Abilities of OspA Proteins from Different Seroprotective Groups of *Borrelia burgdorferi* To Protect Hamsters from Infection

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The ability of vaccination with recombinant OspA from six seroprotective groups of *Borrelia burgdorferi* sensu lato to induce protection against infection with homologous and other Lyme spirochetes was examined in hamsters. Antisera generated against the OspA proteins of *B. burgdorferi* sensu stricto S-1-10 and C-1-11 (seroprotective groups 1 and 2, respectively), *Borrelia afzelii* BV1 (seroprotective group 4), and *Borrelia garinii* LV4 (seroprotective group 5) were able to kill the homologous spirochete in vitro but not other isolates. Surprisingly, antisera against *B. afzelii* PKo (seroprotective group 6) and *B. burgdorferi* sensu lato LV5 (seroprotective group 3) OspA proteins were unable to kill the homologous organism, although LV5 OspA antisera killed the heterologous isolates S-1-10 and LV4. In vivo vaccination studies supported the in vitro findings, confirming that vaccination with a single OspA protein does not provide complete protection against challenge with all Lyme disease spirochetes. In addition, OspA antibodies from some isolates may not protect against the homologous isolate. The induction of protective antibodies against other *B. burgdorferi* proteins may be necessary to insure a comprehensive Lyme disease vaccine.

We (21, 31) and others (11–13, 22, 23, 28) have established that inoculation of experimental animals with *Borrelia burgdorferi* organisms or their components can induce borreliacidal antibodies that provide protection against challenge with the Lyme disease spirochete. These studies have established a role for antibody-mediated protection against infection with *B. burgdorferi* and have provided important information for the potential development of a Lyme disease vaccine.

Several individual *B. burgdorferi* outer surface proteins, OspA (12, 37), OspB (14), OspC (24), and the 39-kDa protein (34), induce a protective antibody response, which has led to the investigation of some of these proteins as Lyme disease vaccine components. Of these, OspA has emerged as the leading vaccine candidate (13, 14, 28). In fact, Connaught Laboratories, Inc., is currently conducting human trials of a Lyme disease vaccine consisting of a single recombinant OspA protein.

Despite the early promise of vaccination with OspA for providing protection against infection with the Lyme disease spirochete, antigenic heterogeneity (16, 40–42) in both North American and European isolates has become a concern. Fikrig et al. (15) demonstrated that vaccination with recombinant OspA from *B. burgdorferi* sensu stricto N40 did not protect mice against challenge with *B. burgdorferi* sensu stricto 25015. In addition, Schaible et al. (29) showed that vaccination against challenge with a single *B. burgdorferi* isolate did not protect against *B. burgdorferi* isolates with other OspA and OspB genotypes.

We recently investigated the extent of antigenic heterogeneity among *B. burgdorferi* sensu lato isolates. To facilitate these investigations, we developed an in vitro assay for detecting borreliacidal antibody. We demonstrated that the ability of

immune serum to protect hamsters from development of Lyme arthritis correlated with the presence of borreliacidal antibodies (21). Borreliacidal antibodies, primarily subclass immunoglobulin G2 (IgG2) (30), were detectable 7 days after infection with *B. burgdorferi*, peaked at weeks 3 to 5, and gradually declined (32). In addition, borreliacidal antibodies have been detected in sera from human patients with Lyme disease (8–10, 25). Passive immunization with case-defined Lyme disease sera containing these borreliacidal antibodies prevented the induction of Lyme arthritis in hamsters (8).

Using the in vitro borreliacidal assay, we identified five seroprotective groups among a large number of *B. burgdorferi* sensu lato isolates, including *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* (19, 20). These findings were confirmed in vivo by passive transfer of immune sera to hamsters and challenge with homologous and heterologous *B. burgdorferi* isolates. In this investigation, we examined the abilities of vaccination with individual recombinant OspA proteins of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* isolates representative of six seroprotective groups to provide protection against infection with isolates of *B. burgdorferi* sensu lato.

MATERIALS AND METHODS

Animals. Inbred LSH/Ss Lak hamsters were obtained from our breeding colony at the Wisconsin State Laboratory of Hygiene. Hamsters weighing 60 to 100 g were housed three or four per cage at an ambient temperature of 21°C.

Bacterial isolates and cultivation. Isolates of *B. burgdorferi* sensu lato used in this study and their sources are described in Table 1. These isolates are representatives of five different seroprotective groups (19, 20). We assigned *B. afzelii* PKo to a sixth seroprotective group because of the inability of antisera generated against the five established seroprotective groups to kill this isolate in vitro. Spirochetes were cultured in Barbour-Stoenner-Kelly (BSK) medium (2, 7) at 32°C for 5 days to a concentration of approximately 10⁶ spirochetes per ml, and 500-μl aliquots were stored in BSK medium with 10% glycerol at –70°C until used. Isolates were obtained from S. M. Callister, R. C. Johnson, B. Wilske, and G. Stanek. *Escherichia coli* DH5α (Bethesda Research Laboratories, Gaithersburg, Md.) was used in all cloning experiments.

Plasmids and amplification of ospA. Plasmids pGEX-2T (Pharmacia, Piscataway, N.J.) and pGEM-3Zf(+) (Promega, Madison, Wis.) were used as appropriate. Plasmid-enriched DNA was isolated from *B. burgdorferi* by standard

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TABLE 1. Isolates used in the study

<i>Borrelia</i> isolate	Geographic location	Origin	Species
LV5	Austria	Human	<i>B. burgdorferi</i> sensu lato
S-1-10	Wisconsin	<i>Peromyscus leucopus</i>	<i>B. burgdorferi</i> sensu stricto
C-1-11	Illinois	<i>Microtus pennsylvanicus</i>	<i>B. burgdorferi</i> sensu stricto
LV4	Austria	Human	<i>B. garinii</i>
PKo	Germany	Human	<i>B. afzelii</i>
BV1	Austria	Human	<i>B. afzelii</i>

techniques (3). The DNA was used as a template for the amplification of the *ospA* gene by the GeneAmp protocol (Perkin-Elmer Cetus, Norwalk, Conn.) of PCR (26). Primers were used at a final concentration of 1.0 μ M with an $MgCl_2$ concentration of 2.5 mM. Thermal cycling parameters were 94°C for 1 min followed by 35 cycles of (i) 94°C for 1 min, (ii) a 2-min ramp to 45°C, (iii) 45°C for 1 min, (iv) a 1-min ramp to 60°C, and (v) 60°C for 6 min. The final extension was done at 60°C for 10 min to fully extend any truncated DNA strands. The amino-terminal primer B1 (5'-GCGTGGATCCATGAAAAAATATTTATTTGGGAA3') and the carboxy-terminal primer B2 (5'-AATTCCTCGGGTTATTTTAAAGCGTTTTTAA3') were used for amplification to create a 5' *Bam*HI and a 3' *Sma*I site for directional ligation of the fragment into the vector.

Cloning of *ospA* genes. Individual *ospA* genes were cloned by standard techniques. Briefly, amplified DNA was purified with GeneClean (Bio 101, La Jolla, Calif.). After digestion with *Sma*I and *Bam*HI (Bethesda Research Laboratories), the purified DNA fragment was ligated into pGEX-2T digested with *Bam*HI and *Sma*I. Some fragments containing internal *Bam*HI and *Sma*I sites were blunt ended with T4 DNA polymerase (Bethesda Research Laboratories) and cloned into pGEM-3Zf(+) digested with *Sma*I. These plasmids were partially digested with *Bam*HI and *Sma*I and subcloned into pGEX-2T. The insert and plasmid were ligated with T4 DNA ligase (Bethesda Research Laboratories), and the ligation mix was used to transform competent *E. coli* DH5 α . Organisms were plated onto 2 $\times$ TY medium (1) containing ampicillin (100 μ g/ml, Sigma Chemical Co., St. Louis, Mo.). Colonies expressing recombinant OspA protein were identified by Western blotting (immunoblotting) with *B. burgdorferi* sensu stricto B31 OspA monoclonal antibody H5332 (4), provided by A. G. Barbour. For identification of *B. afzelii* BV1 and *B. afzelii* PKo *ospA* clones, polyclonal hamster antisera against isolates BV1 and PKo were used.

Western blotting. *E. coli* colonies expressing OspA protein were grown overnight in 2 $\times$ TY broth medium containing ampicillin (100 μ g/ml) and isopropyl- β -D-thiogalactopyranoside (IPTG; 0.1 mM). Cultures were centrifuged and resuspended in phosphate-buffered saline (PBS; pH 7.2). Western blotting was performed as previously described (20). Briefly, samples were boiled in sample buffer for 5 min, and 150 μ g of total protein was loaded onto a 10% polyacrylamide reduced gel for electrophoresis. Protein concentrations were determined with the Bio-Rad protein determination kit (Bio-Rad Inc., Richmond, Calif.). Two slab gels were run simultaneously with a Hoefer SE600 electrophoresis unit (Hoefer Scientific Instruments, San Francisco, Calif.) at 55 mA for 3 h with the buffer system of Laemmli (17). After electrophoresis, proteins were transferred (Hoefer) to nitrocellulose for 3 h at 300 mA under conditions described by Towbin et al. (39). The nitrocellulose was cut into strips and blocked for 20 min at 22°C in PBS and 0.3% Tween 20. Strips were incubated with a 1:60 dilution of pooled immune hamster sera or monoclonal antibodies to OspA (H5332) (4) for 1 h at 22°C and washed three times with PBS-0.05% Tween 20. Horseradish peroxidase-labeled anti-hamster IgG (heavy and light chains; Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.), anti-goat IgG (The Binding Site, San Diego, Calif.), or anti-mouse IgG (Organon-Teknika Corp., Westchester, Pa.) was added, and the strips were incubated for 30 min at 22°C. Following incubation, strips were washed as described above and developed with the TMB Membrane Peroxidase Substrate System (Kirkegaard and Perry).

DNA sequencing and analysis. The DNA sequences of the plasmid inserts were determined by double-stranded sequencing by the Sanger dideoxy-chain termination method (27) modified for use with Sequenase (United States Biochemical Corp.). Primers constructed for amplification were used for initial sequencing, and internal primers were synthesized for continued sequencing. Nucleotide and predicted amino acid sequence alignments were determined with the MacVector 2.0 program (IBI, New Haven, Conn.). Measurements of nucleotide and predicted amino acid sequence homology were determined with the DNASTAR ALIGN and AALIGN programs (DNASTAR, Madison, Wis.).

Recombinant OspA expression and purification. OspA-glutathione S-transferase fusion proteins were produced from six *B. burgdorferi* sensu lato isolates as previously described (1). Briefly, transformed *E. coli* organisms containing each *ospA* gene were grown for 12 h at 37°C in 100 ml of 2 $\times$ TY broth containing 100 μ g of ampicillin per ml. Cultures were diluted 1:10 with broth medium and incubated for an additional 1 h. IPTG (final concentration, 0.1 mM) was added,

FIG. 1. Western blots of purified OspA fusion protein from isolates S-1-10 (lane 1), LV5 (lane 2), LV4 (lane 3), C-1-11 (lane 4), PKo (lane 5), and BV1 (lane 6). Monoclonal antibody H5332 (OspA) was used for LV5, S-1-10, LV4, and C-1-11 OspA fusion proteins. Immune sera generated against the *B. afzelii* isolates PKo and BV1 were used with isolates PKo and BV1 OspA fusion proteins.

and cultures were incubated for an additional 5 h. After incubation, bacteria were centrifuged, resuspended in PBS (pH 7.4), and lysed by three 30-s pulses with a sonicator (Model W-350; Branson Sonic Power Co., Danbury, Conn.). Sonicated *E. coli* organisms were mixed with Triton X-100 (10%), diluted 10-fold with PBS, and centrifuged to remove insoluble material. The supernatant was mixed with a 50% slurry of glutathione-Sepharose beads (Pharmacia) for 5 min at room temperature and washed three times with ice-cold PBS. Fusion proteins were eluted by mixing beads with 1 ml of 50 mM Tris-HCl (pH 8.0) containing 5 mM reduced glutathione for 2 min and collected after centrifugation for 1 min at 500 $\times$ g. The elution procedure was repeated four times. Fractions were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting with specific hamster sera, goat anti-GST serum, or monoclonal antibody H5332.

Immunization of hamsters and collection of serum. Hamsters were injected intramuscularly in each hind leg with 0.2 ml of an inoculum of 100 μ g of OspA fusion protein diluted 1:10 in 2% aluminum hydroxide (Alhydrogel; Superfos, Vedbaek, Denmark). Five weeks after the primary immunization, the hamsters were injected with an additional 50 μ g of OspA fusion protein. Two weeks after the booster vaccination, groups of three hamsters each were challenged with individual *B. burgdorferi* sensu lato isolates. Other hamsters were anesthetized by inhalation of ether contained in a nose-and-mouth cup and bled by intracardiac puncture. After the blood clotted, the serum was separated by centrifugation at 500 $\times$ g and pooled, and 1.0-ml aliquots were dispensed into 1.5-ml screw-cap tubes (Sarstedt, Newton, N.C.) and frozen at -20°C until used. Concomitantly, pooled normal serum was obtained from uninfected hamsters.

Infection of hamsters with *B. burgdorferi*. A frozen vial containing a suspension of *B. burgdorferi* organisms was thawed, and an aliquot was inoculated into BSK medium. Cultures were incubated for 5 days at 32°C before being diluted with fresh BSK to approximately 5×10^5 spirochetes per ml. Spirochetes were quantitated with a Petroff-Hausser counting chamber. Groups of three immunized or normal hamsters were each injected subcutaneously into each hind paw with 0.2 ml of the *B. burgdorferi* suspension.

Assessment of protection. Eighteen days after challenge, hamsters were sacrificed by inhalation of carbon dioxide. The spleen, left kidney, and urinary bladder were removed aseptically and homogenized separately with 1 ml of fresh BSK. Tissue suspensions (0.6 ml) were dispensed into duplicate tubes containing 5.4 ml of BSK medium and incubated in the dark at 32°C. Cultures were monitored weekly for spirochetes by dark-field microscopy. If cultures failed to grow spirochetes after 6 weeks, 2-ml aliquots were subcultured into 4 ml of fresh BSK medium and examined for an additional 3 weeks.

Borreliacidal assay. Borreliacidal activity was determined by a modification of a previously described procedure (18). Briefly, normal and immune sera were heat inactivated at 56°C for 30 min, diluted 10-fold with fresh BSK medium, and sterilized by passage through a 0.22- μ m filter (Acrodisc; Gelman Sciences, Ann

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      10      20      30      40      50      60
B31  ATGAAAAAATTTATTGGG AATAGGTCTAATATTAGCCT TAATAGCATGTAAGCAAAAT
LV5  .....>
S-1-10 .....>
C-1-11 .....T.....>
LV4  .....>
PKo   .....C.....>
BV1   .....C.....>

      70      80      90      100     110     120
B31  GTTAGCAGCCTTGAGAGAGAA AAACAGCGTTTCAGTAGATT TGCCTGGTGAATGAAAGTT
LV5  .....>
S-1-10 .....>
C-1-11 .....T..A..T.....>
LV4  .....T..A..T.....A.....G.....C.....>
PKo   .....T..A..C.....G.....>
BV1   .....T..A..C.....G.....>

      130     140     150     160     170     180
B31  CTTGTAAGCAAGAAAAA CAAAGACGGCAAGTACGATC TAATTGCAACAGTAGACAAG
LV5  .....>
S-1-10 .....>
C-1-11 .....C.....G.....AG.....G.....>
LV4  .....T.....G.....T..A..AG.....GAG.....>
PKo   .....T.....G.....AG.....AG.....>
BV1   .....T.....G.....T.....AG.....AG.....>

      190     200     210     220     230     240
B31  CTTGAGCTTAAGGAAGCTTC TGATAAAACAATGGATCTG GAGTACTTGAAGCGCTAATAA
LV5  .....>
S-1-10 .....>
C-1-11 .....A.....G.....>

      A
      I
LV4  .....C..T.....AC.....T.A.....>
PKo   A.....A.....G.....T.....G..G.....TAC.....>
BV1   A.....A.....G.....G.....G..G.....TAC.....>

      250     260     270     280     290     300
B31  GCTGACAAAGTAAGTAAAT ATTAACAATTCTGACGATC TAGGTCAAACACACTTGAA
LV5  .....>
S-1-10 .....>
C-1-11 .....C.....G.....A.CAC.....>
LV4  A.....G.....T..C.....A.....T.A.T.....>
PKo   A.....C.....G.....A.....A.....T.C.....>
BV1   A.....C.....G.....A.....A.....T.C.....>

      310     320     330     340     350     360
B31  GTTTTCAAAGAGATGGCAA AACACTAGTATCAAAAAAAG TAACCTCCAAGACAGTCA
LV5  .....>
S-1-10 .....>
C-1-11 .....A.....T.....G.....A C.....T.....T.....>
LV4  A.....T.....G.....CCTT.....>
PKo   C.....T.....G.....G.....G.....T.....AA.....>
BV1   C.....T.....G.....G.....G.....T.....AA.....>

      370     380     390     400     410     420
B31  TCAACAGAAAGAAAAATTCAA TGAAAAGGTGAAGTATCTG AAAAAATAATAACAAGAGCA
LV5  .....>
S-1-10 .....>
C-1-11 .....G.....C.....T..GT.....GG.....>
LV4  .....C.....G.....AC.....GT.....>
PKo   .....T..TG.....T..G.....C.....CC..G.....A.....>
BV1   .....T..TG.....T..G.....C.....CC..G.....A.....>

      430     440     450     460     470     480
B31  GACGGAACAGACTTGAATA CACAGGAATTAAGGCGATG GATCTGGAAGGCTAAGAG
LV5  .....>
S-1-10 .....>
C-1-11 A.....T.....A.....A.....C.....A.....>
LV4  A..T..TT.....AC..A.....C.....A.....>
PKo   A..T.....A.....T.....A.....A.....A.....>
BV1   A..T.....A.....T.....A.....A.....A.....>

      490     500     510     520     530     540
B31  GTTTTAAAGGCTATGTCT TGAAGGAACCTAAGTCTG AAAAAACAACATTGGTGGTT
LV5  .....>
S-1-10 .....>
C-1-11 CC.....AA.....G.....>

      CGG
      I
LV4  .....A..T..AC.....G.....C.....AAA.....>
PKo   .....AA..T..AC.....AAG..G..AA.....T.....GT.....AA..A>
BV1   .....AA..T..AC.....AAG..G..AA.....T.....GT.....AA..A>

      550     560     570     580     590     600
B31  AAAGAAGGAAGTGTACTTCT AAGCAAAATATTTCAAAT CTGGGGAAGTTTCAGTTGAA
LV5  .....>
S-1-10 .....>
C-1-11 .....T..GC..C.....A.....AA..C.....>
LV4  ..C.....C.....GT.....G..C.....T.....C..A..A..AA.....C>
PKo   .....C.....C.....T..GG.A..G.....A.....AA.....CT>
BV1   .....C.....C.....T..GG.A..G.....A.....AA.....CT>

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      610     620     630     640     650     660
B31  CTTAATGACACTGACAGTAG TGCTGCTACTAAAAAACTG CAGCTTGAATTCAGGCACT
LV5  .....>
S-1-10 .....>
C-1-11 .....A.....C.....AA.....GGAAA..G..G.....>
LV4  ..G.....T.....C..C..CAG.....G.AAA..T..G.....AAG.....>
PKo   .....A.....C..C..CAG.....GC..A..G.....AAA.....>
BV1   .....A.....C..C..CAG.....GC..A..G.....AAA.....>

      670     680     690     700     710     720
B31  TCAACTTTAACAATTACTGT AAACAGTAAAAAACTAAAG ACCTTGTGTTTACAAAAGAA
LV5  .....>
S-1-10 .....>
C-1-11 .....AC.....C.....A.....A.....C.....>
LV4  ..C.....G.....G..T..CC.....C..A.....A..C.....>
PKo   ..T.....G.....T.....C.....C..C..A.....T.....C.....>
BV1   ..T.....G.....T.....C.....C..C..A.....T.....C.....>

      730     740     750     760     770     780
B31  AACACATTACAGTACAACA ATACGACTCAATGGCACCA AATTAGAGGGTTCAGGAGTT
LV5  .....>
S-1-10 .....>
C-1-11 G.....TC.....A.....GC..A..A..C..G..A..CA.....C>
LV4  G.....A.....A.....GCA.....TC.....A..CAA.....C>
PKo   G.....A..T.....A.....CGCA..T.....T.....A..CA.....C>
BV1  G.....A..T.....A.....CGCA..T.....T.....A..CA.....C>

      790     800     810     820
B31  GAAATTACAAAACCTTGATGA AATTAAGAAACGCTTTAAAT AA
LV5  .....>
S-1-10 .....>
C-1-11 .....A..C.....C.....>
LV4  .....C.....A..A..T.....>
PKo   .....A..C.....C.....>
BV1  .....A..C.....C.....>

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FIG. 2. Nucleotide sequence of LV5, S-1-10, C-1-11, LV4, PKo, and BV1 *ospA* genes aligned against that of isolate B31 (5). Periods indicate identity with the B31 *ospA* nucleotide sequence, and capital letters indicate differences with it.

combined with 20 μ l of sterile guinea pig complement (200 50% hemolytic complement units per ml; Rockland, Gilbertsville, Pa.) and added to *B. burgdorferi* organisms. Suspensions were gently shaken and incubated at 32°C for 24 h. All assays were performed in duplicate or triplicate. Controls included samples containing normal and immune sera with viable spirochetes and heat-killed spirochetes (56°C for 30 min) in BSK medium with and without complement. After incubation, 100 μ l was removed from each assay and diluted 1:5 with PBS, and 50 μ l of acridine orange (5.4×10^{-9} M; Sigma) was added. Borrelial activity was detected with a FACScan flow cytometer (Becton Dickinson Immunocytometry Systems, Mountain View, Calif.). Viable and heat-killed *B. burgdorferi* organisms were differentiated from BSK, serum, and complement particles with side scatter and acridine orange fluorescence and spirochetes were gated during data acquisition. Data were acquired for 1 min and analyzed with FACScan LYSIS II software. Borrelial activity caused a significant decrease in the number of events per minute and a $\geq 16\%$ increase in fluorescence intensity (9, 18) compared with values obtained with normal serum. The borrelial antibody titer was the last dilution of serum that killed spirochetes compared with normal serum.

Nucleotide sequence accession numbers. The *ospA* nucleotide sequences were submitted to GenBank and assigned accession numbers U20356, U20357, U20358, U20359, and U20360.

RESULTS

Cloning and expression of *ospA* genes. *ospA* genes from the *B. burgdorferi* sensu lato isolates S-1-10, C-1-11, LV5, LV4, and BV1, representative of five seroprotective groups (19, 20), and the *B. afzelii* isolate PKo, representative of a sixth seroprotective group, were cloned into an expression vector for the production of recombinant OspA fusion proteins. Analysis by Western blot indicated that the molecular mass of each of the six affinity-purified fusion proteins was approximately 58 kDa. This is consistent with the predicted mass of a native 31-kDa OspA protein and a 27.5-kDa glutathione *S*-transferase protein (Fig. 1). The OspA fusion proteins were also highly reactive with anti-OspA monoclonal antibody and hamster anti-OspA serum. Anti-GST monoclonal antibody also reacted strongly with the 58-kDa proteins (data not shown).

Nucleotide and predicted amino acid sequence analysis of the *ospA* genes. The nucleotide and predicted amino acid sequences of the six *ospA* genes were compared and contrasted with the known nucleotide and amino acid sequences of the

Arbor, Mich.). Frozen suspensions of spirochetes were thawed, inoculated into fresh BSK medium, incubated at 32°C for 72 h, and diluted with fresh BSK medium to a concentration of 10^6 organisms per ml. Aliquots (100 μ l) of the *B. burgdorferi* cultures (10^5 spirochetes) were added to individual wells of a 96-well microtiter plate (GIBCO Laboratories, Grand Island, N.Y.). Subsequently, samples (100 μ l) of sera from vaccinated hamsters or their twofold dilutions were

TABLE 3. Recovery of *B. burgdorferi* isolates from tissues of hamsters vaccinated with OspA

Challenge (no. of organisms)	Rate of recovery with ^a :		
	Adjuvant alone	S-1-10 OspA + adjuvant	LV5 OspA + adjuvant
S-1-10 (10 ⁴)	3/3	0/3	0/3
LV4 (10 ⁴)	3/3	3/3 ^b	0/3
LV5 (10 ³)	3/3	3/3	3/3

^a Results are given as the ratio of the total number of tissues containing *B. burgdorferi* to the total number tested. The spleen, left kidney, and urinary bladder were cultured. There were three hamsters per group.

^b Although S-1-10 OspA antisera killed *B. garinii* LV4 in vitro (1:40), the killing activity was eightfold lower than that against the homologous isolate.

B. burgdorferi sensu lato LV5 or *B. burgdorferi* sensu stricto S-1-10 recombinant OspA proteins. Vaccinated animals were then challenged with LV5, S-1-10, or *B. garinii* LV4. Immunization with S-1-10 OspA provided protection against challenge with 10⁴ homologous organisms (Table 3). In contrast, these animals became infected when challenged with 10⁴ *B. garinii* LV4 organisms. In addition, vaccination with LV5 OspA provided protection against 10⁴ *B. garinii* LV4 or 10⁴ *B. burgdorferi* sensu stricto S-1-10 organisms. However, no protection was observed when LV5 OspA-vaccinated hamsters were challenged with homologous LV5 organisms, even with an inoculum of 10³ spirochetes. These in vivo results, especially those obtained with LV5 OspA antisera, correlated with in vitro findings.

DISCUSSION

OspA is currently the leading candidate for use in vaccination against infection with *B. burgdorferi* sensu lato. The immune response to this lipoprotein has been shown to confer protection against *B. burgdorferi* by passive transfer of monoclonal antibody (28) and active immunization with recombinant OspA protein (12, 14, 29). However, only the homologous isolate or a small number of closely related isolates were used to evaluate protection in early immunization studies. Recently, Telford et al. (38) used infected ticks as a source of antigenically different isolates of *B. burgdorferi* to demonstrate that vaccination with OspA induced broad protection. Although he demonstrated antigenic variability by reactivity to monoclonal antibodies, the challenge isolates may have been more representative of clonal polymorphism than antigenic variation since all the infected ticks were from the same focus of endemicity.

We have demonstrated the existence of at least five seroprotective groups among *B. burgdorferi* sensu lato isolates, including two distinct groups among North American *B. burgdorferi* sensu stricto isolates (19, 20). These seroprotective groups were identified by determination of the ability of polyclonal sera from hamsters infected with whole *B. burgdorferi* organisms to protect against infection with other *B. burgdorferi* sensu lato isolates. Using this procedure, we also recently identified a sixth, distinct seroprotective group. In this investigation, we determined whether antisera against recombinant OspA proteins from isolates of each of these seroprotective groups provided the same level of protection.

In some instances, the abilities of hamster OspA antisera to kill in vitro the homologous organism and *B. burgdorferi* sensu lato isolates from other seroprotective groups were identical to the protective abilities of antisera generated against whole *B. burgdorferi* organisms (19, 20). Antisera generated against the OspA proteins of S-1-10, C-1-11, LV4, and BV1 were able to

protect against infection with the homologous isolates. In addition, OspA antisera from *B. burgdorferi* sensu lato LV5 and *B. garinii* LV4 killed *B. burgdorferi* isolates from other seroprotective groups in the same previously reported pattern (19, 20). These results were confirmed in vivo by vaccination of hamsters with S-1-10 OspA and demonstration of protection against challenge with the homologous isolate, but not *B. garinii* LV4. Antisera generated against OspA of *B. burgdorferi* sensu stricto C-1-11, like polyclonal antisera (19), also did not kill the heterologous isolates in vitro. Fikrig et al. (15) previously reported that animals vaccinated with recombinant OspA from *B. burgdorferi* sensu stricto B31 were not protected from infection with *B. burgdorferi* sensu stricto 25015. The nucleotide sequence of the 25015 *ospA* gene is highly similar (99%) to that of the *ospA* of C-1-11. Other investigators (42, 43) have also recently identified North American *B. burgdorferi* sensu stricto isolates which appear to be similar to C-1-11 and 25015. Additional studies are necessary to determine the frequency distribution of this *B. burgdorferi* sensu stricto seroprotective group.

There were also some striking differences in in vitro killing between antisera from hamsters vaccinated with OspA and antisera from those infected with whole *B. burgdorferi* organisms. Most significantly, antisera against OspA from *B. afzelii* PKo and *B. burgdorferi* sensu lato LV5 failed to kill the homologous isolate in vitro. By contrast, antiserum raised against the whole organisms killed the homologous isolates (19, 20). These discrepancies were apparently not due to an inadequate anti-OspA response, since LV5 OspA antisera responded strongly by Western blot against LV5, *B. garinii* LV4, and *B. burgdorferi* sensu stricto S-1-10. In addition, LV5 OspA antisera could kill S-1-10 and LV4 in vitro at antiserum dilutions of 1:640 and 1:160, respectively.

These in vitro findings were confirmed by in vivo studies. When hamsters were vaccinated with LV5 OspA, recipient animals became infected with LV5 even after challenge with as few as 10³ organisms. Infection occurred despite development of sufficient OspA antibody titers to protect against challenge with 10⁴ S-1-10 or LV4 organisms. Wilske et al. (41) demonstrated an inability of vaccination of gerbils with OspA from *B. afzelii* PKo to induce protection against PKo, despite the development of significant levels of anti-OspA antibodies. In this investigation, we confirm these findings by also demonstrating that antisera to the OspA of PKo could not kill the homologous isolate in vitro. Additional studies are needed to determine mechanisms responsible for the inability of OspA antibodies to provide protection against infection with homologous *B. burgdorferi* isolates. These results may indicate that OspA, or protective OspA epitopes, may not be exposed on the surface of these spirochetes. To date, OspB (14), OspC (24), and the 39-kDa protein (34) have also been shown to induce protective antibody responses in animals. Whatever the mechanism(s) responsible for the failure of OspA antibodies to provide protection, these results suggest that protective antibodies against other *B. burgdorferi* proteins may be necessary for comprehensive protection.

These results are significant for an additional reason. Several investigators have defined the important immunogenic epitopes of OspA (33, 35, 36). This should make it theoretically possible to predict accurately the abilities of immune OspA antisera to protect against other isolates if the DNA sequence is known. However, this investigation demonstrated several important discrepancies. For example, *B. burgdorferi* sensu lato LV5 and *B. garinii* LV4 had significant differences at the OspA nucleotide level in previously described immunogenic regions (33, 35, 36). LV5 OspA antisera, however, provided complete

protection against *B. garinii* LV4. In addition, *B. burgdorferi* sensu stricto S-1-10 differed from LV5 only at 3 amino acids located in regions of the protein not known as immunogenic epitopes (33, 35, 36), but S-1-10 OspA antisera failed to provide protection against *B. burgdorferi* sensu lato LV5. Bockenstadt et al. (6) also recently demonstrated that a truncated OspA protein, containing the most important immunogenic epitopes (35), could not elicit protective immunity against the homologous isolate. Collectively, these results demonstrate that conclusions concerning the locations of protective epitopes on OspA may be premature, especially since epitope mapping studies have not included isolates from other genospecies. In addition, studies defining important conformational epitopes will likely also be necessary.

This study highlights the important role of heterogeneity in developing a subunit Lyme disease vaccine. Sequence analyses and genotyping did not accurately predict protection patterns in OspA-vaccinated animals. Consequently, in vivo animal studies or the in vitro borreliacidal assay appears necessary to accurately assess the ability of a *B. burgdorferi* protein(s) to provide protection against *B. burgdorferi* sensu lato organisms. In addition, the inability of OspA to protect against even the homologous isolate in some instances casts doubt on the ability of a single *B. burgdorferi* protein to form the basis of a comprehensive vaccine.

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