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Access of Antibody or Trypsin to an Integral Outer Membrane Protein (P66) of *Borrelia burgdorferi* Is Hindered by Osp Lipoproteins

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The outer membrane of *Borrelia burgdorferi*, the Lyme disease agent, contains lipoproteins anchored by their lipid moieties and integral proteins with membrane-spanning regions. We used the techniques of in situ proteolysis, immunofluorescence, in vitro growth inhibition, and cross-linking with formaldehyde to characterize topological relationships between P66, an integral membrane protein, and selected Osp lipoproteins of *B. burgdorferi*. Protease treatment of intact spirochetes cleaved P66 and Osp proteins but not the periplasmic flagellin or the BmpA protein of the cytoplasmic membrane. P66 of cells lacking OspA, OspB, and OspC was more susceptible to trypsin cleavage than was P66 of cells with these Osp proteins. A monoclonal antibody against the surface loop of P66 bound, agglutinated, and inhibited the growth of viable spirochetes lacking OspA, OspB, OspC, and OspD but not of the cells that expressed OspA, OspC, and/or OspD. When cells were fixed, the antibody bound to cells that express OspD and OspC but still not to cells with OspA. The close association of OspA and P66 was confirmed by the crosslinking of the two proteins by formaldehyde. These results show that Osp proteins, particularly OspA, limit the access of antibody or trypsin to the surface loop region of P66. The proximity and possible contact between P66 and OspA (or other Osp proteins) may hinder the effectiveness of antibodies to what otherwise would be an appropriate vaccine target.

Spirochetes, including members of the genus *Borrelia*, have both inner and outer membranes (31). The spirochete outer membrane is generally more fluid than the outer membranes of gram-negative bacteria, such as *Escherichia coli* (8, 16), and it contains comparatively few integral membrane proteins (45, 61). One integral membrane protein that has been identified in several species of *Borrelia*, including *Borrelia burgdorferi*, is P66 (12), also known as Oms66 (57). This protein spans the outer membrane at two places with short lengths of what is predicted to be hydrophobic alpha-helix (12). These membrane-spanning regions flank a ~5-kDa loop region that is exposed at the cell surface. The loop is highly polymorphic in size and sequence among different *Borrelia* spp. (12), an indication that it is under frequency-dependent selection, such as by the immune system.

The immunogenicity of P66, in particular the loop region, has been confirmed by using monoclonal antibodies and sera from patients with Lyme disease (12, 14). P66 is one of the 10 proteins included in the diagnostic set of proteins for Western blot-based serologic assays for *B. burgdorferi* infection (1, 19). Antibodies to P66 develop in animals infected with *B. burgdorferi* naturally or experimentally (4, 11, 28, 40). Although P66 is presumably expressed in human and other mammalian hosts, its actual function is unknown. Native P66 has porin activity in liposomes (57), but P66 is unlike gram-negative bacterial porins in its sequence, size, and predicted secondary structure (12).

Also present in the outer membrane of *B. burgdorferi* are the Osp lipoproteins, which are anchored in the membrane by their lipid moieties. The genome of *B. burgdorferi* is unique among sequenced prokaryote genomes for the large number of genes encoding actual or suspected lipoproteins (27). OspA and OspB are homologous proteins that usually are tandemly expressed in abundant amounts by culture-grown organisms (8, 32, 33). In nature, OspA, and apparently OspB as well, is expressed by spirochetes in the midgut of ticks but is seldom if ever expressed by organisms during early infection of mammals. Vaccination with OspA or OspB protects mice from tick-borne infection (25). OspC, a protein homologous to the Vsp proteins of relapsing-fever *Borrelia* spp. (15), is expressed by spirochetes in the tick after the blood meal and during early infection of mammals (52). OspC is also expressed abundantly in culture-grown organisms that have lost plasmids, including the large linear plasmid bearing the *ospAB* operon (51). OspD, a protein expressed by some but not all low-passage isolates, like OspC, is expressed in larger amounts when OspA expression is off or reduced (39, 48). Other Osp proteins are OspE and OspF, which appear to be expressed in smaller amounts than other Osp proteins by culture-grown organisms (34).

At least one of the Osp proteins, OspA, is a promising basis for a vaccine to prevent Lyme disease of humans and domestic animals. Recombinant OspA as a parenterally or orally administered protein provides immunity against *B. burgdorferi* infection in mice (22, 23, 36, 54). The OspA vaccine-induced immunity works, wholly or in part, through the killing of spirochetes still in the tick midgut. Thus, the OspA vaccine prevents transmission and infection but not necessarily disease once infection is under way (9, 18). Recently, recombinant OspA has been shown in two field trials to be efficacious for protecting humans against *B. burgdorferi* infection (53, 58). OspB and OspC immunizations induce immunity in small rodents, but this is more strain specific than the immunity provided by OspA (24, 42). Recombinant OspD, OspE, and OspF have provided no or only partial protection in mouse models (38, 43).

Given the mix of integral membrane proteins and abundant

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TABLE 1. Immunofluorescent binding of P66- and OspA-specific monoclonal antibodies to *B. burgdorferi* cells with different Osp phenotypes

Isolate	Osp phenotype[a]				Binding of antibody[b] to:				
					Fixed cells		Unfixed cells		
	OspA	OspB	OspC	OspD	H1337	H914	H1337	H914	H5332
B31	+	+	−	−	−	−	−	−	+
HB19	+	−	−	+	−	−	−	−	+
B314	−	−	+	−	+	−	−	−	−
HB19R1	−	−	−	+	+	−	+	−	−
B313	−	−	−	−					
Untreated					+	−	+	−	−
Trypsin treated[c]					ND[d]	ND	+	ND	ND
Proteinase K treated[c]					−	ND	−	−	−

[a] +, expression of an Osp protein as determined by sodium dodecyl sulfate-PAGE and Western blot analysis.
[b] +, binding in IFA.
[c] Trypsin and proteinase K were used at a concentration of 200 μg/ml of cell suspension.
[d] ND, not done.

lipoproteins in *B. burgdorferi*, we wondered about the topological relationships between one of the integral membrane proteins, P66, and Osp proteins. Although a comprehensive understanding of the functions of any of these proteins remains to be achieved, one hypothesis is that a function of the abundant Osp proteins involves shielding the membrane and the integral membrane proteins from hazards in tick or mammalian hosts. Indeed, *B. burgdorferi* cells lacking OspA to OspD but not P66 are more susceptible to complement than are Osp-bearing cells (49). Furthermore, Osp-less cells but not Osp-bearing cells were killed by an antibody directed against the P13 integral membrane protein (49). Another potential hazard in postfeeding ticks for resident or newly ingested microorganisms is posed by proteases (37). One of the features of OspA is its comparative resistance to proteases (7, 20).

For the present study, we used antibodies and proteases to examine clonal populations of *B. burgdorferi* with different Osp protein phenotypes to determine the relationship between selected Osp proteins and the P66 integral membrane protein. Using in situ proteolysis, immunofluorescence, and in vitro growth inhibition, we found that the accessibility of the surface-exposed region of P66 to antibody or protease trypsin is hindered to a greater or lesser extent in *B. burgdorferi* cells expressing Osp proteins. In situ formaldehyde cross-linking further detailed a close association between P66 and OspA of Lyme disease *Borrelia* spp.

MATERIALS AND METHODS

Strains and culture conditions. The strains used in this study were *B. burgdorferi* sensu stricto B31 (ATCC 35210) and HB19 (8) and *B. afzelii* ACAI (2). The Osp phenotypes of these strains, as well as of the derivatives B313 and B314 of the B31 lineage and HB19R1 of the HB19 lineage, were described previously (48, 51) and are summarized in Table 1. Cells were grown in BSK II medium and harvested as previously described (3, 8). Spirochetes were counted in a Petroff-Hauser chamber by phase-contrast microscopy.

Antibodies. The origins of the monoclonal antibodies against OspA (H5332), OspB (H6831), OspD (1C8), flagellin (H9724), and P66 (H914 and H1337) were described previously (6–8, 12, 48). Monoclonal antibodies against OspC and BmpA (55) were provided by Denee Thomas, University of Texas Health Science Center, San Antonio, Tex., and Barbara Johnson, Centers for Disease Control and Prevention, Atlanta, Ga., respectively. For some experiments, the monoclonal antibodies were affinity purified from hybridoma culture supernatants by using a protein A column (Pierce Chemical Co., Rockford, Ill). Production of polyclonal antisera against *B. burgdorferi* B31 and B313 whole cells in mice was described previously (49).

PAGE and Western blot analysis. Cell lysates were subjected to polyacrylamide gel electrophoresis (PAGE) with 10 or 12% acrylamide as described previously (15). For Western blot analysis, proteins were transferred to nitrocellulose membranes (Bio-Rad Laboratories, Richmond, Calif.), which were then blocked with 3% dried nonfat milk in 10 mM Tris (pH 7.4)–150 mM NaCl (milk-TS) for 2 h. The membranes were incubated with hybridoma supernatants diluted 1:10 in 0.3% milk-TS. Alkaline phosphatase-conjugated recombinant protein A/G (Pierce) was the second ligand. The blots were developed with nitroblue tetrazolium and 5-bromo-4-chloro-3′-indolylphosphate *p*-toluidine salts as the substrates (Pierce).

Protease treatment of *Borrelia* cells. Proteins of intact or sonicated borrelias were cleaved with proteinase K or trypsin by a modification of the procedure of Barbour et al. (7). Freshly harvested spirochetes were washed once with phosphate-buffered saline–5 mM Mg (PBS-Mg) and, after centrifugation at 2,000 × *g* for 10 min, resuspended in this buffer at a final concentration of 2×10^9 cells/ml. In some experiments, prior to protease treatment, the cells were sonicated with two 30-s pulses of a 250 Sonifier (Branson Ultrasonics Corp., Danbury, Conn.) at 10% output. To 0.5 ml of intact or sonicated cells in PBS-Mg was added 25 μl of proteinase K (Boehringer Mannheim, Indianapolis, Ind.) in distilled water or 25 μl of trypsin (Sigma Chemical Co., St. Louis, Mo.) in 0.001N HCl to a final concentration of 12.5 to 200 μg/ml or 6.25 to 400 μg/ml, respectively. Distilled water or dilute HCl was added to the cells in negative control tubes. After incubation for 60 min at 20°C, 10 μl of phenylmethylsulfonyl fluoride (Sigma) (50 mg/ml of isopropanol) was added, and the cells were centrifuged at 2,000 × *g* for 10 min and washed twice with PBS-Mg. The pellet was boiled in PAGE sample buffer or was resuspended in PBS-Mg with 2% (wt/vol) bovine serum albumin (BSA) for immunofluorescence studies. The amount of protease that reduced the amount of full-length protein by 50% was determined by densitometry of Western blots with a Microtek scanner and NIH Image version 1.57 (provided by W. Rasband, National Institutes of Health) software.

Immunofluorescence assay. Binding of monoclonal antibodies to unfixed cells was investigated by indirect and direct fluorescence assays (IFA and DFA, respectively). Freshly harvested cells were washed once with PBS-Mg–BSA. Then 10^7 washed spirochetes were suspended either in 0.5 ml of PBS-Mg–BSA containing 15 μg of purified antibody conjugated with fluorescein isothiocyanate (Pierce) or in 0.5 ml of undiluted hybridoma supernatant. For some experiments, the cells were cross-linked with formaldehyde (see below) before being incubated with antibody. The cell mixtures were incubated at room temperature with gentle rotation for 1 h, centrifuged, and washed with PBS-Mg–BSA. For IFA, the cells were suspended in a 1:15 dilution of anti-mouse immunoglobulin–fluorescein $F(ab')_2$ fragment (Boehringer Mannheim) and incubated for 30 min at room temperature. The volume of the cell suspension was adjusted to 0.3 ml with PBS-Mg–BSA. Phase-contrast microscopy and fluorescence microscopy of the cells were performed with Olympus BX60 microscope and PM-30 camera.

For IFA with fixed cells, washed spirochetes were suspended with sheep erythrocytes in 50% PBS–50% fetal calf serum (Gibco BRL, Grand Island, N.Y.). Thin smears of the mixture were made on the glass slides, air dried, and fixed in 100% methanol. The slides were spotted with undiluted hybridoma supernatant and incubated at 37°C for 30 min in a moist chamber. After three washes in PBS, the slides were incubated under the same conditions with the secondary antibody. The slides were washed in PBS, air dried, and mounted with the medium for fluorescence microscopy (Kirkegaard & Perry Laboratories, Gaithersburg, Md.). Microscopy was performed with a Zeiss Axioskop epifluorescence microscope. The images were captured with the Oncor imaging system.

Growth inhibition assay. The growth inhibition assay was described previously (50). Briefly, 100 μl of BSK medium containing 0.5×10^6 borrelias was added to flat microtiter plate wells containing 100 μl of a serially diluted purified monoclonal antibody in a BSK medium. For some experiments, 2 U of guinea pig complement (Sigma) was added to the wells. The plates were covered with adhesive seals and incubated for 72 h at 34°C. Growth in the wells was monitored visually for changes in the color of the phenol red indicator and by examination of wet mounts by phase-contrast microscopy. The MIC was the lowest concen-

tration of antibody that precluded pink-to-yellow discoloration of the wells and represented at least 20-fold fewer cells (49).

Protein cross-linking. Proteins of intact borrelias were chemically cross-linked by a modification of a procedure described by Skare et al. (56). Approximately 1.5×10^9 freshly harvested and washed spirochetes were suspended in 300 μl of 0.1 M sodium phosphate buffer (pH 6.8). Formaldehyde (Sigma) was added to a final concentration of 1%, and the sample was incubated for 30 min at room temperature. The cells were washed once in cold sodium phosphate buffer. The cell pellet was solubilized in PAGE sample buffer at 37°C.

Immunoprecipitation. Proteins of lysed spirochetes were immunoprecipitated by modification of a procedure described previously (7). A total of 2.5×10^{10} cells were washed with PBS-Mg and lysed by suspension in 1.5 ml of 50 mM Tris (pH 7.5)–150 mM NaCl–5 mM EDTA–0.05% sodium azide (TSEA) with 1% Triton X-100 (Sigma) and 50 μg of phenylmethylsulfonyl fluoride per ml and subsequent incubation for 2 h at 37°C. The lysate was centrifuged at 50,000 × *g* for 30 min. To 500 μl of the supernatant was added 200 μg of purified monoclonal antibody in 100 μl of PBS–1% BSA. The reaction mixture was incubated for 1 h at 37°C. Antibody and antigen-antibody complexes were precipitated by the addition of 300 μl of a 50% (vol/vol) aqueous slurry of recombinant protein A immobilized on 6% beaded agarose (Pierce). After a 45-min incubation at room temperature, the immunoabsorbent matrix was centrifuged at 7,000 × *g* for 10 min and the pellet was washed three times with TSEA containing 0.05% Tween 20. The final pellet was suspended in PAGE sample buffer and subjected to electrophoresis followed by Western blot analysis.

RESULTS

Effect of different concentrations of proteinase K on intact and disrupted cells. In previous studies, P66 and Osp proteins were cleaved in situ by treatment of intact *B. burgdorferi* cells with of proteinase K at 200 or 400 μg/ml (7, 41). However, as pointed out by Cox et al., these high concentrations of proteinase K may result in cleavage of proteins that are not surface exposed (17). Therefore, to further address the question of specificity of protease treatment for surface-exposed molecules, we compared the effect of protease on whole and sonicated cells. As a further measure of specificity, we also varied the concentration of proteinase K for a constant number of spirochetes. In the present study, two markers for internal proteins were flagellin, the major constituent of the periplasmic flagella (6), and BmpA, also known as P39, a protein associated with the cytoplasmic membrane (55). Proteolytic products were evaluated by Western blot analysis with monoclonal antibodies against OspA (H5332), OspB (H6831), flagellin (H9724), BmpA, and P66 (H914). H914 binds to an epitope of P66 that is not susceptible to proteinase K treatment (12).

Figure 1 shows that the bulk of P66 and OspB of intact cells were proteolyzed by low levels of proteinase K; i.e., 10 and 12.5 μg/ml cleaved 50% of P66 and 50% of OspB, respectively. Comparable cleavage of OspA required 70 μg of proteinase K per ml; for complete cleavage of OspA from the cells, more than 200 μg of this protease per ml would be needed. While neither flagellin (Fig. 1) nor BmpA (data not shown) in intact cells was detectably affected by high concentrations of proteinase K, these internal proteins were susceptible to this protease when the cells were first sonicated. We estimated that 7 and 25 μg of proteinase K per ml cleaved 50% of the flagellin and BmpA, respectively, in sonicated preparations.

Effect of trypsin on cells with different Osp phenotypes. The above experiment demonstrated the specificity of the protease activity for surface-exposed proteins of intact cells. However, because proteinase K cleaves OspA, albeit only when present at a high concentration, it would not be expected to be useful in assessing the effect of OspA. Accordingly, for the next experiments, we used different concentrations of the more site-specific protease trypsin on derivatives of strains B31 and HB19 with different Osp phenotypes (Fig. 2). In a preliminary experiment, we demonstrated that trypsin cleaved flagellin in sonicated B31 cells but not in intact cells (data not shown).

When the bacteria were producing either OspA (strain B31)

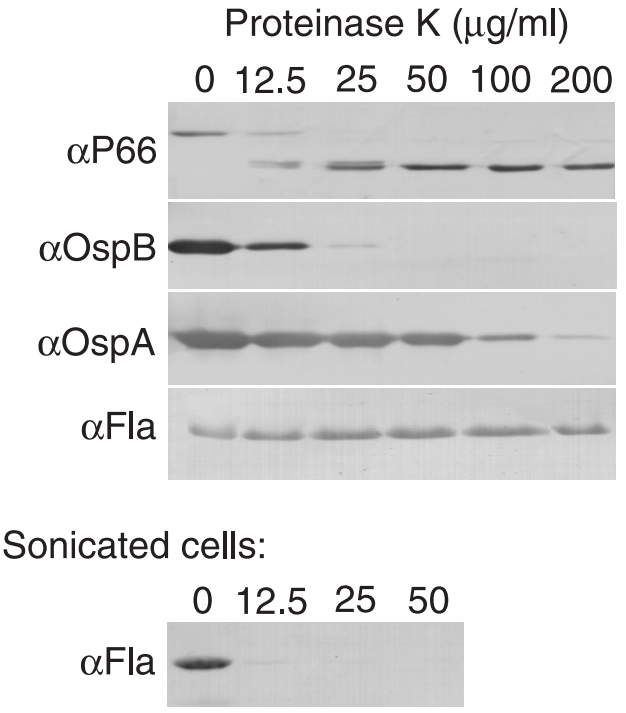

FIG. 1. Western blot analysis of the effect of proteinase K on selected *B. burgdorferi* B31 proteins. Intact (top) or sonicated (bottom) cells were incubated in different concentrations of proteinase K. After being washed, the cells were subjected to PAGE (12% acrylamide), and blots of the cell lysates were probed with the monoclonal antibodies (α) against OspA (H5332), OspB (H6831), P66 (H914), or flagellin (H9724).

or OspC (strain B314), the estimated concentrations of trypsin needed to cleave 50% of P66 were 45 and 17.5 μg/ml, respectively (Fig. 2A and B). For complete cleavage of P66 in either B31 or B314 cells, more than 200 μg of trypsin per ml was needed. In contrast, with cells of isolates B313 and HB19R1, which lack OspA, OspB, and OspC, P66 was completely or nearly completely cleaved by as little as 6.25 μg of trypsin per ml (Fig. 2C). Cells of HB19R1 produce OspD (39, 48), but this protein is cleaved from the cells by trypsin, effectively producing Osp-less cells. OspA and OspC in these studies were resistant to trypsin (Fig. 2A and B).

The ~30-fold-greater susceptibility of P66 to trypsin in Osp-less cells than in cells expressing OspA or OspC may have been the consequence of smaller amounts of available protein in the Osp-less cell suspension. In other words, the amount of potential protease substrate may be greater when cells have OspB proteins, as is the case for isolate B31. Thus, protease otherwise disposed to cleaving P66 would be occupied with OspB or another protein. To assess the magnitude of this effect, we used a 40-fold range of cells (0.4×10^9 to 16×10^9) of strain B31 and trypsin at a final concentration of 50 μg/ml. The amount of cleavage of OspB and P66 was essentially the same over this range of substrate (Fig. 3).

Immunofluorescence with *B. burgdorferi* cells. The results of the trypsin experiments indicated that accessibility of a protease per se, rather than the amount of available protein substrate, was the major determinant of the susceptibility of P66 to the protease. Furthermore, protease access to P66 was limited by the presence of one or more abundant Osp proteins. The pathogenetic significance of protease hindrance is unclear; possible advantages of this phenotype in the tick environment are discussed below. More relevant to our understanding of

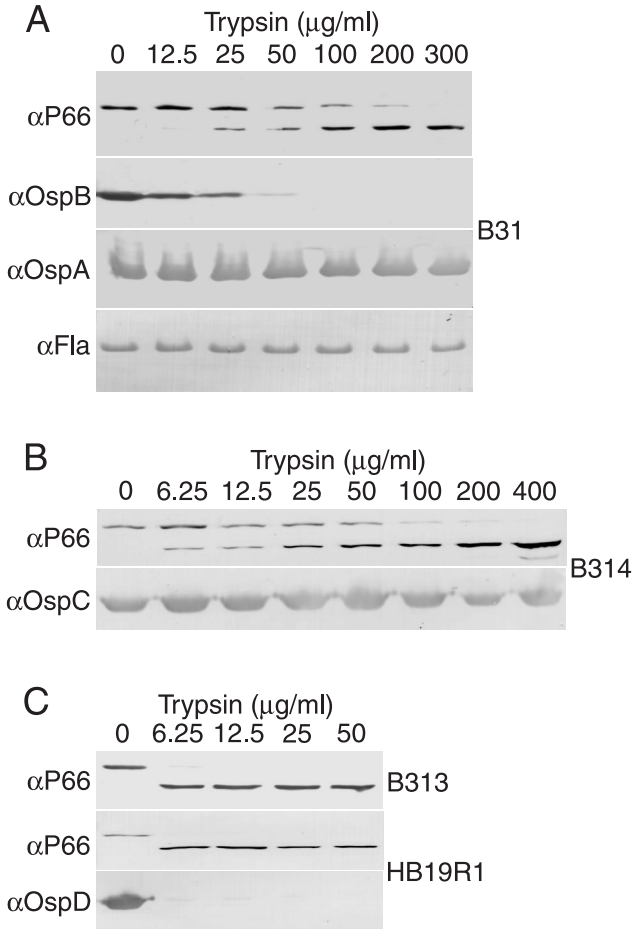

FIG. 2. Western blot analysis of the effect of trypsin on selected proteins of *B. burgdorferi* isolates with different Osp phenotypes. Cells of B31 (A), B314 (B), and B313 and HB19R1 (C) were incubated with different concentrations of trypsin and subjected to PAGE (12% acrylamide). Blots of the cell lysates were probed with the monoclonal antibodies (α) against OspA (H5332), OspB (H6831), OspC, OspD (1C8), P66 (H1337), or flagellin (H9724).

infection of humans would be hindrance of antibody access to P66. Accordingly, we next investigated whether access of antibody to P66 is hindered in the presence of Osp proteins.

For the immunofluorescence experiments, we used *B. burgdorferi* cells with different outer membrane protein phenotypes

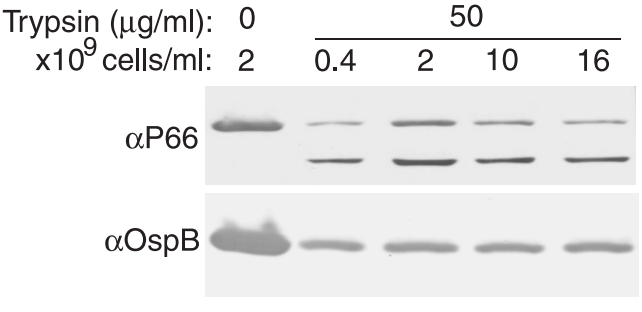

FIG. 3. Western blot analysis of the binding of monoclonal antibodies (α) for P66 (H1337) or OspB (H6831) to *B. burgdorferi* B31 cells. Different number of cells (range, 0.4×10^9 to 16×10^9) were incubated in buffer with or without trypsin at 50 μg/ml. The cells were then subjected to PAGE (12% acrylamide), and blots of the cell lysates were probed with antibodies.

and two P66-specific monoclonal antibodies: H1337, which binds to the surface loop of the protein, and H914, which binds to nonexposed region of the protein. The cells for the antibody binding studies were prepared in three different ways: (i) no fixation of viable cells in buffer, (ii) methanol fixation of dried cells on glass slides, and (iii) formaldehyde fixation of suspended cells. These different preparations provide different presentations of the in situ antigen to the antibody and thus allow assessment of the effects of outer membrane perturbation on antibody access. Monoclonal antibody H5332, which binds to OspA, served as a positive control for binding to the surface of both fixed and unfixed borrelias (8). A negative control was monoclonal antibody, H9724, which is specific for the periplasmic flagella and would bind to fixed cells of all phenotypes. The results of the immunofluorescence experiments are presented in Table 1 and Fig. 4.

When cells expressed OspA (B31 and HB19), antibody H1337 did not bind to unfixed or fixed cells (Fig. 4; Table 1). H1337 did bind to fixed and unfixed Osp-less B313 cells, as well as to HB19R1 cells, which express OspD but not OspA, OspB, or OspC. Binding of H1337 antibody to B313 was prevented by prior treatment with proteinase K but not with trypsin. (Although the surface loop of P66 is cleaved by trypsin, the epitope for H1337 in the loop is not affected by trypsin cleavage [12].) Even after removal of OspB by trypsin treatment, H1337 did not bind to B31 cells (data not shown), an indication that the presence of OspA is sufficient to hinder binding. Binding of antibody H1337 to viable B314 cells expressing OspC in suspension was not detected (Table 1). In contrast, detectable binding of the antibody to methanol-fixed B314 cells did occur, although more weakly than the binding to Osp-less cells (Fig. 4). Antibody H914 did not bind fixed or unfixed cells of any phenotype (Fig. 4; Table 1). As expected (6), H9724 bound to the cells after fixation (Fig. 4).

When the spirochetes were exposed to 1% formaldehyde and then washed before being subjected to IFA under the same conditions described above, H1337 was again found to bind to B313 cells but not B31 cells (data not shown). When the experiments were repeated with antibodies H1337, H914, and H5332 that had been labeled with fluorescein, the results in this DFA were the same as with unlabeled antibodies in the IFA (data not shown).

In the immunofluorescence assay, antibody H1337 agglutinated B313 cells within 30 min and before the addition of the second fluorescein isothiocyanate-labeled ligand (Fig. 5). There was no agglutination of B31 or B314 cells by H1337. Although H1337 bound to OspD-expressing HB19R1 cells (Table 1), it did not agglutinate the cells (Fig. 5).

Inhibition of *B. burgdorferi* growth by antibodies. Having found that H1337 binding is associated with agglutination of Osp-less cells but not OspD-expressing cells, we examined the functional outcomes of different types of binding (Table 2). For this study, spirochetes were grown in the presence of different concentrations of purified antibodies and the effect on growth was assessed by monitoring the change in an acid-base indicator and by microscopy (50). The anti-OspB antibody H6831 served as a positive control for this experiment (47). The results are shown in Table 2.

Antibody H1337 did not affect growth of B31 cells at the highest concentration used, even when complement was added. This antibody also did not inhibit isolates expressing OspA, OspB, OspC, and/or OspD (Table 2). As expected, monoclonal antibody H6831 inhibited the growth of B31 cells in the absence of complement but did not inhibit the growth of the Osp-less B313 spirochetes. The only population that H1337 inhibited was the Osp-less B313. H914, the P66-specific anti-

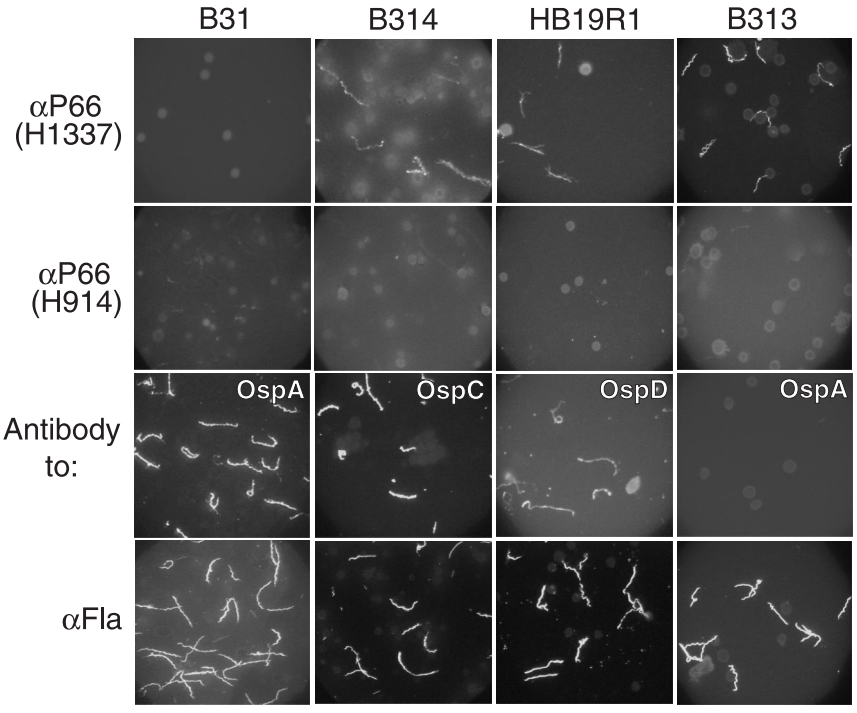

FIG. 4. Photomicrographs under UV illumination of binding of monoclonal antibodies to methanol-fixed *B. burgdorferi* cells with different Osp phenotypes (Table 1). The isolates examined were B31, B314, HB19R1, and B313. Smears of spirochetes with sheep erythrocytes were incubated first with monoclonal antibodies (α) against P66 (H1337 or H914), OspA (H5332), OspC, OspD (1C8), or flagellin (H9724) and then with fluorescein-conjugated sheep anti-mouse immunoglobulin. Magnification, ×80.

body against a non-surface-exposed epitope, did not inhibit B313 or cells of any other phenotype. Fab monomer fragments of H1337 bound unfixed B313 cells, as indicated by IFA, but did not inhibit their growth (data not shown), an indication that growth inhibition with complete immunoglobulin was the consequence of cell agglutination rather than a direct bactericidal activity (47).

In situ cross-linking of *Borrelia* proteins. The preceding studies revealed that access of a protease or antibody to the surface loop of P66 was limited to various extents in *B. burgdorferi* cells expressing OspA, OspC, and/or OspD. We next used cross-linking to further define the topological relationships between P66 and other surface structures. Intact or trypsin (200 μg/ml)-treated cells were suspended in dilute formaldehyde or buffer alone, washed, lysed, and either directly subjected to Western blot analysis or first incubated with anti-P66 antibody H1337 and then with protein A in an immunoprecipitation step. Western blots of the cell lysates or antibody-antigen complexes were probed with the monoclonal antibodies against P66 and OspA.

In the absence of formaldehyde, the two antibodies detectably bound only to OspA or P66 of *B. burgdorferi* B31 (Fig. 6A). When the cells were first treated with formaldehyde, both antibodies bound to a new polypeptide in the lysates of approximately 120 kDa. A similar-size polypeptide that reacted with both an anti-OspA and an anti-P66 antibody was also noted in a blot of formaldehyde-treated *B. burgdorferi* HB19 and *B. afzelii* ACAI cells (data not shown). For the ACAI cells, the anti-P66 antibody was H914, which is specific for P66 of Lyme disease *Borrelia* spp. The 120-kDa polypeptide was also present when the lysate was immunoprecipitated with anti-P66 antibody H1337 before being subjected to Western blotting with anti-OspA antibody H5332 (Fig. 6A). The size of this polypeptide is consistent with a cross-linked complex of one P66 molecule and two OspA molecules. When the cells were first trypsinized and subsequently treated with formaldehyde, both H5332 and H1337 antibodies detected another polypeptide, of approximately 83 kDa, in the lysates (Fig. 6A). The size of this product is consistent with a heteromer of one truncated P66 polypeptide of approximately 52 kDa (12) and one OspA molecule. The monomers of these truncated P66 and OspA molecules were also detectable in the blots. Western blot analysis also revealed products that reacted weakly with anti-OspA but not anti-P66 antibody and could represent OspA complexed to itself. An anomalously migrating P66 band was probably the result of an intramolecular cross-linking (44).

Figure 6B shows a Western blot analysis in which cross-linking of proteins of Osp-less B313 and OspC-expressing B314 cells was examined. Both isolates had the same anomalously migrating P66 band, and OspC dimers were observed in B314 cells. There was a faint, larger anti-P66 reactive band in the lane of OspC-bearing B314 cells, but a similarly migrating band was not identified with the anti-OspC antibody. Thus, there was no evidence of cross-linking between OspC and P66 or of oligomerization of P66 in Osp-less cells. Neither was there evidence of cross-linking of P66 to OspB or OspD when blots of formaldehyde-treated B31 or HB19R1 cells were probed with antibodies to OspB or OspD (data not shown).

Polyclonal antisera to *B. burgdorferi* cells with different Osp phenotypes. The preceding experiments suggested that antibody access to the surface loop of P66 is hindered in the presence of Osp proteins. It was also possible that the presence of OspA (or other Osp protein) had an effect on the immune response to P66. To address this question, we used pooled antisera previously developed against whole cells of B31 and B313 (49). The mice had been immunized with whole cells

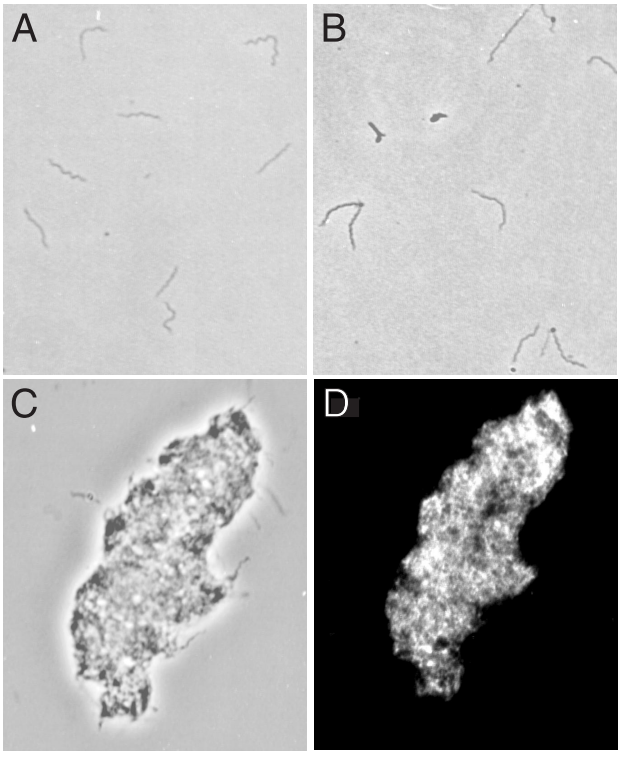

FIG. 5. Photomicrographs under phase-contrast (A to C) or UV (D) microscopy of unfixed, suspended cells of *B. burgdorferi* isolates HB19R1 (A) or B313 (B to D) incubated with monoclonal antibodies H1337 (A, C, and D) or H914 (B). After incubation with the antibody, the cells were washed and incubated with fluorescein-conjugated sheep anti-mouse immunoglobulin. Magnification, ×400.

mixed with adjuvant and then boosted by subcutaneous injection of viable cells. Figure 7 shows the Western blot with the antisera reacted with whole-cell lysates of B31 and B313. The location of P66 in the blot was confirmed with antibody H1337. For most proteins visible in the blot, there was no difference in response between each pool. As expected, mice immunized with B31, but not those immunized with B313, had reactive antibodies to OspA and OspB. B31 has the genes for OspA and OspB and expresses them; B313 does not. Less expected was the difference in reactivity to P66. Both B31 and B313 express P66, as indicated by the blot probed with H1337. However, only the antisera from mice immunized with the Osp-less B313 produced prominent bands of P66 in the blot. (The identity of another band of about 20 kDa that was present in both lysates but differentially bound by antibodies in the anti-B313 sera is not known.)

DISCUSSION

A commonly held view is that surface structures of pathogens are appropriate candidates for vaccines, especially if immunity to infection is dependent on antibodies. A given surface protein may, in comparison to other cellular proteins, be more variable in sequence between strains, but this evidence of selection by the immune system often justifies a choice for vaccine development. In our previous studies, we identified and characterized P66, an integral membrane protein of *B. burgdorferi* with a surface-exposed loop that was polymorphic between species (7, 12–14). These features suggested to us that P66 could be an appropriate target of immunization for protection against Lyme disease, perhaps as a second antigen in combination with OspA. However, in the course of the present investigation of this possibility, we discovered that the surface of *B. burgdorferi* was not as easily defined as we thought, at least with regard to what antibodies can react with. These findings led to a more detailed examination of the topological relationships between different outer membrane proteins, in particular between P66 and selected Osp proteins.

TABLE 2. MIC of P66- and OspB-specific monoclonal antibodies against *B. burgdorferi* cells with different Osp phenotypes

Isolate[a]	MIC (μg/ml) of:		
	Anti-P66		Anti-OspB (H6831)
	H1337	H914	
B31	≥40	≥40	0.31
HB19	≥40	ND[b]	ND
B313	5.0	≥40	≥40
B314	≥40	ND	ND
HB19R1	≥40	ND	ND

[a] Refer to Table 1 for the Osp phenotypes of the isolates.
[b] ND, not done.

For these studies, we used derivatives of the B31 and HB19 strains that differ in Osp phenotype on the basis of plasmid content from each parent and from other members of these lineages. (A system for stable allelic exchange of most of the genes considered here has not been achieved for *Borrelia* spp.) A drawback to plasmid mutants is that other proteins besides the selected Osp protein may be missing. For instance, the absence of the 54-kb linear plasmid of strain B31 means the loss not only of the *ospAB* operon but also of the genes for decorin binding proteins (5, 30). We cannot rule out the effects of other proteins that are present or absent in the isolates under study. However, given their comparatively smaller amounts in the cell, we have assumed that any contribution of these less abundant proteins to antibody or protease access is smaller than the effects of the more abundant proteins, such as OspA or OspC, studied here.

Access of antibodies is obviously relevant to questions about immunity and its evasion, but what of the role of proteases? The protease trypsin was used as a probe, because the Osp proteins varied in susceptibility to this protease and because the site of trypsin cleavage of P66 in situ had been identified (12). The first series of experiments showed the specificity of proteinase K for surface proteins, such as OspA and OspB, which have been confirmed by other procedures to be surface exposed (7, 8). As Cox and colleagues have argued, some or many of the Osp proteins of a cell may be located in the periplasm rather than at the outer surface of the cell (17). However, even if a subsurface compartment of OspA, for instance, went undetected in the present study, this protein pool would not be expected to affect the access of antibody to the exterior of the cell.

The in situ susceptibility of P66 to trypsin varied with the Osp phenotype of the *B. burgdorferi* cells in the reaction. The concentration of trypsin required to cleave P66 in the absence of OspA to OspD or the presence of only OspD was 30-fold lower than when trypsin-resistant OspA was present. This phenomenon was not attributable to a smaller amount of potential substrate with Osp-less cells; the same susceptibility was observed when the trypsin concentration was held constant and the cell concentration was varied. Tryptic cleavage of P66 was also less efficient in the cells expressing trypsin-resistant OspC.

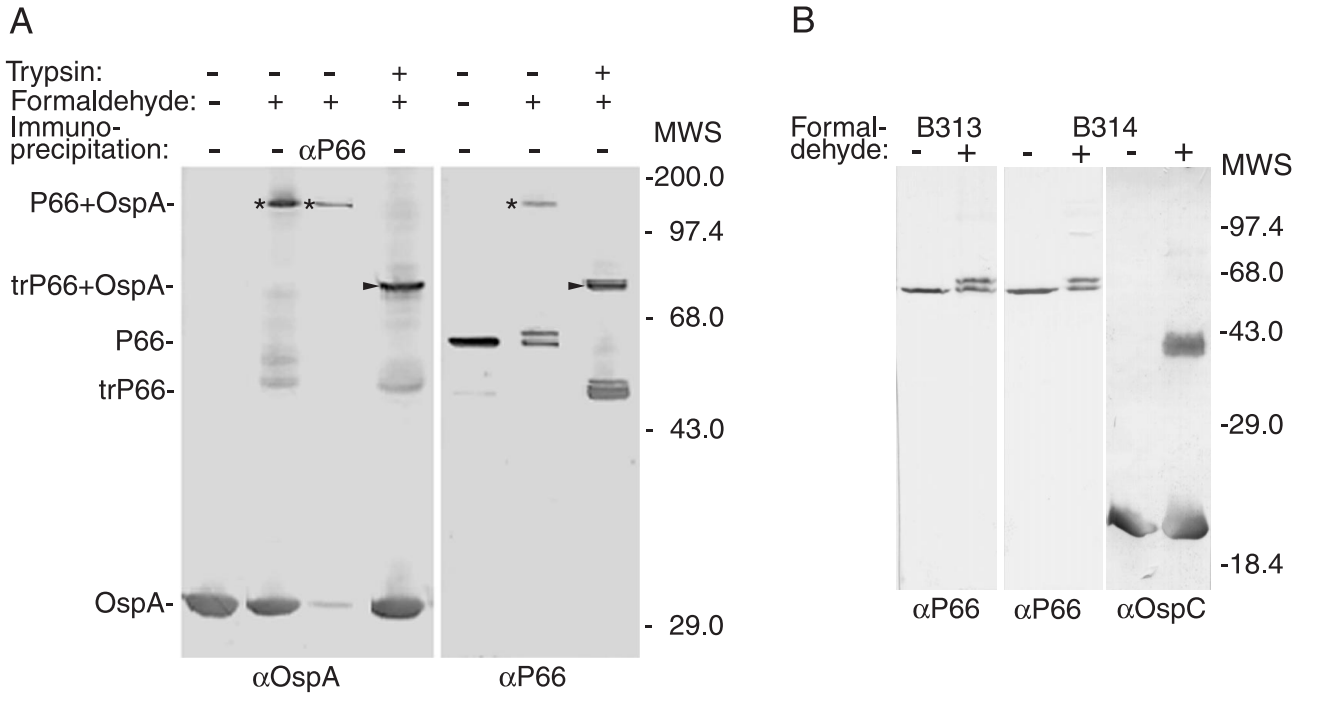

FIG. 6. Western blot analysis of cross-linked proteins of *B. burgdorferi* B31 (A) and B313 and B314 (B). Intact or trypsin-treated cells were incubated in 1% (vol/vol) formaldehyde or buffer alone, washed, and lysed. The lysates were either directly subjected to Western blot analysis of a 10% acrylamide gel or first immunoprecipitated with monoclonal antibody H1337. The blots were probed with monoclonal antibodies H5532 (αOspA) or H1337 (αP66). Positions of monomers of OspA and full-length and trypsin-truncated (tr) P66 are indicated. Positions of putative heteromers of OspA and the full-length or truncated P66 are indicated by an asterisk and arrowhead, respectively. Molecular weight standards (MWS) in thousands (shown to the right) are myosin H-chain (200.0), phosphorylase B (97.4), bovine serum albumin (68.0), ovalbumin (43.0), carbonic anhydrase (29.0), and β-lactoglobulin (18.4).

These findings indicated that an important variable for the in situ susceptibility of P66 was the access of trypsin to this protein.

Hindrance of access was also demonstrated with a monoclonal antibody directed to the surface loop of P66. The antibody bound, agglutinated, and inhibited the growth only of cells that lacked OspA to OspD by genotype or trypsin treatment. The antibody did not bind to cells that had been first treated with proteinase K, which cleaves the antibody epitope (12). When either OspA or OspC was expressed, the anti-P66 antibody did not bind to viable cells and growth was not inhibited. The antibody bound to cells expressing OspD but did not agglutinate or inhibit their growth. Hindrance of antibody access was also noted with fixed, dried cells on a slide. This was observed with the anti-P66 antibody but not with the anti-flagellin antibody, which bound equally well to Osp-bearing and Osp-less cells. Thus, even when the cell is permeable to an antibody to a periplasmic structure, i.e., flagella, the anti-P66 antibody still did not bind. This effect was most pronounced with OspA- and OspB-bearing cells. When OspC or OspD was expressed, the antibody against the loop of P66 bound to fixed cells with similar efficiency to that in the absence of OspA to OspD.

The findings with antibody and trypsin suggested that different Osp proteins hindered access to different extents. While OspD had the least effect and OspA had the greatest effect, OspC was intermediate in hindrance. Although OspB is usually expressed with OspA, it had minimal or no additional effect to OspA. These differences in hindrance between the Osp proteins were not associated with differences in molecular copy number: the molar ratio of P66 to OspA to OspC to OspD is approximately 1:7:7:7. Thus, other factors besides Osp protein density determine antibody access to other surface components.

The complete protection afforded by OspA to P66 against antibody and trypsin was evidence of an association between these two proteins, the former spanning the outer membrane and the latter anchored in the outer membrane by its lipid moiety. The cross-linking experiment confirmed a close topological relationship between P66 and OspA. Although OspC was nearly as effective as OspA in blocking trypsin and antibody, there was no evidence of cross-linking of this protein to P66. The degree and nature of the contact between OspA and P66 remains to be determined, but the three-dimensional structure of OspA suggests a possible contact point (35). Contained within the predominantly alternating beta-sheet structure of OspA is a deep cleft of unknown function that may be a site of interaction between the two molecules. The apparent cross-linking of two OspA molecules with one P66 indicates that there are two points of potential contact for OspA on P66. At least one of these points is likely to be surface exposed, since cross-linking of the OspA molecule to it was prevented by trypsin treatment. On the other hand, when trypsin did cleave P66 in the presence of OspA, the site of cleavage was the same as in the absence of OspA, a finding that suggests that any contact with OspA is not measurably affecting the conformation of P66.

The common occurrence of antibodies to P66 among patients with Lyme disease, especially those with late disseminated disease, is evidence of the immunogenicity and in vivo expression of P66. P66-specific antibodies in these patients are commonly directed against the surface-exposed loop of the protein (14). However, this finding is seemingly at odds with the results shown in Fig. 7, namely, that antibodies against P66 are effectively produced only in mice immunized with Osp-less

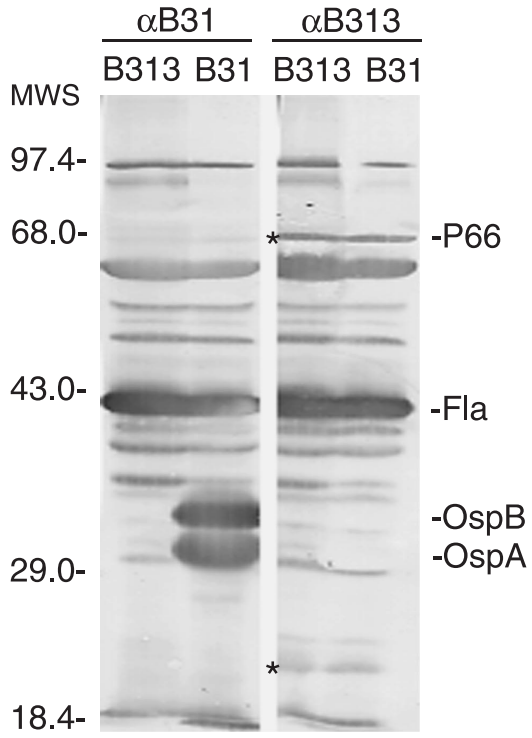

FIG. 7. Western blot analysis of the polyclonal antisera from mice immunized with whole-cell *B. burgdorferi* B31 and B313. B31 and B313 cell lysates were separated by PAGE (12% acrylamide), blotted, and probed with pooled antisera. Asterisks mark the positions of P66 and an approximately 20-kDa protein detected exclusively by antisera against Osp-less B313 cells. Molecular weight standards (MWS) in thousands (shown to the left) are the same as in Fig. 6.

whole cells. The monoclonal antibodies to the surface loop of P66, as well as to the P13 outer membrane protein, were the product of intravenous immunizations with Osp-less cells (12, 49). Antibodies with these specificities had not been noted in previous batteries. The latter findings suggest that the presence of OspA not only limits access of antibody but also limits access to P66 during antigen presentation. The finding that humans and other naturally infected animals commonly have an anti-P66 response is further evidence that OspA is not expressed in most infections of mammals.

Hindrance of antibody access has been noted in other pathogens. In gram-negative bacteria, such as *Neisseria gonorrhoeae*, *E. coli*, and *Brucella* spp., lipooligosaccharides or lipopolysaccharides shield porin proteins from antibody (21, 29, 60). *B. burgdorferi* lacks these molecules (59) but instead expresses abundant Osp proteins that appear to hinder the access to other outer membrane proteins. To our knowledge, hindrance between two surface proteins has not been previously reported for bacterial pathogens.

A closer analogy to the hindrance phenomenon is found in the eukaryotic parasite *Trypanosoma brucei*. Ziegelbauer and Overath reported that variant surface glycoproteins of these extracellular, blood-borne pathogens provide a barrier against both trypsin and polyclonal antibodies for other surface glycoproteins that are less polymorphic (62). In contrast to collection of *B. burgdorferi* with different Osp phenotypes, there was no variant surface glycoprotein-negative mutant available for study. Moreover, the investigators found that formaldehyde fixation served to expose the invariant protein to antibody. In the present study, access of antibody remained hindered after cross-linking.

Trypanosomes and borrelias share a dependence on arthropod vectors for transmission between mammals. One of the challenges for arthropod-borne organisms is their exposure to midgut proteases involved in digestive processes in ticks and insects after the blood meal (10, 37, 46). OspA is expressed in the tick midgut (52); it is also protease and acid resistant (20). One of its functions may be the protection it provides to other proteins in the outer membrane against proteases.

Another constituent of the tick gut after feeding is host antibody. Antibody to OspA kills or otherwise inhibits *B. burgdorferi* in the tick, but this has been noted only after the animal has been passively or actively immunized with OspA (18, 26). In the natural state, few reservoir animals, such as *Peromyscus leucopus*, have antibodies to OspA, because OspA is seldom expressed during infections of the reservoir (11). On the other hand, infected mice frequently have antibodies to P66 (11). Chronically infected mice with antibody to P66 would pass that antibody to the tick. In the absence of a barrier, such as that provided by OspA, this anti-P66 antibody might kill or inhibit *B. burgdorferi*. Immunization of animals or people with P66 could lead to the same situation: the antibody, effective in the absence of OspA or OspC, is ineffective in their presence. If P66 or other shielded membrane protein, such as P13 (49), is to be effective as a vaccine, it may be effective only in combination with antibody to OspA or OspC.

In conclusion, the findings suggest to us that the definition of surface for a pathogen depends on the nature of the probe. With respect to antibody and the protease trypsin, P66 was effectively "subsurface" when OspA and, to a lesser extent, OspC was present in the outer membrane. The surface of a pathogen may be metaphorically closer to a rain forest with its shadowed ground than to a Sonoran desert with its multitude of sunlit cacti.

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