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Protective Immunity Is Induced by a *Borrelia burgdorferi* Mutant That Lacks OspA and OspB

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A mutant of virulent *Borrelia burgdorferi* 297 was apparently selected for by long-term storage at 5°C. This mutant was found to lack the plasmid which encodes outer surface protein A (OspA) and OspB. In addition to the loss of the OspA and OspB proteins, the mutant lacked two lipoproteins, of 20 and 7.5 kDa, that were observed in the wild type. Since the mutant was not recovered from the tissues or blood of hamsters injected with the mutant, the mutant was determined to be noninfectious. Hamsters vaccinated with noninfectious mutant 297 were protected completely from challenge with virulent wild-type 297 spirochetes. Prechallenge sera from hamsters vaccinated with mutant 297 lacked antibodies to OspA and OspB, while those from hamsters vaccinated with virulent wild-type 297 or avirulent 297 exhibited antibodies to these proteins. Hamsters vaccinated with virulent wild-type 297 or mutant 297 elicited antibodies to OspC and a 39-kDa protein (P39), whereas hamsters vaccinated with avirulent 297 lacked these antibodies. These results suggest that OspC and/or P39 are important for the development of a protective immune response. Study of this mutant may elucidate factors important to the development of a Lyme disease vaccine.

Lyme disease remains the most common arthropod-borne infection in the United States. The causative agent of the disease was recognized as a spirochete (8, 12, 49) and described as a new *Borrelia* species (29). The spirochete, *Borrelia burgdorferi*, is transmitted following the bite of an infected ixodid tick (12). In humans, Lyme disease is a multisystem illness that primarily affects the skin, nervous system, heart, and joints (48).

Considerable effort has been invested in the development of a Lyme disease vaccine. The feasibility of vaccination as a method for the prevention of Lyme disease was evident when hamsters vaccinated with killed whole-cell *B. burgdorferi* were protected against challenge with a virulent homologous strain (27). Subsequent research has been directed towards the development of a Lyme disease vaccine with other animal models and has focused on the identification of *B. burgdorferi* immunoprotective antigens (1, 7, 14, 36, 37, 41, 42, 51).

A sizeable amount of information has been published pertaining to outer surface protein A (OspA), which has an apparent molecular mass of 31 kDa, as determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and its effectiveness in eliciting protective immunity. In active immunization studies, vaccination with recombinant OspA in Freund's adjuvant and from strain N40 was reported to protect immunocompetent mice from challenge with strain N40 (18, 19). In contrast, recombinant OspA in $Al(OH)_3$ and derived from *B. burgdorferi* PKo did not protect gerbils from infection with strain PKo (37). Also, posttranslational lipid attachment was found to be a critical determinant for OspA immunogenicity (17).

However, immunological heterogeneity (6, 20, 55) and molecular heterogeneity (30, 34, 57) of OspA exist. Antigenic variations in other outer surface proteins of *B. burgdorferi* have been observed (11, 31, 36, 43, 44, 52, 54). In addition, antibodies against OspA are developed late in the course of disease in tick-infected humans (3, 33, 53, 56).

The selection of antigenic variants (or escape variants) of *B. burgdorferi* induced by antibodies was recently reported (15, 16, 39). In each of these reports, monoclonal antibodies (MAbs) or polyclonal antisera were used to inhibit the growth of wild-type *B. burgdorferi* and select for antibody-resistant escape variants or mutants. Of the four classes of antibody-resistant mutants described by Sadziene et al. (39), class I mutants lacked OspA and OspB and the linear plasmid that encodes them, while class II mutants had truncated OspA and/or OspB proteins. Neither the animal infectivity of the class I mutants nor the use of these antibody-resistant mutants of *B. burgdorferi* in active immunization studies has been reported. An OspB mutant of *B. burgdorferi* Sh.2 was recently shown to have reduced invasiveness in vitro and reduced infectivity in *scid/scid* mice (38).

In this paper, we describe a mutant of virulent *B. burgdorferi* apparently selected for by long-term storage at 5°C and lacking OspA and OspB. We describe the immunogenicity of the mutant in hamsters. Also, we report studies to determine whether vaccination with this mutant confers protection against infection in hamsters.

MATERIALS AND METHODS

Strains and culture conditions. Borreliae were cultured in Barbour-Stoenner-Kelly (BSK) medium (2) with minor modifications (9) at 30°C. The virulence of wild-type *B. burgdorferi* 297 (designated hereafter as virulent wild-type 297), a human spinal fluid isolate from Connecticut (49), was maintained by periodic passage through golden Syrian hamsters (28). Borreliae were recovered from the bladder, spleen, heart, or blood of animals infected with this organism. Avirulent 297 has been maintained in continuous in vitro culture since 1983 and is noninfectious for hamsters injected

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with 10^8 cells. Mutant 297 was originally isolated from the heart tissue of a hamster that had been experimentally infected with virulent wild-type 297. The culture tube had been placed at 5°C and stored for 6 months, at which time the culture was subcultured to fresh medium and grown at 30°C. Subcultures were examined for protein content and DNA plasmid profile (see below). Cultures were cloned by limiting dilution (4).

Growth curves were determined from triplicate cultures for virulent wild-type 297, avirulent 297, and mutant 297. Inocula for growth curve studies consisted of 5×10^6 early-logarithmic-phase cells per 5 ml of medium, yielding a final cell concentration of 1×10^6 cells per ml. At designated time intervals, 0.1 ml of culture was removed from each tube, and live motile spirochetes were counted in a Petroff-Hausser chamber by dark-field microscopy.

DNA characterization. In situ-lysed DNA samples from each variant were examined by contour-clamped homogeneous electric field pulsed-field gel electrophoresis (CHEF PFGE) as described previously (24). Lambda phage DNA concatamers (FMC BioProducts, Rockland, Maine) and a 5-kb DNA ladder (Bio-Rad Laboratories, Hercules, Calif.) were used as DNA size standards. The DNA was transferred from the agarose gels to a membrane support (GeneScreen Plus; NEN Research Products, Boston, Mass.) by the method of Southern (47).

The probe for the *ospAB* locus was prepared by polymerase chain reaction amplification (21). Polymerase chain reaction primers represented nucleotides 788 to 812 and 920 to 944 of the *ospAB* locus sequence (GenBank accession number X14407) (10). Approximately 1 ng of total *B. burgdorferi* virulent wild-type 297 DNA was amplified for 40 cycles under the following conditions: denaturation at 95°C for 5 min, annealing for 5 min at 50°C, and extension for 1 min at 72°C; the subsequent 39 cycles were done at 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min; the final elongation was 5 min at 72°C. Amplitaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) was used as directed by the manufacturer. Polymerase chain reaction products were purified by elution from a Sephadex G-25 column (Quick-Spin; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) and labeled with digoxigenin-11-dUTP by the random-primed method with a commercially available kit (Genius System; Boehringer). Hybridization and subsequent washings were done essentially as previously described (24). Digoxigenin-labeled nucleic acids were detected by use of the Genius Chemiluminescent System as suggested by the manufacturer.

Antibodies. MAbs, their protein specificities, and their sources were as follows: H9724 (flagellin), H5332 (OspA), H5TS (OspB), and anti-P39 (39 kDa) were provided by T. Schwan, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Mont.; CB312 (72 kDa), CB625 (22 kDa), and CB49 (19 kDa) were provided by J. Benach, New York Department of Health, Stony Brook; 240.7 (7.5 kDa) was provided by G. Habicht, State University of New York, Stony Brook; o31a (OspA) and o62a (62 kDa) were provided by T. Masuzawa, University of Shizouka, Shizuoka, Japan; H68 (OspB) and H614 (OspB) were provided by A. Barbour, University of Texas, San Antonio; D4 (82 to 93 kDa) was provided by D. Volkman, State University of New York, Stony Brook; L22 1F8 (OspC) was provided by B. Wilske, Pettenkofer-Institut, University of Munich, Munich, Germany; and LA2 (OspA) and LA5 (OspA) were provided by M. M. Simon, Max Planck Institute for Immunobiology, Freiburg, Germany.

SDS-PAGE and Western immunoblotting. Proteins of the borreliae studied were separated by SDS-PAGE with the buffer systems of Laemmli (32). Early-log-phase borreliae were harvested by centrifugation at 4°C. The pellet was washed three times with ice-cold 10 mM phosphate-buffered saline (PBS; pH 7.2). The final suspension in 0.0625 M Tris was vortexed, and aliquots were stored at −70°C. The protein content was determined by a detergent-compatible protein assay (Bio-Rad). Linear gradient gels (7.5 to 15.0%) (22) were poured at 4°C in a multiple-gel caster (Hoefer Scientific Instruments, San Francisco, Calif.) according to the manufacturer's instructions and stored at 5°C for later use. A 3.75% stacking gel was prepared on the day of electrophoresis. Protein samples were diluted appropriately in sample buffer (0.0625 M Tris, 2% SDS, 15 mM dithiothreitol, 27% sucrose, 0.002% bromophenol blue) and boiled for 2 min. Electrophoresis was carried out at a 35-mA constant current per gel at room temperature. For Western immunoblots, proteins were transferred to Immobilon P (Millipore Corp., Bedford, Mass.) at a 1-A constant current for 30 min with the buffers of Towbin et al. (50). To examine the protein profiles of the borreliae, gels were stained for 1 h in Coomassie brilliant blue, destained overnight, and then photographed.

For Western immunoblots, blots were quenched for 1 h in 0.5% instant dry milk–Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl [pH 7.5]) and then washed for 45 min with 0.1% Tween 20–TBS. The immunoblots were incubated for 1 h with hamster sera (1:100) or with MAbs (various dilutions), washed twice with 0.1% Tween 20–TBS, and incubated with alkaline phosphatase-conjugated goat anti-hamster immunoglobulin G (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) diluted 1:1,000 or alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G (Kirkegaard and Perry) diluted 1:1,000 for 1 h. The immunoblots were washed twice with 0.1% Tween 20–TBS, twice with TBS, and once with barbital buffer (150 mM sodium barbital [pH 9.6] with glacial acetic acid). Color was developed at 22 to 23°C with a developing solution consisting of 300 μg of Nitro Blue Tetrazolium per ml, 150 μg of 5-bromo-4-chloro-3-indolylphosphate per ml, and 813 μg of $MgCl_2 \cdot 6H_2O$ per ml in barbital buffer.

Vaccine preparation and vaccination procedure. *B. burgdorferi* virulent wild-type 297, avirulent 297, and mutant 297 were grown to a cell density of 10^8 spirochetes per ml in 50-ml tubes. Borreliae were harvested by centrifugation (10,000 × *g* for 30 min), washed two times with 10 mM PBS, suspended in 30 ml of 10 mM PBS, and lyophilized. Vaccines were prepared by suspending the appropriate dry weight of cells in sterile saline containing a 1:10,000 dilution of thimerosal and then mixing the suspension with an adjuvant (Ribi Immunochem Research, Inc., Hamilton, Mont.). Vaccine preparations contained nonviable bacteria, as lyophilized preparations did not grow in BSK medium. Male hamsters, 4 weeks old, were administered 0.2-ml doses of vaccine subcutaneously at days 0 and 14. Each dose of vaccine contained, as the adjuvant, 50 μg each of monophosphoryl lipid A (MPL) and synthetic trehalose dicorynomycolate (TDM). Fourteen days after the final vaccination, hamsters were challenged by intraperitoneal injection of 10^8 or 10^6 cells of virulent wild-type 297. These challenge doses represent approximately 100,000 or 1,000 50% infective doses of this spirochete, respectively (26). Serum samples were collected and pooled for each group of hamsters before challenge and 14 days postchallenge. At 14 days postchallenge, the hamsters were sacrificed, and the bladder, spleen, heart,

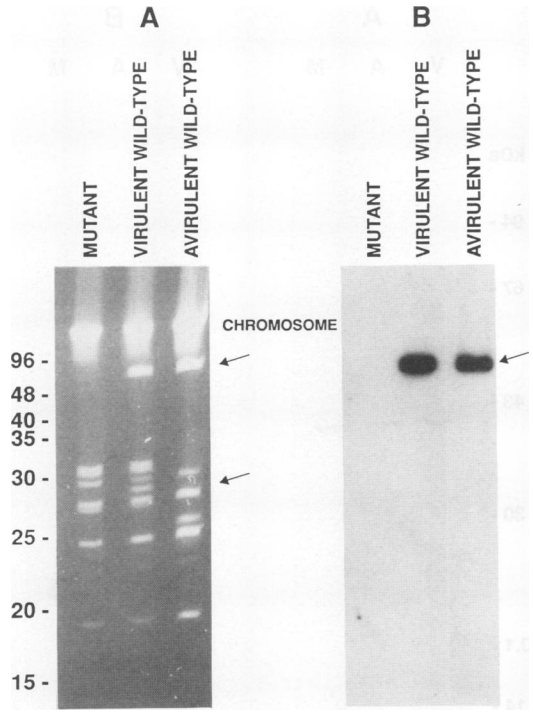

FIG. 1. CHEF PFGE of *B. burgdorferi* mutant 297, virulent wild-type 297, and avirulent 297 DNAs. (A) In situ-lysed DNA samples were separated at 200 V with 1.5-s pulses for 2 h, with 0.6-s pulses for 8 h, and then with 0.8-s pulses for 8 h. Lambda DNA concatamers and a 5-kb DNA ladder were loaded as standards. Arrows indicate the positions of the *ospAB* plasmid and the 28.4-kb plasmid, both of which were absent in the mutant. (B) Autoradiograph of a Southern blot of a pulsed-field gel following hybridization with a DNA probe specific for the *ospAB* locus. The arrow indicates the position of the *ospAB* plasmid. The numbers at left are in kilobases.

and blood from each animal were cultured. Each hamster organ was placed in 6 ml of BSK medium and homogenized with a Stomacher Lab-Blender (Tekmar Co., Cincinnati, Ohio). After the larger tissue debris was allowed to settle, duplicate 1:10 dilutions of the supernatant were made in BSK medium containing 0.15% agarose (SeaKem LE; FMC). Cultures were examined by dark-field microscopy after 3 weeks of incubation at 30°C.

RESULTS

Characterization of wild-type 297 and mutant 297. A mutant of virulent wild-type *B. burgdorferi* 297 was apparently selected for by long-term storage at 5°C. In situ-lysed DNA samples from virulent wild-type 297, avirulent 297, and mutant 297 were analyzed by CHEF PFGE to determine plasmid content (Fig. 1A). Electrophoresis parameters were chosen to optimize the separation of the plasmid bands. The 60-kb plasmid, which corresponds to the linear plasmid that is 49 kb in size in conventional agarose gel electrophoresis (5, 13) and that encodes the OspA and OspB proteins, was absent from the mutant plasmid profile and present in both the virulent wild-type and avirulent plasmid profiles. In addition, a plasmid of approximately 28.4 kb was absent from the mutant and avirulent plasmid profiles. The absence of this plasmid from the profiles may reflect the loss of the DNA from the genome or a DNA rearrangement event. The avirulent 297 plasmid profile also lacked a plasmid doublet at approximately 30 kb and a plasmid at approximately 26.8 kb but gained a plasmid at approximately 25 kb. The addition of the plasmid band in the avirulent 297 plasmid profile may reflect a plasmid rearrangement or, most likely, the loss of DNA from a larger plasmid band.

The loss of the OspA and OspB genes from the mutant genome was verified when a DNA probe specific for the *ospAB* locus failed to hybridize to the DNA of the mutant (Fig. 1B). These genes were present on the 60-kb plasmid of both virulent 297 and avirulent 297. The mutant was cloned by limiting dilution. The DNAs from nine in situ-lysed clones were examined by CHEF PFGE. The *ospAB* plasmid and the 28.4-kb plasmid were also absent from the plasmid profiles of the mutant clones (data not shown).

Growth curve experiments indicated that mutant 297 and virulent wild-type 297 have similar generation times, approximately 24 h. In contrast, avirulent 297 grew with a generation time of approximately 11 h. Hamster-passaged, virulent wild-type 297 has a high level of infectivity. This strain has a 50% infective dose for hamsters of 10^3 spirochetes when needle injected by the intraperitoneal route (26). In contrast, mutant 297, which was derived from virulent wild-type 297, had lost its infectivity. Spirochetes could not be recovered from the tissues or blood of hamsters that had been administered 10^7 to 10^9 spirochetes intraperitoneally.

TABLE 1. Results of Western immunoblot analysis with MAbs to proteins of *B. burgdorferi* 297

MAb	Protein (kDa)	Reactivity[a] with 297:		
		Virulent	Avirulent	Mutant
D4	82–93	+	+	+
CB312	72	+	+	+
o62a	62	+	+	+
H9724	41	+	+	+
Anti-P39	39	+	+	+
H5TS	34	+	+	−
H68	34	+	+	−
H614	34	+	ND	−
H5332	31	+	+	−
LA2	31	+	+	−
LA5	31	+	+	−
o31a	31	+	+	−
L22 1F8	24	+	+	+
CB625	22	+	+	+
CB49	19	+	+	−
240.7	7.5	+	+	−

[a] +, reactive; −, nonreactive; ND, not done.

Antigenic characterization. The results of Western immunoblot analysis with a variety of MAbs against *B. burgdorferi* 297 proteins are shown in Table 1. As expected, MAbs specific for OspA and OspB did not recognize these proteins in the mutant. In addition, MAbs specific for a 19- or 20-kDa lipoprotein and a 7.5-kDa lipoprotein of virulent wild-type 297 and avirulent 297 did not react with corresponding proteins in the mutant, suggesting that the genetic information for these proteins has been lost or that these proteins are not expressed in the mutant. The 22-kDa, 24-kDa (OspC), 39-kDa, 41-kDa (flagellin), 62-kDa, 72-kDa, and 82- to 93-kDa proteins were recognized by their specific MAbs in virulent wild-type 297, avirulent 297, and mutant 297.

Coomassie brilliant blue-stained SDS-polyacrylamide gels illustrate differences in the protein profiles among virulent wild-type 297, avirulent 297, and mutant 297. The expression

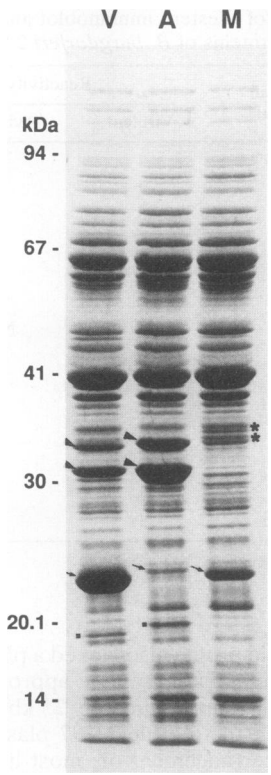

FIG. 2. Protein profiles of *B. burgdorferi* virulent wild-type 297 (V), avirulent 297 (A), and mutant 297 (M). Twenty micrograms of protein was applied to each lane of a 7.5 to 15% linear gradient gel, separated by electrophoresis, and stained with Coomassie brilliant blue. Molecular mass markers are indicated on the left. Arrows indicate the positions of OspC. Arrowheads indicate the positions of OspA (31 kDa) and OspB (34 kDa). Asterisks indicate the positions of proteins (34 to 35 kDa) expressed by mutant 297 but absent in virulent wild-type 297 and avirulent 297 profiles. Squares indicate the positions of proteins (19 or 20 kDa) present in virulent wild-type 297 and avirulent 297.

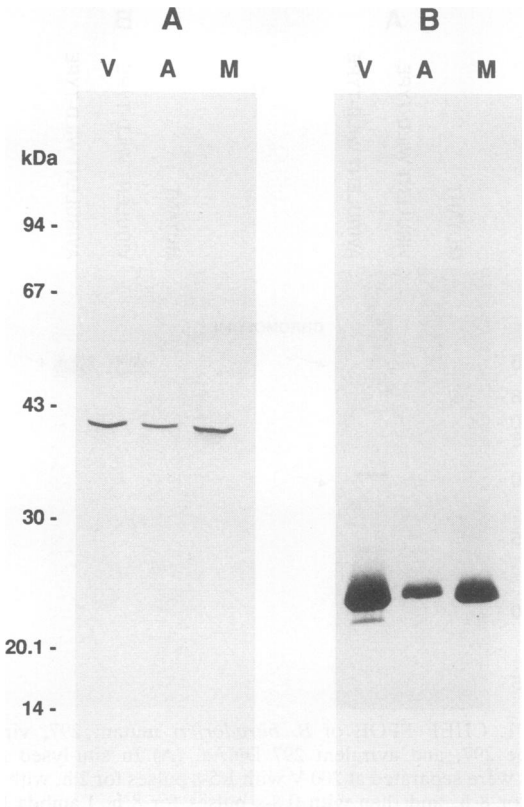

FIG. 3. Reactivity of *B. burgdorferi* virulent wild-type 297 (V), avirulent 297 (A), and mutant 297 (M) with MAbs specific for P39 and OspC. Ten micrograms of protein was loaded into each lane of a 7.5 to 15% linear gradient gel, separated by electrophoresis, transferred to a membrane, and probed with anti-P39 MAb (A) and with L22 1F8 MAb, specific for OspC (B). Molecular mass markers are indicated on the left.

of OspA, OspB, and OspC varied in the three organisms (Fig. 2). The protein profile of the mutant shows the absence of OspA (31 kDa) and OspB (34 kDa) and the addition of proteins in the 34- to 35-kDa region. Avirulent 297 expresses a larger amount of OspA and OspB than virulent wild-type 297, whereas virulent wild-type 297 expresses OspC to a greater degree than avirulent 297 or mutant 297. Variations were also observed in the 19- or 20-kDa region. Virulent wild-type 297 expresses a protein that has an apparent molecular mass of 19 kDa and that is not present in avirulent 297 or mutant 297. Avirulent 297 expresses a protein that has an apparent molecular mass of 20 kDa and that is not present in virulent wild-type 297 or mutant 297.

MAb reactivity with P39 and OspC is shown in Fig. 3. Equal amounts of protein were loaded per lane. While relatively similar amounts of P39 were present, the amounts of OspC varied greatly in these three organisms. Virulent 297 expresses the largest amount of OspC, whereas avirulent 297 expresses the smallest amount of OspC.

Vaccination. Increased in vitro passage of *B. burgdorferi* has been associated with decreased animal infectivity (44). We wanted to determine whether loss of virulence (or loss of infectivity) would interfere with the ability of *B. burgdorferi* to elicit protective immunity. Hamsters were vaccinated with either virulent wild-type 297 or avirulent 297 and challenged with virulent wild-type 297. Both virulent wild-type 297 and avirulent 297 express the OspA and OspB proteins. Active immunization of hamsters with virulent wild-type 297 protected against challenge with an inoculum of 10^8 spirochetes, but immunization with avirulent 297 did not protect all animals against challenge (Table 2). We also confirmed that the incorporation of an adjuvant containing MPL and TDM into the vaccine increased vaccine immunogenicity (25).

Since both virulent wild-type 297 and avirulent 297 express OspA and OspB, we next evaluated the ability of a mutant that lacks OspA and OspB to elicit immunoprotection. The mutant of wild-type 297 was used to vaccinate hamsters (Table 3). Eighty-six percent of the hamsters vaccinated with as little as 12.5 μg of whole-cell mutant 297 vaccine in an adjuvant were protected from challenge with 10^6 virulent wild-type 297 spirochetes. Vaccination with 12.5 μg of whole-cell virulent wild-type 297 vaccine provided 100% protection from infection. In contrast, vaccination with up to 100 μg of whole-cell avirulent 297 vaccine offered no protection from infection.

Western immunoblotting. We wanted to determine which antibodies were circulating at the time of challenge (infection) and which additional antibodies were produced in response to infection in both vaccinated and nonvaccinated hamsters. Pooled hamster sera from groups of hamsters

TABLE 2. Vaccination of hamsters with virulent and avirulent *B. burgdorferi* 297

Vaccine[a]	Total vaccine dose (μg, dry wt)	No. of culture-positive animals/total no. of animals (% protection)
Adjuvant only[b]		0/3
Adjuvant only		7/7
Virulent 297, whole cell, with adjuvant	2.5	3/7 (57)
	5	4/7 (43)
	10	4/7 (43)
	20	0/7 (100)
	40	0/7 (100)
Virulent 297, whole cell, without adjuvant	5	4/7 (43)
	10	6/7 (14)
	20	2/7 (71)
	40	3/7 (57)
	80	2/7 (71)
Avirulent 297, whole cell, with adjuvant	20	4/7 (43)
	40	4/7 (43)
	80	4/7 (43)

[a] The adjuvant contained MPL-TDM emulsion. Hamsters were challenged 14 days after the last vaccination with an inoculum of 10^8 virulent wild-type 297, sacrificed 14 days later, and assayed for infection by culturing as described in Materials and Methods.
[b] Negative control group; no challenge was administered to this group.

vaccinated with virulent wild-type 297, avirulent 297, or mutant 297 were examined by Western immunoblotting for antibodies reactive with the virulent challenge organism (Fig. 4). Data are shown for hamster groups receiving a 100-μg dose. As expected, prechallenge sera from hamsters vaccinated with mutant 297 lacked antibodies to OspA and

TABLE 3. Vaccination with virulent, mutant, and avirulent *B. burgdorferi* 297

Vaccine[a]	Total vaccine dose (μg, dry wt)	No. of culture-positive animals/total no. of animals (% protection)
Adjuvant only[b]		0/2
Adjuvant only		7/7
Virulent 297, whole cell	12.5	0/6 (100)
	25	0/7 (100)
	50	0/7 (100)
	75	0/7 (100)
	100	0/7 (100)
Mutant 297, whole cell	12.5	1/7 (86)
	25	0/7 (100)
	50	0/7 (100)
	75	0/7 (100)
	100	0/6 (100)
Avirulent 297, whole cell	50	5/5 (0)
	75	5/5 (0)
	100	5/5 (0)

[a] The adjuvant contained MPL-TDM emulsion. Hamsters were challenged 14 days after the last vaccination with an inoculum of 10^6 virulent wild-type 297, sacrificed 14 days later, and assayed for infection by culturing as described in Materials and Methods.
[b] Negative control group; no challenge was administered to this group.

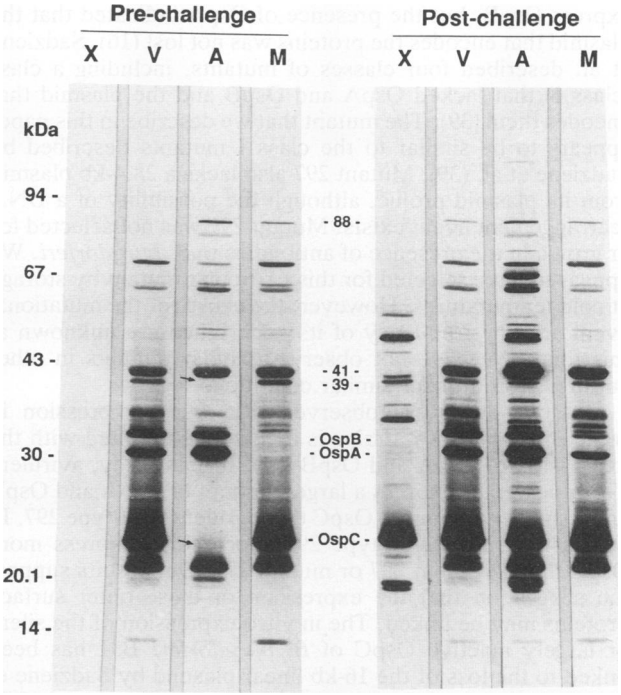

FIG. 4. Western immunoblots of hamster sera. Hamsters were vaccinated with adjuvant alone (X) or adjuvant with 100 μg of virulent wild-type 297 (V), avirulent 297 (A), or mutant 297 (M). Prechallenge hamster sera were collected 14 days after the final vaccination and pooled. Postchallenge sera were collected 14 days after the challenge and pooled. Five micrograms of virulent wild-type *B. burgdorferi* 297 protein was loaded into each lane. Blots were probed with a 1:100 dilution of hamster sera. Molecular mass markers are shown on the left. Arrows indicate the absence of reactivity with P39 and OspC in the sera from hamsters vaccinated with avirulent 297.

OspB, while sera from hamsters vaccinated with virulent wild-type 297 or avirulent 297 exhibited antibodies to these proteins. Interestingly, hamsters vaccinated with virulent wild-type 297 or mutant 297 elicited antibodies to OspC and the 39-kDa protein, whereas hamsters vaccinated with avirulent 297 failed to produce these antibodies. Also, sera from hamsters vaccinated with avirulent 297 did not react with P39 of avirulent 297 (data not shown). The 19- or 20- and 7.5-kDa lipoproteins that are missing from the mutant protein profile do not appear to be antigenic in the lyophilized vaccine, since hamsters vaccinated with either virulent wild-type 297 or avirulent 297 do not produce antibodies directed against these lipoproteins. Immunoblots of postchallenge sera revealed an expanded antibody profile in all three groups of vaccinated hamsters. After challenge with virulent wild-type 297, hamsters vaccinated with avirulent 297 produced antibodies to OspC and P39. Hamsters vaccinated with mutant 297 produced antibodies to OspA and OspB after challenge with virulent wild-type 297.

DISCUSSION

Mutants and antigenic variants of *B. burgdorferi* have been described in the literature (11, 15, 16, 39, 43). A number of these mutants or variants were selected for following incubation of the culture in the presence of antibodies (15, 16, 39). Coleman et al. described a variant that failed to

express OspB, but the presence of OspA indicated that the plasmid that encodes the proteins was not lost (16). Sadziene et al. described four classes of mutants, including a class (class I) that lacked OspA and OspB and the plasmid that encodes them (39). The mutant that we describe in this paper appears to be similar to the class I mutants described by Sadziene et al. (39). Mutant 297 also lacks a 28.4-kb plasmid from its plasmid profile, although the possibility of a DNA rearrangement event exists. Mutant 297 was not selected for or grown in the presence of antibodies to *B. burgdorferi*. We appear to have selected for this particular mutant by storage at cold temperatures. However, the cause for the mutational event and the frequency of its occurrence are unknown at this time. We have not observed similar mutants in other cultures stored under similar conditions.

Margolis and Rosa observed that OspC expression in some *B. burgdorferi* strains is inversely correlated with the expression of OspA and OspB (35). In this study, avirulent 297 appeared to express a larger amount of OspA and OspB and a smaller amount of OspC than virulent wild-type 297. In contrast, virulent wild-type 297 appeared to express more OspC than avirulent 297 or mutant 297. Our results support the suggestion that the expression of these outer surface proteins may be linked. The in vitro expression of the silent or largely inactive OspC of *B. burgdorferi* B31 has been linked to the loss of the 16-kb linear plasmid by Sadziene et al. (40). The right telomere of the 16-kb plasmid of strain B31 was shown to hybridize to the 18-kb plasmid of strain 297 (23). The left telomere of the 16-kb plasmid of strain B31 did not hybridize to the 18-kb plasmid of strain 297. These results suggested that although plasmids of different sizes may share terminal sequence similarities, they may not share extensive sequence homology over their entire lengths. Our findings that the 18-kb plasmid is present in virulent wild-type 297, avirulent 297, and mutant 297 and that OspC is expressed in all three organisms indicate that OspC expression is not dependent on the loss of this linear plasmid.

The class I mutants described by Sadziene et al. expressed an abundant amount of a protein at 28 kDa, in comparison with the wild type, and another protein, at 36 kDa, which was not detectable in the wild type (39). The mutant 297 that we describe in this paper does not express an overabundance of the 28-kDa protein. However, we did note the expression of additional proteins in the 34- to 35-kDa range in the mutant profile. The expression of other *B. burgdorferi* proteins may be derepressed or upregulated in the absence of the *ospAB* plasmid.

The mutant that was derived from low-passage virulent wild-type 297 and that we describe in this paper is no longer infective for golden Syrian hamsters. Sadziene et al. did not report the infectivity of their class I mutants (39). However, their wild-type isolates had been passaged continuously in culture from 1982 and 1983 and are most likely avirulent. The loss of plasmids in *B. burgdorferi* as a result of continuous in vitro cultivation has been documented and has been linked to the loss of infectivity in rodents. Schwan et al. reported that the 7.6-kb circular and 22-kb linear plasmids of *B. burgdorferi* Sh-2-82 were not detectable in noninfectious spirochetes (44). They suggested that the gene(s) which encodes factors responsible for infectivity may be present on one or more of these plasmids. When the plasmid profiles for noninfectious mutant 297 and avirulent 297 are compared with the plasmid profile for virulent wild-type 297, the loss or rearrangement of the 28.4-kb plasmid in the noninfectious spirochetes suggests that this plasmid may encode factors responsible for the infectivity of *B. burgdorferi* 297.

OspA is highly regarded as a candidate for a Lyme disease vaccine (18, 45). The present study demonstrates that a vaccine prepared from a mutant of *B. burgdorferi* 297 that lacks OspA and OspB will protect hamsters from challenge with virulent wild-type 297. Our results suggest that antigens other than OspA and OspB are involved in protective immunity.

The absence of circulating antibodies against OspC and P39 in hamsters vaccinated with avirulent 297 in addition to the lack of protection from challenge offered by this vaccine suggest that either one or both of these proteins may serve as protective antigens. The lack of protection against infection occurs even though both OspC and P39 are present in the avirulent 297 vaccine. It is possible that the OspC and P39 proteins have been modified in the avirulent 297 organism to render them nonimmunogenic. Preac-Mursic et al. have shown that vaccination with recombinant OspC protects gerbils from infection (37).

The immunogenicity of P39 has been correlated with the viability and infectivity of inocula by Simpson et al. (46). They indicated that inocula must be viable and infectious in the absence of an adjuvant to elicit antibody production in mice and suggested that virulence may be dependent on P39 expression. While this appears to be true for virulent wild-type 297 and avirulent 297, mutant 297 is not infectious for hamsters, yet its P39 is immunogenic in the presence of an adjuvant. This does not appear to be due to differences in the quantity of P39 present in the vaccines, since MAb analysis revealed similar amounts of P39 in all three 297 organisms. In addition, rabbits immunized with viable mutant 297 produce antibodies to P39 (26). These results suggest that P39 (and possibly other proteins) may serve as protective antigens but are not associated with infectivity.

The findings of this study encourage the development of a Lyme disease vaccine that incorporates not only OspA but also OspC and possibly P39 as well.

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