Antibody-resistant Mutants of *Borrelia burgdorferi*: In Vitro Selection and Characterization

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Summary

We used polyclonal antisera and monoclonal antibodies (mAbs) to inhibit the growth of clonal populations of two strains of *Borrelia burgdorferi*, the Lyme disease agent, and thereby select for antibody-resistant mutants. mAbs were directed at the outer membrane proteins, OspA or OspB. Mutants resistant to the growth-inhibiting properties of the antibodies were present in the populations at frequencies ranging from 10^{-5} to 10^{-2}. The several escape variants that were examined were of four classes. Class I mutants were resistant to all mAbs; they lacked OspA and OspB and the linear plasmid that encodes them. Two other proteins were expressed in larger amounts in class I mutants; mAbs to these proteins inhibited the mutant but not the wild-type cells. Class II mutants were resistant to some but not all mAbs; they had truncated OspA and/or OspB proteins. Class III mutants were resistant only to the selecting mAb; they had full-length Osp proteins that were not bound by the selecting antibody in Western blots. In two class III mutants resistant to different anti-OspA mAbs, missense mutations were demonstrated in the *ospA* genes. Class IV mutants were likewise resistant only to selecting antibody, but in this case the selecting antibody still bound in Western blots.

In vitro selection of antigenic variants or mutants with antibodies has proven a valuable strategy for identifying or further characterizing antigens of infectious agents. Several different viruses have been studied by this approach: poliovirus (1), cytomegalovirus (2), coxsackie B (3), hepatitis A (4), hepatitis B (5), visna (6), rabies (7), Herpes simplex (8), influenza A (9, 10), and Newcastle disease (11) viruses among others. In vitro selection of antibody escape variants has also been successfully carried out from populations of parasites including *Plasmodium chabaudi* (12), *P. berghei* (13), and *Trypanosoma equiperdum* (14) in recent years.

Although in vitro selection of variants with antibodies was first demonstrated with bacteria (reviewed by Hadley in 1926 in reference 15), this experimental approach has seldom been applied to pathogenic bacteria since the 1950's. This is ironic in the heyday of molecular microbiology, because several of these early studies were directly or indirectly concerned with the bacterium's hereditary material. Dawson's finding in 1928 (16) that "S" forms of pneumococci could be selected from clonal populations of "R" forms by adding anti-"R" serum to the broth medium was one of the steps toward the elucidation of the identity of the transforming substance in pneumococci as DNA. Leidy et al. (17) used in vitro selection in their studies of transformation of *Hemophilus influenzae* in 1953. A year later, Uetake et al. (18) demonstrated that antisera in the growth medium selected for alternate flagella in the biphasic variation of *Salmonella*. In recent decades the only consistent application of this approach for bacteria has been the studies of antigenic variants of *Leptospira interrogans* by Shimono and Yanagawa (19), and in these studies the genetic basis for the escape mutations had not been determined.

Notwithstanding infrequent application for bacterial studies presently, there were compelling reasons to use in vitro antibody selection with *Borrelia burgdorferi*, the agent of Lyme disease. First, we had shown for the related species, *B. hermsii*, that an antiserum specific for one serotype could select for new serotypes in an isogenic population undergoing in vitro growth (20). The ability of polyclonal antisera and mAbs to agglutinate (21) and inhibit the growth of *B. burgdorferi* (21a) indicated that this was also possible with the Lyme disease agent. Second, previous studies had shown antigenic differences in outer membrane proteins, OspA and OspB, between strains (21–26) and also true antigenic variation of these proteins within a strain (25, 27–30). If borrelias did "escape"

killing (31), growth inhibition (32), or agglutination (21) by antibodies, it was likely that this was the consequence of antigenic variation. Third, the success of passive immunization with either polyclonal antisera or mAbs in protecting animals indicated that an in vitro study of antibody effects was relevant for studies of pathogenesis and immunoprophylaxis of Lyme disease (33–38).

In the present study we demonstrate in vitro selection of antibody-resistant mutants of *B. burgdorferi*. Among the several escape mutants evaluated, four major phenotypic classes were identified, and for two classes genotypes were determined.

Materials and Methods

Strains and Culture Conditions. *B. burgdorferi* isolates were the following: B31 (35210; American Type Culture Collection, Rockville, MD) (39) and HB19, a human blood isolate (30, 40). Cultures of strains B31 and HB19 had been passed continuously from 1982 and 1983, respectively, and each had been cloned at least twice by limiting dilution or colony plating (30). For some of the experiments these strains were cloned again by colony plating. Borrelias were grown in broth BSK II medium and harvested by methods previously described (41, 42). Cells were counted in a Petroff-Hauser chamber by phase-contrast microscopy. In some experiments borrelias were also grown as isolated in solid medium (42a). In brief, borrelias were added to BSK II medium with 1% low-gelling temperature agarose (SeaPlaque; FMC Corporation, Rockland, ME) kept liquid at 37°C. The cell suspension was then poured as a thin layer over solid BSK II medium containing 1.5% agarose in small polystyrene petri dishes (30). The plates were incubated at 34°C in a candle jar for 7–10 d, by which time colonies were visible to the naked eye.

Antibodies. The origins of the OspA-specific mAb H5332 (IgG2A; 42) and H3TS (IgG3; 22); the OspB-specific mAbs H6831 (IgG2A; 21), H614 (IgG3; 30), and H68 (IgG2A; 30); the VmpC-specific mAb H4825 (IgG2A; 21); and the Vmp21-specific mAb H10022 (IgG3; 43) have been given. In previous studies the following Western blot results were obtained: H5332, H3TS, H614, and H68 bound to B31 and HB19 (22, 30, 42, and our unpublished data); H6831 bound to B31 but not HB19 (21); and H4825 only bound to BhC (21). H10022 bound only to serotype 21 cells of *B. hermsii* strain HS1 (43). The OspA-specific mAb 6A4B (IgG2b) was the gift of Denee Thomas (University of Texas) (Comstock et al., manuscript submitted for publication). Specificities and reactivities of mAbs were confirmed by indirect immunofluorescence assay as previously described (42).

Additional mAbs were produced for this study. Adult female BALB/c mice were inoculated intraperitoneally on days 1 and 21 with 2×10^8 viable borrelias in PBS. On day 53, the mice were injected intravenously with 0.5×10^8 viable borrelias in PBS. Fusion of the mouse spleen cells with NS1 myeloma cells were performed on day 57 by a modification of the method of Oi and Herzenberg (44). Hybridoma supernatant fluids were screened by ELISA (45, 46) and then by Western blot analysis (see below). Hybridomas were cloned by limiting dilution, and ascites from the hybridomas were produced as described previously (47). The isotypes of antibodies were determined using a commercial kit (Immunotype; Sigma Chemical Co., St. Louis, MO).

6–8-wk-old female Lewis rats (LEW/N; Harlan-Sprague-Dawley, Indianapolis, IN) were immunized with viable HIB19 cells suspended in PBS as described previously (21a). In brief, for the first immunization the cell suspension was emulsified with CFA, and for the booster immunization at 4 wk the suspension in PBS alone was used. Serum was obtained during weeks 5 and 6 and pooled.

Mutant Selection in Broth and Solid Media. Conditions for growth inhibition with polyclonal antisera or mAbs in sealed, 96-well, flat-bottomed microtiter plates were essentially as described (21a). The initial inocula of spirochetes in each well varied by experiment. The antisera and ascitic fluids did not contain preservatives or antibiotics, and were heat-inactivated (56°C for 30 min) and filter sterilized before use. In general, the dilution of antisera and ascitic fluids used for selection were at least twice the minimal concentrations required for growth inhibition (21a). A previous study had shown that the growth medium did not contain rabbit complement sufficient to detectably enhance growth inhibition by antibody. Growth at 34°C in a 1% CO_2 atmosphere was monitored visually for changes in the color of the phenol red indicator and by phase contrast microscopy of wet-mounts of culture samples. In some experiments selection was carried out in tightly capped, 13 × 100-mm polystyrene culture tubes (Falcon Labware, Lincoln Park, NJ) containing 6 ml medium. For selection of antibody-resistant mutants in solid medium, the antiserum or ascitic fluid was diluted 1:100 in the top agarose at the time of the addition of the spirochetes. The bottom agarose did not contain antibody. Resistant colonies appeared on the plates within 7–10 d. Once cultures or colonies with antibody-resistant borrelias were identified, the populations were serially passed in medium first with and then without the selecting antibody. Variants were cloned by limiting dilution or single-colony plating (30).

In Situ Protease Treatment. Cleavage of surface-exposed proteins of viable borrelias with proteinase K (Boehringer Mannheim, Indianapolis, IN) was a modification of a previously described method (21). A 10-μl volume of an aqueous solution of proteinase K (20 mg/ml) was added to 490 μl of a suspension containing 10^9 washed borrelias/ml of PBS. After incubation of the cells with protease for 40 min at 22°C, the reaction was stopped by addition of PMSF. To assess the specificity of the protease for surface accessible proteins, the motility of the spirochetes after protease treatment was observed by phase-contrast microscopy. Under the conditions described here, >95% of the spirochetes were motile after protease exposure.

PAGE and Western Blot Analysis. Whole-cell lysates were subjected to PAGE with 12.5% acrylamide as previously described (42, 47). For Western blot analysis, proteins were transferred to nitrocellulose membranes, which were then blocked with 3% (wt/vol) dried, nonfat milk in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl (milk/10 mm Tris-HCl-150 mm NaCl [TS]) for 2 h. After the membranes were washed with milk/TS, they were incubated with mAbs in ascitic fluid diluted 1:100 in milk/TS. After washing with milk/TS, the blots were incubated for 1 h with alkaline phosphatase–conjugated, recombinant protein A/G (Immunopure; Pierce Chemical Co., Rockford, IL) diluted in milk/TS. The blots were developed using nitro blue tetrazolium chloride/5-bromo-4-chloro-3′-indolylphosphate *p*-toluidine salt (NBT/BCIP) (Pierce Chemical Co.).

DNA Extraction and Southern Blot Analysis. Extractions of total DNA and plasmid-enriched DNA from the borrelias, standard agarose electrophoresis, Southern blot analysis, and direct gel hybridization of low-percentage agarose gels were performed as described previously (22, 30, 48, 49). The probes for the *ospA* and *ospB* genes of HIB19 and B31 were recombinant plasmids pTRH43 and pTRH46, respectively (50–52). The probe DNA was labeled with α-[^{32}P]dATP by nick translation using a commercial kit (Bethesda Research Laboratories, Gaithersburg, MD). Hybridiza-

tion fluid contained 50% formamide; the blots were incubated with the probe at 37°C overnight. The blots were washed with 15 mM NaCl, 1.5 mM sodium citrate, 1 mM EDTA, 0.1% SDS at 64°C.

DNA Sequencing. The *ospAB* loci of selected variants, which had been cloned by single colony plating, were amplified by PCR essentially as described previously (53, 54) and directly sequenced using the dideoxy method and a dsDNA Cycle Sequencing kit (Bethesda Research Laboratories). PCR primers represented nucleotides 1–20 and 1896–1915 (opposite strand) of the *osp* locus sequence (52; GenBank accession number X14407). Approximately 100 ng of total borrelial DNA was amplified for 20 cycles with the following conditions: 94°C for 1 min, 50°C for 30 s, and 70°C for 3 min. Taq polymerase (Perkin-Elmer-Cetus, Norwalk, CT) was used and the buffer conditions were as recommended by the supplier. Before sequencing, PCR fragments were purified by precipitation in 2 M ammonium acetate with an equal volume of isopropanol. Sequencing primers were located approximately every 200 nucleotides throughout the *osp* operon and were labeled by T4 kinase with ^{32}P. Approximately 10 ng amplified DNA was used per sequencing reaction, and cycle sequencing conditions were as follows: 20 cycles at 95°C for 30 s, 55°C for 30 s, and 70°C for 1 min. Nucleic acid sequences were deposited into the EMBL database.

Results

Selection of Antibody-resistant Mutants. During development of the in vitro growth inhibition assay (21a), we observed microscopically that, even when overall growth was clearly inhibited and the majority of spirochetes were aggregated, blebbed, and nonmotile, there were rare spirochetes in the antibody-treated cultures that remained isolated, smooth surfaced, and motile. We reasoned that if the persisting, motile spirochetes were antigenic variants, upon subsequent passages into fresh, antibody-containing medium, the proportion of motile spirochetes would further increase. Alternatively, if motile spirochetes were antibody bound but had yet to aggregate or cease movement, then we would expect that the proportion of motile cells in the cultures would not increase during serial passage in the presence of the same antibody. To distinguish between these explanations, we serially passed *B. burgdorferi* isolates in medium containing antisera and mAbs.

In our first experiments we used broth cultures containing a total inoculum of 10^8 spirochetes and either rat polyclonal serum or mAbs to OspA and OspB, such as H5332 and H614. After 3–7 d of incubation, we blindly transferred 0.1 ml to 6 ml fresh medium containing the same antibody concentration. This was repeated after another 3–7 d of incubation. At least three different tubes were used for each selecting antibody. Control tubes either without antibody or with irrelevant antibodies were passed in parallel.

When the first cultures were compared with the second and third cultures, the proportion of motile cells in the tube increased from <1% in the first cultures to >90% in the third culture. After two to three passages, the cell counts at entry into stationary phase of "resistant" cultures in antibody-containing medium were as high as the wild-type strain grown in the absence of antibody or in the presence of irrelevant antibodies. The resistant phenotypes remained after cloning by limiting dilution or single-colony plating and through at least 6–20 serial passages in the absence of selecting antibody. After the demonstration with broth cultures of the selection of resistant variants, we studied the selection phenomenon further using broth medium in microtiter plates and solid medium as described below.

Many resistant variants were obtained during the course of the foregoing studies and have been stored for further analysis. Full description of each of the variants and precise determination of the frequency of each type of mutation is beyond the scope of this report. Nevertheless, a survey of randomly selected variant populations from both broth and solid medium was carried out for this study. All of the variants catalogued here had stable resistance phenotypes through serial passage in the absence of antibody and after cloning by either limiting broth dilution or colony plating. Each of these variants were subjected to PAGE and Western blot analyses; some were further characterized by in situ treatment with protease, Southern blot analysis, and DNA sequencing.

Although it is possible that additional types of variants will be revealed by analysis of the complete set of variants, the analysis to date reveals considerable heterogeneity of the phenotypes conferring antibody resistance. Under these selection conditions, we isolated four major classes of escape mutants. These are summarized in Table 1 and described below. Mutants of class I are characterized in the greatest detail here.

Biochemical Characterization of Class I Mutants. The first class of mutant was the predominant phenotype after selection of 10^5 to 10^8 HB19 cells with anti-HB19 polyclonal antiserum from rats immunized with whole borrelias. Not only was this type of variant not inhibited by the selecting polyclonal antiserum, it also was resistant to growth inhibition by all of the relevant mAbs to OspA and OspB. This resistance phenotype was unchanged after 20 passages (~300 generations) in the absence of antibody.

Fig. 1 shows the PAGE gel of the wild-type isolate and one of the polyclonal antibody–resistant variants, in this case R1, before and after protease treatment. The escape variant did not have detectable OspA or OspB protein. The absence of Osp proteins in either full-length, truncated, or fused forms was confirmed by Western blot analysis with mAbs H5332, H3TS, H614, and H68 (data not shown). In place of OspA and OspB were an abundant protein of apparent M_r 28,000 (28K), which seemed to be present in small amounts in the wild-type population, and another protein of 36K, which was not detectable in the antibody-susceptible population. Treatment of the cells with proteinase K provided evidence that the 28K and 36K proteins, like Osp and 66K proteins (21), are exposed at the cell's surface.

Mutants lacking OspA and OspB were also selected from newly cloned populations of HB19 with the OspB-specific mAb H614 or the OspA-specific antibody H5332 in broth or solid medium. Using the latter antibody and also the OspB-specific mAb H6831, which binds to B31 but not HB19, we selected a mutant lacking OspA and OspB from strain B31.

mAbs to the R1 type of HB19 mutant were produced by immunizing mice with viable whole cells; both were IgG3. One of the mAbs, 1C8, bound in Western blots to the 28K protein of R1 and also to a smaller amount of the protein

Table 1. *Classes of Antibody-resistant Mutants of B. burgdorferi*

Class	PAGE phenotype	Antibody resistance		Western blot reactivity	
		Selecting antibody	Plus other antibodies	Selecting antibody	Plus other antibodies
I	$OspA^-OspB^-$	+	+	–	–
II	ΔOspA/ΔOspB*	+	+	–	–
III	$OspA^+OspB^+$	+	–	–	+
IV	$OspA^+OspB^+$	+	–	+	+

* Truncation of OspA and/or OspB.

in the population of wild-type cells of HB19 (Fig. 2). mAb 6B5 was specific for the 36K of the Osp less-resistant variant. This latter mAb did not detectably bind to any protein in the wild-type cells (Fig. 2).

Antibodies 1C8 and 6B5 were used in twofold dilutions in the growth inhibition assay in the absence of complement (21a). Whereas antibodies 1C8 and 6B5 individually inhibited the growth of the Osp-less mutant at dilutions up to 1:2,048 and 1:8,192, respectively, they did not inhibit the growth of the wild-type cells at dilutions as low as 1:8.

The investigations of this group of mutants indicated that OspA and OspB are apparently not required for in vitro growth and that other proteins may take the place of the Osp proteins at the cell surface. Furthermore, antibodies specific for the new proteins inhibit mutant but not wild-type cells.

Genetic Characterization of Class I Mutants. Absence of both OspA and OspB could be explained by either failure of expression of the *ospAB* operon or loss of the genes. Inasmuch as the *osp* genes are plasmid borne (48), it was also reasonable to attribute the phenotype to absence of the entire linear plasmid. Although loss of other linear and supercoiled plasmids from *B. burgdorferi* had been documented (28, 49), spontaneous or provoked loss of the 49-kb plasmid, which encodes OspA and OspB, had not been noted previously. Nevertheless, this possibility was first examined by extraction of total DNA and performance of plasmid analysis. Fig. 3, *EB*, shows the absence of the 49-kb linear plasmid from two different clones of HB19 mutants with the $OspA^-OspB^-$ phenotype, R1 and R2, and the presence of this plasmid in wild-type HB19 (*W*). In other respects the plasmid profiles were the same. Southern blot analysis (Fig. 3, *SB*) with probes for both *ospA* and *ospB* genes provided evidence that these genes were absent from the remaining plasmids of $OspA^-OspB^-$ variants R1 and R2 while still present in the 49-kb plasmid of the antibody-susceptible, $OspA^+OspB^+$ wild type. To determine whether the *osp* genes were present on the chromosome, a Southern blot analysis of total DNA, including chromosomal and plasmid DNA, which had been digested by restriction enzymes known to cut within the *ospAB* operon, was carried out. There were no hybridizing bands in the DNA from the resistant variants R1 and R2 (Fig. 4).

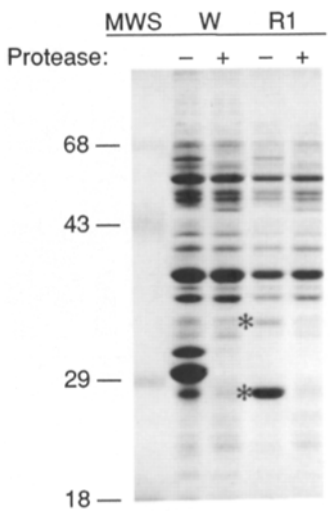

Figure 1. Coomassie blue-stained protein of wild type (*W*) and resistant mutant R1 of *B. burgdorferi* strain HB19. Spirochetes were (+) or were not (–) treated with proteinase K (Protease) before cell lysis. The asterisks indicate proteins of 28K and 36K that were present in detectably increased amounts in R1. Molecular weight standards (*MWS*; ×1,000) were BSA (68), OVA (43), carbonic anhydrase (29), and β-lactoglobulin (18).

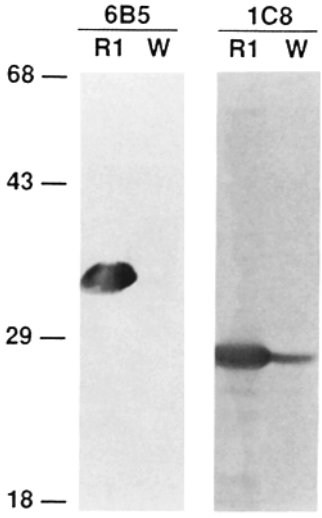

Figure 2. Western blot analysis of resistant mutant R1 and wild-type (*W*) cells of *B. burgdorferi* HB19 with mAbs to the 28K (1C8) and 36K (6B5) proteins of R1. The molecular weight standards, the locations of which are shown to the left, are described in the legend of Fig. 1.

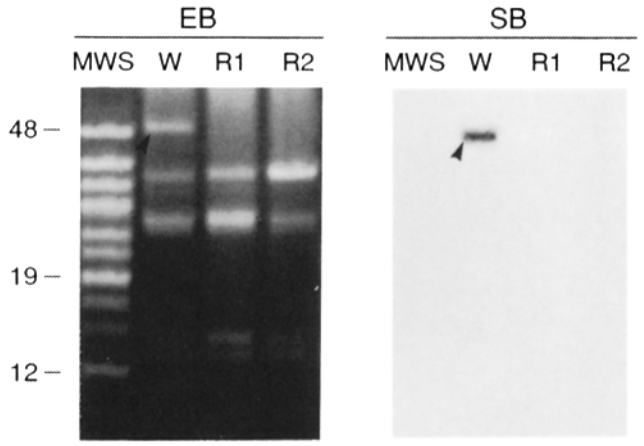

Figure 3. Resistant mutants R1 and R2 lack the 49-kb linear plasmid of wild-type (*W*) *B. burgdorferi* HB19. (*Left*) Ethidium bromide–stained (*EB*) 0.2% agarose gel of plasmid-enriched DNA from spirochetes. (*Right*) Southern blot analysis (*SB*) of dried gel shown on the left; the probe for *ospA* gene was pTRH43 (51). Arrowheads indicate the location of the 49-kb plasmid in W cells. The locations in the gel of selected high molecular weight standards of linear DNA (*MWS*; Bethesda Research Laboratories) are shown to the left and are in kilobases.

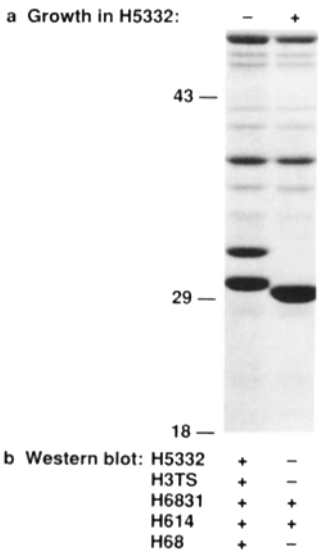

Figure 5. An example of a class II mutant of *B. burgdorferi*. A mutant selected for growth (+) in the presence of the OspA-specific mAb H5332 was compared with the susceptible (–) wild-type parent B31. (*a*) Coomassie blue–stained protein of the mutant and wild type; the molecular weight standards are described in legend for Fig. 1. (*b*) Western blot analysis results with mAbs to different epitopes of OspA (H5332 and H3TS) and OspB (H6831, H614, and H68).

Plasmid analysis and Southern blot analysis was also carried out with four other Osp-less mutants of HB19 and three Osp-less mutants of B31, selected with mAbs H5332, H614, or H6831. These mutants, like R1 and R2, lacked the 49-kb linear plasmid (data not shown).

Class II Mutants. The second type of escape mutants, among B31 as well as HB19 cells, was observed with mAbs but not polyclonal antisera. An example of this type of mutant is shown in Fig. 5. This escape mutant was selected with the OspA-specific mAb H5332. Instead of the OspA and OspB proteins of the wild-type B31, a single Osp-like protein of slightly smaller apparent size is expressed by the escape mutant. The partial identity of this protein with Osp proteins was demonstrated by the binding in Western blots with Osp-specific mAbs. Two OspB-specific antibodies, H6831 and H614, bound to the new protein in a Western blot. As expected, H5332, the antibody used for selection, did not bind to the mutant in the blot. Two other mAbs, H3TS and H68, which were not used for selection, also did not bind to the mutant in the blot. H68 binds to an epitope in OspB that is distinct from the epitopes recognized by H6831 and H614 (30). H3TS binds to an OspA that is different from the epitope bound by H5332 (22). Thus, more than one antibody epitope was missing from the mutant cells. These variants appeared to represent truncations and/or fusions of Osp proteins (54a).

A similar phenotype of this class were escape mutants of HB19 and B31 that by PAGE lacked OspB but still expressed full-length OspA proteins (data not shown). These mutants were not bound by any of OspB-specific antibody but were still recognized by both OspA-specific mAbs H5332 and H3TS in blots. In this respect these variants resembled spontaneous OspB$^-$ variants observed by Schwan and Burgdorfer and Bundoc and Barbour previously (27, 30).

Class III Mutants. The third type of escape mutant was obtained when mAbs were used in either broth or solid media but not when polyclonal antisera were used. An example of this class of mutant is shown in Fig. 6. Although the B31 variant resistant to growth inhibition by antibody H6831 has OspA and OspB of the same size as the susceptible wild-

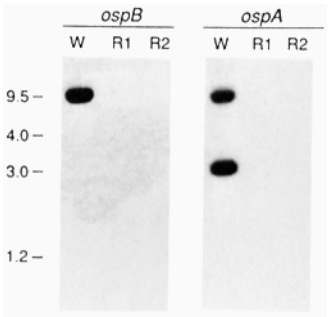

Figure 4. Southern blot analysis of total DNA from wild-type (*W*) and resistant mutants R1 and R2 of *B. burgdorferi* HB19. DNA was digested with EcoRI and the fragments were separated on a 0.7% agarose gel. After transfer to membrane, blots were probed for *ospB* or *ospA* using pTRH46 and pTRH43 (51), respectively. The locations of molecular weight standards of 4.0 and 1.2 kb and of the 9.5- and 3.0-kb hybridizing fragments are shown on the left. The *ospA* gene but not the adjacent *ospB* gene contains an EcoRI site (52).

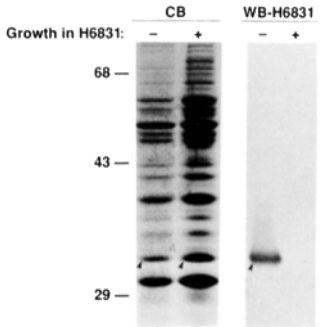

Figure 6. An example of a class III mutant of *B. burgdorferi*. A mutant selected for growth (+) in the presence of the OspB-specific mAb H6831 was compared with the susceptible (–) wild-type parent B31. (*Left*) Coomassie blue–stained (*CB*) proteins of the mutant and wild type. The migrations of the OspB proteins, which are indicated by arrowheads, of the mutant and wild type are identical. (*Right*) Western blot analysis (*WB*) of gel shown to left; the blot was probed with antibody H6831. The molecular weight standards are described in the legend for Fig. 1.

Table 2. *Class III Antibody-resistant Mutants of B. burgdorferi B31*

Selection	Western blot analysis					
	H5332	6A4B	H3TS	H614	H6831	H68
None	+	+	+	+	+	+
H5332	−	+	+	+	+	+
6A4B	+	−	+	+	+	+
H614	+	+	+	−	+	+
H6831	+	+	+	+	−	+

type, H6831 did not bind to the resistant mutant in a Western blot. Similar findings were made with mutants selected with other antibodies. That is, OspA and OspB were unchanged in apparent size but the selecting antibody did not bind in Western blots. However, unlike the situation with class I and II mutants, mAbs to other epitopes of OspA or OspB still bound to the escape mutants in blots. A summary of B31 variants that represented this third type of resistance is shown in Table 2. Mutants resistant to antibodies H5332, 6A4B (OspA), H6831, or H614 (OspB) were no longer recognized by the selecting antibody in blots, while battery antibodies that were not used for selection still bound to Osp protein and inhibited growth.

We asked whether mutants of this class had changes limited to the epitope site, perhaps the result of point mutations, and, accordingly, examined by DNA sequence analysis two of the B31 mutants, A1 and A3. These mutants, both of which had been cloned again after selection, were resistant to the growth inhibition by the OspA-specific mAbs H5332 or 6A4B, but had an OspA of the same apparent size as the wild type and was still bound by other OspA-specific antibodies as well as OspB-specific antibodies (Table 2). Other relevant mAbs still inhibited the growth of the mutants. The *ospA* genes from the antibody-resistant mutants were amplified by PCR and the amplified product was directly sequenced. In the case of the H5332-resistant mutant, the *ospA1* gene had a single base change, a transition from G to A, at nucleotide 616 of reference 52 (Fig. 7). The effect of this transition would be the replacement in OspA1 of the position 156 glycine, which has a small, aliphatic side chain, with an arginine, which has a bulky, basic side chain. The mutant resistant to antibody 6A4B, which is represented by *ospA3* and OspA3 in Fig. 7, also had a G to A missense mutation, in this case changing the glycine residue at position 219 of OspA to aspartate with its acidic side chain.

Class IV Mutants. Other escape variants of HB19 grew in the presence of antiserum to HB19. PAGE and Western blot showed that the variant possessed apparently full-length OspA and OspB proteins, which were bound by antibodies in polyclonal serum and by the battery of all specific mAbs used in the study (data not shown). This type of escape variant was also found among the resistant population of HB19 and B31 strains selected with OspA-specific mAb H5332 and the OspB-specific mAbs H614 and H6831. In these cases the antibody neither inhibited the growth nor agglutinated the cells of the mutant, but in Western blots it still bound to the Osp protein against which the selecting antibody was directed. All other relevant antibodies were still effective at inhibiting growth and agglutinating the mutants. The selecting antibodies also still bound to the antibody-resistant borrelias in the indirect immunofluorescence assay, for which whole cells were dried and fixed on slides (42).

Frequency of Mutants in Broth Medium. Although the primary goal of this study was an evaluation of the phenotypic

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                                   150                                 160
OspA:   AspGlyThrArgLeuGluTyrThrGlyIleLysSerAspGlySerGlyLysAlaLysGlu
ospA:   GACGGAACCAGACTTGAATACACAGGAATTAAAAGCGATGGATCTGGAAAAGCTAAAGAG
OspA1:                                                 Arg
ospA1:                                                 A
OspA3:
ospA3:

                                   170                                 180
OspA:   ValLeuLysGlyTyrValLeuGluGlyThrLeuThrAlaGluLysThrThrLeuValVal
ospA:   GTTTTAAAAGGCTATGTTCTTGAAGGAACTCTAACTGCTGAAAAAACAACATTGGTGGTT
OspA1:
ospA1:
OspA3:
ospA3:

                                   190                                 200
OspA:   LysGluGlyThrValThrLeuSerLysAsnIleSerLysSerGlyGluValSerValGlu
ospA:   AAAGAAGGAACTGTTACTTTAAGCAAAAATATTTCAAAATCTGGGGAAGTTTCAGTTGAA
OspA1:
ospA1:
OspA3:
ospA3:

                                   210                                 220
OspA:   LeuAsnAspThrAspSerSerAlaAlaThrLysLysThrAlaAlaTrpAsnSerGlyThr
ospA:   CTTAATGACACTGACAGTAGTGCTGCTACTAAAAAAACTGCAGCTTGGAATTCAGGCACT
OspA1:
ospA1:
OspA3:                                                          Asp
ospA3:                                                          A

                                   230                                 240
OspA:   SerThrLeuThrIleThrValAsnSerLysLysThrLysAspLeuValPheThrLysGlu
ospA:   TCAACTTTAACAATTACTGTAAACAGTAAAAAAACTAAAGACCTTGTGTTTACAAAAGAA
OspA1:
ospA1:
OspA3:
ospA3:
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Figure 7. Nucleotide and deduced amino acid sequences of two class III mutants of *B. burgdorferi* B31. The *osp* operons of both mutants were sequenced and compared with the sequence of Bergström et al. (52) for wild-type *osp* genes for this strain. Sequences corresponding to amino acids 140–240 of unprocessed OspA are shown. OspA and *ospA* represent the wild-type protein and gene. OspA1/*ospA1* and OspA3/*ospA3* are the deduced protein/nucleotide sequences of mutants resistant to OspA-specific mAbs H5332 and 6A4B, respectively.

Table 3. *Frequency of Mutants Resistant to Selected mAbs among Two Populations of B. burgdorferi in Broth Medium*

Estimated cells per well at t_0	HB19		B31		
	H10022	H614	H10022	H5332	H614
1	22/48* (0.6)§	0/48	28/48 (0.9)	–‡	–
10	96/96	14/96 (3×10^{-2})	96/96	–	–
10^2	–	43/48 (4×10^{-2})	–	0/48	0/48
10^3	–	–	–	16/96 (2×10^{-4})	4/96 (0.5×10^{-4})
10^4	–	–	–	47/48 (5×10^{-4})	–
10^5	–	–	–	–	–
10^6	–	–	–	–	–

* Wells with growth/total wells inoculated per microtiter plate.
‡ Not done.
§ Frequency of cells with growth potential at t_0.

diversity of the antibody-resistant mutants, we also estimated the frequency of antibody-resistant mutants in populations with selected antibodies. For this series of experiments we again started with tube cultures, in this case inoculating tubes in triplicate or quadruplicate with 10^1 to 10^7 spirochetes per tube instead of the 10^8 we had used in the initial selections. Incubation was continued for 14–21 d. If growth was detected in at least one of the three to four tubes containing a given inoculum of cells, the prevalence of variants in the original population was estimated to be the reciprocal of that inoculum. With strain B31 and mAbs H5332, H6831, or H614, resistant mutants were found in at least one of the tubes containing 10^4 borrelias; with $\geqslant 10^5$ cells per tube, all tubes showed growth. This provided an estimate of the prevalence or the frequency of resistant mutants in the B31 population of 10^{-4} for each of the mAbs. The prevalence of escape variants among HB19 cells was 10^{-2} for both H5332 and H614, but 10^{-5} with polyclonal antiserum to HB19. In another experiment in which dilutions calculated to yield an average of one HB19 cell per tube were used, 7 of 10 tubes without antibody and 1 of 20 tubes with H614 showed growth after 14 d of incubation.

The prevalence of antibody escape mutants among HB19 and B31 populations was estimated more precisely using multiple samples of the populations (Table 3). Cultures were serially diluted in fresh medium and placed as 200-μl volumes in each of 48–96 individual microtiter plate wells; ascitic fluids were added for 1:100 dilutions. Antibody H10022, which is directed at a surface protein of *B. hermsii*, was the control. Growth in the wells was followed by observation of change in the indicator from pink to yellow and by phase contrast microscopy. If wells showed growth, the culture medium was transferred to tubes of medium containing the selecting antibody. After growth in the tubes the phenotype of the resistant mutant was examined by PAGE, Western blot, and plasmid analysis. The HB19 and B31 populations had been cloned again by single-colony plating or limiting dilution of these experiments. The prevalence or frequency of resistant mutants, i.e., the number of noninhibited cells per well at the time of inoculation, was calculated using tables for the Poisson distribution.

Table 3 shows that the frequency of H614-resistant mutants was again approximately two orders of magnitude higher with HB19 cells than with B31 cells. The 14 of 96 wells with growth were passed and characterized as to phenotype by PAGE, Western blot, and plasmid analysis. Each of the resistant populations had the class I phenotype; all lacked the 49-kb linear plasmid. The frequency of antibody-resistant mutants among B31 population was $2–5 \times 10^{-4}$ for antibody H5332 and 0.5×10^{-4} for antibody H614 (Table 3). This was similar to what had been observed with tube cultures. When the phenotypes of the mutants was evaluated we found that of the 16 H5332 escape mutants, 1 was class I and 15 were class IV. The 4 H614-resistant mutants were class III.

Frequency of Mutants on Solid Medium. Current procedures for cultivation of *B. burgdorferi* in solid medium plates yield efficiencies of plating between 50 and 100% (42a). Therefore, colony growth in the presence of antibodies was more likely to represent antibody resistance and not factors leading to improved growth on solid medium. Furthermore, in the process of cloning individual variant populations and in preliminary studies of selecting mutants on solid medium, we

had observed that mutants representing each phenotype class grew in solid medium conditions. Thus, solid medium was suitable for providing another estimate of variant frequency.

For this study we used the same newly cloned B31 population of the previous experiment. Antibody H614 was added to the top agar of the medium for a concentration 10 times the minimum inhibitory level. In studies with broth medium we had demonstrated that addition to the medium of 12% (vol/vol) ascitic fluid containing an irrelevant mAb did not inhibit borrelial growth. To control for the possibility of greater sensitivity of colony growth to ascitic fluid in general, we included an ascitic fluid control, H10022, in this study as well as the no antibody control. The results of colony selection for resistance to antibody H614 at various inocula of B31 cells per plate are shown in Table 4. In this experiment the efficiency of plating was between 50 and 100% in plates with either no antibody or the irrelevant ascitic fluid. There as no difference between efficiency of plating between the two controls. On the plates containing the anti-*B. burgdorferi* antibody, well-demarcated colonies were observed within the same time interval that colonies were noted on control plates. The colonies growing on the antibody plates had morphologies that were similar to colonies on control plates. Colonies were detected on H614 antibody plates at estimated cell inocula of 5,000 or greater. The frequency of resistance to H614 in B31 on solid medium was estimated to be 0.8–2.9 $\times 10^{-4}$, a value similar to the results using broth medium. When the phenotypes of the 18 colonies of B31 growing on plates containing H614 were determined, 5 were found to be class II and 13 were class III.

Discussion

The exact mechanism by which antibodies alone inhibit the growth of the borrelias is not yet known (21a). Whatever the mechanism, *B. burgdorferi* had a variety of ways to escape this inhibition under the experimental conditions of this study. Ultimately, the significance of each of these strategies for human and domestic animal disease will need to be evaluated in vivo. Evidence to date suggests that antigenic variation occurs during the course of *B. burgdorferi* infection in both animals and humans, but the phenotypes of the putative variants have not been characterized (reviewed in reference 55). For further studies of this phenomenon, the present study provides an elementary "playbook" with which to analyze the hide and seek between the pathogen and the host's immune system. It may prove easier to follow the action in vivo when the strategies are known ahead of time.

Table 4. *Frequency of Antibody-resistant Mutants in a B. burgdorferi B31 Population Plated on Solid Medium*

Estimate of cells/plate	Mean no. colonies/plate*		
	None	H10122	H614
10^1	6	4	0
5×10^1	28	40	0
10^2	72	91	0
5×10^2	250	185	0
10^3	tntc‡	tntc	0
5×10^3	tntc	tntc	1
10^4	tntc	tntc	3
5×10^4	tntc	tntc	8
10^5	tntc	tntc	10
5×10^5	tntc	tntc	40

* In triplicate.
‡ Too numerous to count.

After our first experiments we were stuck by the analogy between use of antibodies to achieve growth inhibition and use of antibiotics for the same aim. Knowing the value of antibiotic-resistant mutants for understanding a number of different cellular functions, we wondered whether antibodies could be used to isolate mutants in the surface antigens of the borrelias. The methodologies for undertaking genetic studies of borrelias are limited, but certainly one that had not been utilized to any great extent was the isolation of mutants in traits of interest (46).

We have applied the terms "mutation" and "mutant" to the phenomena described here. In the case of two classes, I and III, an actual change in the genome was documented. It is likely that the variants grouped in classes II and IV also represent actual change in the DNA. Evidence for this assertion was the stability of the resistance phenotypes during passage in absence of selecting agent. Furthermore, the experiments with populations divided into small samples in microtiter plates indicated that the resistant cells were present before exposure to the inhibiting antibody. Only a fraction of the wells at certain inocula showed growth of resistant forms. If resistance was an adaptation in response to antibodies, then one would expect to see growth in each of the wells (56).

Strictly speaking, we can at this point only say that the observed changes in the protein components or in the genome itself were associated with antibody resistance. To prove the cause-and-effect relationship between the altered DNA and antibody resistance, the candidate mutation would have to be successfully transferred as a well-defined DNA packet to an antibody-susceptible *B. burgdorferi*, thereby conferring antibody resistance. As transformation or transduction is not yet possible with borrelias, the relationships will remain associations by precise definition. Nevertheless, the changes we observed in the Osp proteins and the *osp* genes themselves after selection with mAbs were, in their specificities for the selecting antibody, compelling evidence for cause-and-effect relationships.

In terms of attribution, the least ambiguous group of resistant mutants were those lacking both OspA or OspB proteins and so placed in class I. All mutants with this phenotype, whether of HB19 or B31 origin, did not have the 49-kb plasmid that bears the *osp* operon. Moreover, there was no evidence that this operon had transposed elsewhere in the genome. Variants not expressing OspB had been observed

previously, but there had not been detectable change in the 49-kb plasmid or even in the *osp* genes themselves (27, 28, 30). There have been strains, such as FI from Sweden and DN27 from California, of *B. burgdorferi* that did not express either the OspA or OspB protein, but in both isolates the large plasmids bearing the *osp* genes were still present (49). In addition, both FI and DN27 retain the potential to produce OspA (25, 29; A. G. Barbour and S. Bergström, unpublished observations).

While the OspA$^-$OspB$^-$ mutants of this study have lost this potential, the HB19 and B31 lineages examined here did express one or two different surface proteins that were approximately equivalent to OspA and OspB in abundance. One of these proteins, with an apparent size of 28K, was present in small amount in the wild-type population. The other protein, one of 36K apparent size, was not detectable in the same wild-type population by Western blot analysis with specific antibody. mAbs with specificities for these new proteins with surface exposure inhibited the growth of the OspA$^-$OspB$^-$ mutants but not the growth of the wild type. These findings suggest that when the 49-kb plasmid is absent, expression of other Osp-like genes are derepressed or otherwise upregulated. This may also occur when the *osp* operon is still present but not expressed. Strains DN27 or FI produce a protein of ~20–22K when there is no apparent expression of the *osp* operon (22, 25, 29, 49).

OspA$^-$OspB$^-$ mutants were first obtained with polyclonal antisera. The rat antiserum to HB19 contained antibodies to a variety of other proteins besides OspA and OspB (21a). Although the studies with mAbs showed that antibodies to OspA or OspB are sufficient to inhibit growth, it is possible that the polyclonal antiserum contained antibodies to other, less abundant components encoded by the 49-kb plasmid, and that these antibodies, alone or in concert, also inhibit borrelial growth.

Single mAbs to OspA or OspB were also effective for selecting OspA$^-$OspB$^-$ mutants from a newly cloned population of HB19. The frequency of mutants with this phenotype was $\sim 10^{-2}$ with mAb H614 (Table 3). The frequency of OspA$^-$OspB$^-$ mutants in the B31 population was 100-fold lower. A previous study had shown that in B31 the 49-kb plasmid is very stable; it was the only linear plasmid left after ~7,000 generations of in vitro cultivation, and attempts to cure the cell of plasmids with agents specific for DNA or its replication (42a). In the present study the plasmid was cured using antibody selection. The 49-kb plasmid and the Osp proteins it encodes are dispensable for in vitro growth in both HB19 and B31.

The next characterized class of antibody resistance mutants was III. The frequency of class III mutations was $\sim 10^{-4}$ in B31 and HB19 populations. The examples of this class made what appeared to be full-length OspA and OspB proteins, and the selecting antibody alone, among the battery's antibodies, did not bind to the mutant in Western blots. The findings suggested that the putative changes in the Osp protein were limited to the epitope for the selecting antibody. In the two mutants examined in detail for this study, the missense mutations, G to A transitions, result in the replacement of a glycine residue with a charged amino acid. The two mutations were in different parts of the OspA sequence, as would be expected for the different epitopes for the two mAbs (Comstock, et al., manuscript submitted for publication). The amino acid change in the H5332-resistant mutant occurred in a 13-residue region of B31's OspA that differs by one and two amino acids from the deduced OspA proteins of isolates ACAI and Ip90 from Sweden and Russia, respectively (26). H5332 inhibits the in vitro growth of B31 but not ACAI or Ip90 (21a; and unpublished findings).

The phenotype of some class II mutants, for example the mutant of Fig. 5, is that of a single chimeric OspA/B protein. We have observed spontaneously arising variants of *B. burgdorferi* in which deletion of part of the *osp* locus generates *ospA/B* gene fusions that encode a single OspA/B protein (54a). Such deletions could be the consequence of homologous recombination between *ospA* and *ospB*, which share direct repeats (52). The extent to which the fusion protein antigenically resembles OspA or OspB reflects the crossover point along the genes. Class II mutants that appear to completely lack OspB but express full-length OspA may represent fusion in which the crossover occurred between the extreme 3′ regions of *ospA* and *ospB*. Alternatively, they could have point mutations in *ospB* that prematurely terminate the protein, thereby producing the OspB$^-$ phenotype. We have also observed this type of variant (54a).

The least understood class of mutants was IV. These mutants, while no longer inhibited in their growth or agglutinated by the selecting antibody, were still recognized by the antibody by Western blot and an immunofluorescence assay using dried, fixed cells. This phenomenon is similar to what was observed among antigenic variants of Newcastle disease virus; some virus variants, while still capable of binding the selecting antibody in solid phase assays, were only slightly neutralized by it (11). These characteristics suggest that in mutants of this class the linear epitope for the antibody is not substantially altered but access of the antibody to the epitope has been hindered, at least when the proteins are in situ and in a native state. If a class IV mutant has a missense mutation, it is not likely to be in that part of the gene corresponding to the linear epitope. The mutation could also be in a protein closely associated with the Osp protein target in the cell. The effect of the latter type of mutation would be to alter or reduce exposure of the epitope. Whatever the mechanism, this phenomenon would be unrecognized without a function-oriented assay, such as the growth inhibition or agglutination. A patient or experimentally infected animal may have antibodies that bind in an immunofluorescence or Western blot assay but not to viable cells. Such antibodies may make little contribution to an effective immune response.

The findings encourage further investigation and use of the antibody-resistant mutants. The class I mutants lacking the genes for OspA and OspB can be used to study the role of the proteins in pathogenesis. Such plasmid-less mutants could also serve as appropriate hosts for genetic transfer experiments. In many cases class II mutants appear to be the

result of deletions between direct repeats in the *osp* operon (54a). The role of this type of mutation in avoiding the immune response could be evaluated directly by applying the PCR assay to blood and tissue samples from experimentally infected animals and Lyme disease patients. Class III mutations will be useful for identifying the linear epitopes of mAbs against Osp and other proteins that elicit growth-inhibiting antibodies. Finally, class IV mutations, while poorly understood at present, may prove useful for study of the conformation of Osp proteins in the cell and their interactions with other cell components.

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