or Fab fragments on susceptible borrelias was similar to what was observed with MAbs to OspB of *B. burgdorferi* and Vmp33 of *B. hermsii* (50, 53). The concentration of MAb 15G6 at which growth inhibition and cell disruption occurred was 20 ng/ml, 10-fold lower than what was observed with MAb H6831 against *B. burgdorferi* and the same as that for H4825 against *B. hermsii* (53). The failure of MAbs to p13 to inhibit the growth of Osp-bearing cells is consistent with lack of surface exposure of the protein, or at least impairment of the antibody's access to its target. The cloaking or obstruction could be from OspA, OspB, or a complex of the two. It was also possible that p13 was not in the outer membrane at all in B311 cells; in those cells, it may have been in the periplasmic space or in the cytoplasmic membrane. Evidence against this latter possibility was cleavage of anti-p13 MAbs epitopes from Osp-bearing cells by in situ treatment with proteinase K.

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