

Expression and Gene Sequence of Outer Surface Protein C of *Borrelia burgdorferi* Reisolated from Chronically Infected Mice

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OspC from *Borrelia burgdorferi* reisolated from mice persistently infected with cloned spirochetes was examined. In all cases, the sequence of the *ospC* gene was identical to that of the original inoculant. We conclude that variation of *ospC* is not necessary for evasion of the host immune system.

Lyme borreliosis is a multisystem disorder of humans and other mammals which involves the skin, musculoskeletal, cardiac, and neurological systems. This disease has been identified throughout the world and is caused by spirochetes of the group *Borrelia burgdorferi* sensu lato (19). Left untreated, Lyme borreliosis tends to be self limiting but is often seen to recur intermittently (19), indicating that remission of symptoms does not necessarily coincide with clearance of the infectious organisms from the body. Intermittent patterns of disease expression during persistent infection have also been observed in experimentally infected immunocompetent mice, and spirochetes have been consistently reisolated from these animals for up to at least 1 year after inoculation (3). The mechanism by which *B. burgdorferi* is able to persist in the body and cause recurrent bouts of disease is unknown.

When grown in culture, *B. burgdorferi* synthesizes a wide variety of proteins, including large quantities of the outer surface proteins OspA and OspB (9). Rather surprisingly, antibodies against these externally exposed proteins have only rarely been detected in the early phase of infection during naturally occurring Lyme borreliosis in humans or in animals infected in the laboratory by tick bite or by intradermal syringe inoculation with a small dose of bacteria (less than 10⁴) (2, 3, 8, 11, 14, 17, 24). In addition, antibodies against OspA or OspB could not be found in chronically infected mice until after several months of infection (3), suggesting that the immune system does not recognize these two proteins during the early course of infection.

In contrast, antibodies directed against outer surface protein C (OspC) have repeatedly been seen in natural and laboratory infections (11, 13, 17, 22). This highly antigenic protein has been used successfully in vaccine studies to protect laboratory animals from *B. burgdorferi* infection (13). In a previous study (3), *B. burgdorferi* was cultured from tissue sites of chronically infected mice 1 year after inoculation with a clonal population of *B. burgdorferi*. In this study, we examined the expression of OspC and the sequence of the corresponding *ospC* gene among late reisolates of *B. burgdorferi* compared to those of the original, clonal inoculant.

Reisolation of *B. burgdorferi* from chronically infected mice has been reported previously (3). Briefly, random-sex C3H/HeN mice were inoculated intradermally with 10⁴ cloned N40 bacteria. The clonal isolate N40 of *B. burgdorferi* was derived

from the original isolate N40 via repeated terminal dilutions and passage through mice. One year postinoculation, the mice were killed, and various tissues, including blood, urinary bladder, and ear punches, were collected aseptically and cultured in modified Barbour-Stoener-Kelly (BSK II) medium (1) at 33°C. Viable *B. burgdorferi* was identified via dark-field microscopy. Late-term reisolates of *B. burgdorferi* selected for study were obtained from the blood of three of the mice (mice 36, 43, and 44), from skin biopsies from two of the spirochetemic mice (mice 43 and 44), and from the skin and bladder of a fourth mouse (mouse 39).

It has been reported that North American isolates of *B. burgdorferi* are unable to synthesize OspC when grown in culture (23), yet all isolates examined to date contained the *ospC* gene (12, 18, 20, 22). To determine if isolate N40 produced OspC in vitro, we prepared immunoblots of a whole-cell lysate of N40 (3) with monoclonal antibody L221F8, which is directed against the OspC protein of *B. burgdorferi* sensu lato isolate PKo (10) (provided by B. Wilske). This antibody specifically labelled a single band corresponding to a protein of a molecular mass of 22 to 24 kDa (Fig. 1, lane A), indicating that isolate N40 is indeed capable of the synthesis of OspC.

To determine whether infected mice produced antibodies against OspC during the course of the infection, a recombinant OspC protein was generated from isolate N40 to serve as a target for immunoblotting. By use of the cloned *ospC* gene of N40 (see below) as a PCR template, a DNA fragment was synthesized to encode a truncated OspC protein in which the postulated leader-peptidase signal sequence (7) was deleted. We have observed during the expression of other *B. burgdorferi* outer surface proteins that the elimination of the signal sequence facilitated the enzymatic cleavage of the protein product from its fusion partner and allowed for the production of a soluble protein. The PCR oligonucleotide primers used were 5'-CGCGGATCCAATAATTCAGGG-3' and 5'-CGC GAATTCTTAAGGTTTTTTTGG-3', complementary to the regions encoding the amino terminus and the carboxy terminus, respectively, of the open reading frame of the *ospC* gene of isolate PKo (7). The amplified PCR product was ligated into expression vector pGEX-2T (Pharmacia, Piscataway, N.J.), to produce the desired protein linked to glutathione *S*-transferase (GT) via a thrombin cleavage site. Recombinant plasmids were transformed into *Escherichia coli* DH5 α , and the synthesis of GT-OspC was induced by 0.5 mM isopropyl-1-thio- β -D-galactopyranoside (IPTG) for 2 h. GT-OspC was purified over a glutathione-agarose column, and the recombinant OspC protein was cleaved from the bound GT with thrombin according

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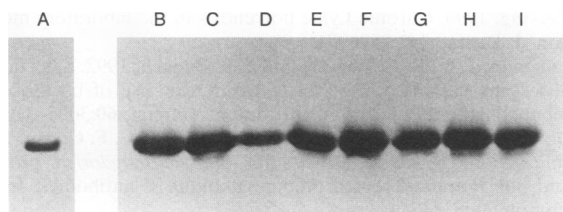


FIG. 1. Lane A, analysis of OspC expression by *B. burgdorferi* N40 by immunoblot using anti-OspC monoclonal antibody L221F8 (10); lanes B through I, OspC of N40 and seven 1-year reisolates identified by immunoblot with anti-N40 OspC polyclonal serum. Lanes: B, N40; C, 36 blood; D, 39 skin; E, 39 bladder; F, 43 blood; G, 43 skin; H, 44 blood; I, 44 skin (numbers identify the mice from which the samples were taken).

to the manufacturer's protocol (Pharmacia). Thrombin was extracted from the recombinant OspC eluate with antithrombin III-coated agarose beads (Sigma, St. Louis, Mo.). The resultant OspC appeared as a single band on Coomassie brilliant blue staining of a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and was recognized by monoclonal antibody L221F8. Immunoblots of the recombinant OspC protein, using serum collected from animals infected as described above, indicated that the mice produced antibodies against OspC within 30 days of inoculation.

One possible explanation for persistent *B. burgdorferi* infection could be a mutation which resulted in truncation of antigenic proteins or affected the ability to express such proteins. Variants of *B. burgdorferi* which are able to escape immune clearance through a mutation which results in a truncation of OspB have been detected (6). Additionally, bacteria which fail to express OspA or OspB have been isolated *in vitro* by growth in the presence of antibodies directed against these proteins (4, 16). Analysis of the protein content of the original, cloned N40 and of the 1-year reisolates indicated no obvious variations in the relative sizes or concentrations of any proteins visible by SDS-PAGE, including the major outer surface proteins OspA and OspB (3). Whole-cell lysates (3) were produced from the seven late reisolates of *B. burgdorferi*, grown for one passage in BSK II medium. The lysates were diluted to a final protein content of 0.85 mg/ml, and equal amounts were separated by SDS-polyacrylamide gel electrophoresis. Proteins were transferred to a nitrocellulose membrane as described elsewhere (3) and immunoblotted by use of anti-OspC polyclonal antiserum (prepared by hyperimmunization of C3H/HeN mice with recombinant OspC [immunoblot titer > 1:5,000]). The results (Fig. 1, lanes B through I) indicated that all of the late reisolates had retained the ability to synthesize OspC and that their OspC proteins were indistinguishable in size from the original N40 inoculant.

An infectious organism may be able to stay ahead of the host immune response if the gene for an externally exposed protein is capable of rapid mutation. Mutants which arise from clonal *B. burgdorferi* isolates and which express variant outer surface proteins and are resistant to antibodies against the original isolate have been identified (4, 6, 16). Additionally, recombination between outer surface protein genes has been observed (15). OspC is a highly variable protein, differing by up to 30% among isolates of the same species collected from the same geographic area (10, 20, 21, 22). It may be possible that these extensive differences are results of past antigenic variation within mammalian hosts.

We cloned and sequenced the *ospC* gene from N40 and the seven late reisolates to explore the possibility that persistent *B.*

burgdorferi infection was made possible by mutations which allowed the bacteria to avoid immune clearance. Portions (1 ml) of an N40 culture and of each of the initial reisolate cultures (unpassaged) were harvested by centrifugation, washed two times with phosphate-buffered saline, and finally lysed by vortexing in 30 μ l of distilled water and heating to 94°C for 5 min. Two microliters of each bacterial lysate was used as a template for PCR amplification with oligonucleotide primers based on the published sequence of the *ospC* gene of isolate PKo (7): 5'-CGCGGATCCTGCAAAGAAAATTGTTGGAC-3' and 5'-CGCGGATCCCCAGTTACTTTTTTAAACAA-3', complementary to DNA flanking regions approximately 30 bp upstream and downstream of the gene, respectively. PCR conditions consisted of 30 cycles of 1 min at 94°C, 1 min 30 s at 45°C, and 1 min 30 s at 74°C. We have also used these same primers and PCR conditions to amplify and clone the *ospC* genes from a number of other, unrelated isolates of *B. burgdorferi* (20). As a negative PCR control, all reactions were accompanied by mock amplifications containing primers, nucleotides, PCR buffer, and polymerase-lacking template. Efficiency and purity of amplification were determined by 0.7% agarose gel electrophoresis of a sample of the completed PCR mixtures, and in all eight cases a single band of approximately 700 bp was detected. No bands from any of the control amplifications lacking an *ospC* template were detected, indicating that there had been no contaminating source of template. The PCR products for each isolate were cloned by the TA Cloning System (Invitrogen, San Diego, Calif.). The cloned DNAs were purified by use of a Qiagen plasmid kit (Qiagen, Chatsworth, Calif.), and both strands were sequenced in entirety with a 373A DNA sequencer (Applied Biosystems, Foster City, Calif.). Each clone included an open reading frame of 627 nucleotides, capable of producing a protein of 209 amino acids with a molecular mass of 22.4 kDa (Fig. 2) and having a high degree of identity to the *ospC* gene of isolate PKo (7, 20). In every case, the nucleotide sequence of the *ospC* gene of each late reisolate was 100% identical to that of the original inoculant N40. This absolute conservation of sequence extended to the cloned upstream and downstream noncoding regions as well as the *ospC* open reading frame.

These results do not exclude the possibility that *ospC* variants of *B. burgdorferi* could have arisen during the course of the chronic infections, nor can it be assumed that the reisolates examined are representative of all the spirochetes within each tissue. It may have been that within the tissues were *ospC* variants which we failed to amplify, clone, and sequence. What is obvious from these data, however, is that mutational variation of the *ospC* gene is not required for the persistence of *B. burgdorferi* infection. Even though mice produced antibodies against OspC early in the *B. burgdorferi* infection, seven of seven *B. burgdorferi* late reisolates examined had an *ospC* gene which was absolutely identical to that of the original inoculant. This is perhaps most surprising for the three reisolates which were cultured from the bloodstream, as these bacteria would be expected to have been in full view of all of the components of the immune system.

A number of explanations can be suggested to account for the lack of variation in *ospC* during chronic infection. OspC may be expressed only intermittently during the course of infection, such that complete clearance of the bacteria by the immune system is made unlikely. Alternatively, OspC may be expressed continuously on the outer surface of the spirochete but is masked by host proteins such that antibodies directed against OspC are unable to interact appropriately with the protein. Such an interaction has been speculated to take place between the heavy chain of IgM and OspA and OspB (5).

TAATAATTCATAAATAAAAAGGAGGCCACAAATTA
 ATGAAAAAATACATTAAGTGAATTAATTAAGTACTTTATTTTATTATATCTTGTAAAT
 M K K N T L S A I L M T L P L F I S C N
 AATTGAGAAAAGATGGGAATGCATCTGCAAAATCTGCTGATGAGTCTGTTAAAGGSOCT
 N S G K D G N A S A N S A D E S V K G P
 AATCTTACAGAAATAAGTAAAAAATTCAGAAATCTAAGCAGTTGTTCTGGCOGTGAAA
 N L T E I S K K I T E S N A V V L A V K
 GAAGTGGAGACTTACTTGCATCTATAGATGAATCTGCTACCAAGCTATTGTTAAAAA
 E V E T L L A S I D E L A T K A I G K K
 ATAGGCAATAATGGTTTAGAGGCCAATCAGAGTAAAAACACATCAITGTTATCAGGAGCT
 I G N N G L E A N Q S K N T S L L S G A
 TATGCAATATCTGACTAATAGCAGAAAAATTAATGTATTGAAAAATGAAGAATTAAG
 Y A I S D L I A E K L N V L K N E E L K
 GAAAAGTTGATACAGCTAAGCAATGTTCTCAGAAATTTACTAATAAACTAAAAAGTGA
 E K I D T A K Q C S T G F T N K L K S E
 CATGCGTCTGGTCTGGCAATCTTACTGATGATAATGCACAAAGAGCTATTTTAAAA
 H A V L G L D N L T D D N A Q R A I L K
 AAACATGCAAAATAAGGTTAGGGTCTGCGAATCTGAAAAGTTAATTTAAAGCGGTAGAA
 K H A N K D K G A A E L E K L F K A V E
 AACTTATCAAAAGCAGCTCAAGACACATTAAAAAATGCTGTTAAAGAGCTTACAAGTCT
 N L S K A A Q D T L K N A V K E L T S P
 ATGTGGCAGAAAGTCCAAAAAACCTTAAATTAAGATCAATATTATAGATTAAAT
 I V A E S P K P P stop

FIG. 2. The nucleotide sequence of the *ospC* gene of *B. burgdorferi* N40 and the predicted amino acid sequence of the corresponding protein, having a molecular mass of 22.4 kDa. The proposed start and stop codons are underlined.

Additionally, the location of the *B. burgdorferi* bacteria during chronic infection is unclear. It is possible that during early Lyme borreliosis some of the spirochetes find hiding places in tissues where they can reside and avoid the immune system. Recurrence of the disease state could then occur by the occasional reemergence of some of these sequestered bacteria with subsequent evasion of the immune system as postulated above. It might be that some of the bacteria we cultured from the tissues of the chronically infected mice were in a state of dormancy within those mice. Having neither grown nor been exposed to the immune system since they initially disseminated during the early stage of infection of the mice, these bacteria would not have been under any pressure to mutate.

Nucleotide sequence accession number. The sequence shown in Fig. 2 has been submitted to the GenBank/EMBL sequence data library and given the accession number U04240.

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