Presence of Multiple Variants of *Borrelia burgdorferi* in the Natural Reservoir *Peromyscus leucopus* Throughout a Transmission Season

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ABSTRACT

White-footed mice (*Peromyscus leucopus*) serve as the principal reservoir for *Borrelia burgdorferi* and have been shown to remain infected for life. Complex infections with multiple genetic variants of *B. burgdorferi* occur in mice through multiple exposures to infected ticks or through exposure to ticks infected with multiple variants of *B. burgdorferi*. Using a combination of cloning and single strand conformation polymorphism (SSCP), *B. burgdorferi* *ospC* variation was assessed in serial samples collected from individual *P. leucopus* during a single transmission season. In individuals with *ospC* variation, at least seven *ospC* variants were recognized at each time point. One to four of these variants predominated at each time point; however, the predominant variants seldom remained consistent in an individual mouse throughout the entire sampling period. These results confirmed that mice in southern Maryland were persistently infected with multiple variants of *B. burgdorferi* throughout the transmission season. However, the presence of multiple *ospC* variants and the fluctuations in the frequency of these variants indicates that either new *ospC* variants are regularly introduced to this mouse population and predominate while the existing infections are cleared, or that the variation detected in the genetic profile at different time points reflects a complex mixture of *B. burgdorferi* populations whose relative frequencies may continually change. **Key Words:** *Borrelia*—Ixodes—Lyme disease.

INTRODUCTION

PEROMYSCUS LEUCOPUS, the white-footed mouse, serves as a host for larval and nymphal *Ixodes scapularis*, the vector of *Borrelia burgdorferi* and etiologic agent of Lyme disease in eastern North America. *Peromyscus leucopus* and other rodents remain infected for 1 year or longer when infected with *B. burgdorferi*, making them extremely successful reservoirs for continuing the enzootic cycle of this bacterium (Johnson et al. 1984, Duray and Johnson 1986, Donahue et al. 1987, Barthold et al. 1993, Hofmeister et al. 1999). In field studies, *B. burgdorferi* is detected throughout the year in *P.*

leucopus, with the highest prevalence (~75%) occurring between June and August (Anderson et al. 1987). In mice with existing infections, new infections can be introduced through subsequent tick bites (Bunikis et al. 2004).

Recent studies concluded that *B. burgdorferi* persistence is related to pathogen diversity (Hefty et al. 2002). *Borrelia burgdorferi* from mixed strain infections in mice can be successfully transferred to xenodiagnostic ticks. However, in almost all cases, only one strain is detected in the tick, suggesting that interference exists between the experimental infecting strains (Derdakova et al. 2004). Elias et al. (2002) hypothesized that *in vitro* growth of

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clonal *B. burgdorferi* strains may result in a heterogeneous population due to instabilities in the genome under the *in vitro* conditions.

The outer surface protein C (*ospC*) gene is frequently analyzed as a marker of infection but is of additional interest due to the high levels of diversity at both global and local scales (Wang et al. 1999). *OspC* is a highly polymorphic gene with 22 recognized groups where differentiation is defined by less than 2% sequence divergence within and greater than 8% sequence divergence between groups (Seinost et al. 1999, Wang et al. 1999). Between samples, the amino acid sequence identity of *ospC* is only 70–74% (Jauris-Heipke et al. 1993), with *B. burgdorferi* sensu stricto isolated in the United States being more heterogeneous than European isolates, suggesting that *B. burgdorferi* s.s. originated in the United States (Marti Ras et al. 1997).

Pathogenicity and degree of invasiveness have been associated with certain *ospC* groups (Seinost et al. 1999, Lagal et al. 2003). However, Lagal et al. (2003) determined that the clinical presentation of Lyme disease cannot be reliably predicted by the *ospC* sequence. In C3H mice, the severity and dissemination of *B. burgdorferi* depends on the isolate used for the infection which is not necessarily associated with the *ospC* sequence (Wang et al. 2001, 2002). Based on *ospC* sequences and original isolate source, Seinost et al. (1999) suggested three categories for *B. burgdorferi* sensu stricto: (1) isolates not from humans, (2) isolates from erythema migrans in humans, and (3) isolates from disseminated infections in humans, with this last group considered as the invasive group. Recent studies indicated that these groupings may not be accurate since two-thirds of human isolates in the mid-Atlantic region did not cluster with these invasive groups when sequenced (Alghaferi et al. 2005).

Multiple *B. burgdorferi* strains can exist in an individual mouse or tick at a given time point (Hofmeister et al. 1999, Wang et al. 1999). In mice experimentally infected with a mixture of *B. burgdorferi* clones, the relative dominance of the clones shifts during infection (Hofmeister et al. 1999). The mechanism behind this variance is not understood, but in some individu-

als, only one clone from the original heterogeneous mixture was detected at different time points, suggesting that competition or immune pressure from the host may be involved in selection of the dominant clones.

Previous cross-sectional studies in our laboratory have confirmed high diversity in *ospC* among rodent samples collected in southern Maryland, but the maintenance of this diversity within individuals throughout a transmission season had not been explored. The current study investigated whether the *ospC* variation observed in samples was attributable to the presence of multiple *B. burgdorferi* variants at each time point. We hypothesized that the observed *ospC* variant changes could be the result of a persistent infection complicated by the introduction of new variants, the resolution of the original infection and the subsequent introduction of new infectious variants, or the fluctuation in the relative frequency of individual variants in a complex infection.

METHODS

Sample collection and extraction

Peromyscus leucopus were trapped once a month from May through October 2002 in southern Maryland. One hundred Sherman traps (H.B. Sherman Traps, Tallahassee, FL) were placed in a 50 m × 200 m grid for 3 consecutive nights each month. At the time of first capture for each trapping period, ear tissue punch biopsies (2 mm) were collected. Tissue samples were extracted using the QIAamp DNA MiniKit (Qiagen, Inc., Valencia, CA) following the manufacturer's protocol with a single modification. After step 3 of the Tissue Protocol, samples were centrifuged for 3.5 min at 13,000 rpm to pellet the undigested hair, continued at step 4 using the supernatant, and eluted in 50 µL of high-performance liquid chromatography (HPLC) water.

PCR-based pathogen detection

Samples were obtained from mice that were sampled at least twice and were positive by polymerase chain reaction (PCR) for *B. burgdor-*

feri ospC, following the protocol of Wang et al. (1999) with modifications. The protocol was modified to use 1.5 μ L (~22 ng) of template DNA for the first round of PCR, and the annealing temperature was increased from 54°C to 60°C for the second round of PCR.

Cold single strand conformation polymorphism

Single strand conformation polymorphism (SSCP) was performed twice to analyze the genetic diversity of *ospC* (Orita et al. 1989, Norris et al. 1999). The first round was to determine whether the *ospC* fragment variation changed between sampling times in an individual mouse. The second round was performed after cloning amplicons from individuals whose *ospC* banding pattern varied at different time points in the first SSCP. For each positive clone, 5 μ L of PCR product was added to 7 μ L of denaturing loading mix (90% deionized formamide, 2% 1M NaOH, 8% ddH₂O, 0.005 g xylene cyanol, 0.005 g bromophenol blue) and heated to 95°C for 7 min before snap annealing on ice. A 10- μ L volume of each mixture was loaded on a nondenaturing 38.5% polyacrylamide gel and electrophoresed at 4°C at constant 25 mA for approximately 3 h on a vertical gel unit (16 $\times$ 18 cm, SE600 series; Hoefer Scientific Instruments, San Francisco, CA). Gels were stained using SYBR Green I Nucleic Acid Stain (Cambrex Bio-Science, Rockland, ME) and photodocumented.

Cloning and data analysis

ospC samples from four individuals with varying banding patterns and from two individuals with constant banding patterns were cloned using the TOPO TA Cloning Kit (Invitrogen Corp., Carlsbad, CA). The *ospC* amplicon vector fusion was transformed into OneShot TOP10 chemically competent *Escherichia coli* cells. A minimum of 94 transformed colonies were selected and screened by PCR using the *ospC* inner forward and outer reverse primers. For PCR, 1 μ L of the sample diluted in 50 μ L HPLC water was used as template. A minimum of 72 of the randomly selected 94 transformed clones from each original sample were used for analysis using SSCP. Banding patterns ob-

served on SSCP gels were analyzed for differences within an individual. The number of different variants and changes in the frequency of those variants in individual mice were examined over the sampling period.

RESULTS

Tissue samples were collected from 59 *P. leucopus* captured during two or more trapping periods. Of these, 28 (48%) were positive for *B. burgdorferi* infection by *ospC* PCR during at least two sampling periods. *ospC* diversity was analyzed using SSCP for 26 individuals; two individuals were not completed due to poor PCR results. The SSCP banding patterns for 18 individuals (18/26; 69%) remained the same at all sampling times (Fig. 1A). When samples from individuals with consistent banding patterns were cloned and examined using SSCP, the banding patterns were identical in all clones from a single sample and between all samples from that individual, confirming that only one *ospC* variant was present in each of these individuals. Banding patterns that differed at subsequent time points were observed in 31% (8/26) of individuals (Fig.

FIG. 1. Single strand conformation polymorphism (SSCP) gels illustrating banding patterns of *ospC*-positive *P. leucopus* that were sampled in multiple months. (A) SSCP gel illustrating conserved banding pattern across time points for a single animal. (B) SSCP gel illustrating variable banding patterns across time points for a single animal.

1B). Each of these individuals was captured and sampled between two and five times during the 6-month trapping period. High *ospC* diversity was observed on SSCP within any single sample from individuals with changing banding patterns (Fig. 2).

In a subset of four individuals whose *ospC* banding pattern changed over time, SSCP analysis revealed 21–41 *ospC* variants per individual. At any time point, however, the number of variants observed in an individual was 7–19 (Fig. 3). The majority of these variants represented less than 10% of the clones analyzed in a single sample; variants that were present in more than 10% of the clones analyzed were considered to be predominant. In any one individual, the total number of predominant variants from all samples analyzed was 7–9 (Table 1), with one to four variants predominating (frequencies of 10–87%) at each time point (Table 2). The relative dominance of the *ospC* variants present in an individual mouse shifted in serial samples, often appearing at a much lower frequency or not detectable at all. Conversely, a variant detected at a low frequency in 1 month could be present in the majority of the clones analyzed from a sample collected in a later month. In addition, variants that were not detected at all during one sampling period could be detected in a majority of clones in another sample from that same individual. Due to the nature of this study and the large number of SSCP variants observed, variation was quantified based on banding patterns rather than on sequence data.

DISCUSSION

This study was undertaken to determine whether the *ospC* changes observed by SSCP in serial samples from individual mice were the result of concurrent infections with multiple variants of *B. burgdorferi* or whether they were evidence of successive infection with different *ospC* variants. This study is the first to our knowledge to examine the temporal genetic variation of *B. burgdorferi* within individuals by intensively studying serial samples from mice. The small sample size ($n = 4$) limits the conclusions that can be made regarding *B. burgdorferi* infections in all *P. leucopus* populations. However, a wide number of *ospC* variants were present in an individual mouse at different time points. Furthermore, some variants appeared in or disappeared from the individual at different time points. In all of the individuals displaying *ospC* variation, minor variants were always present with the major or predominant variants.

This study investigated the number of *B. burgdorferi ospC* variations detected within persistently infected individual *P. leucopus* and whether the frequency of those variants changed or remained constant during a transmission season. Although nearly 70% of the individuals appeared to maintain an infection with a single *ospC* variant, the remainder displayed infections of a far more complex nature. This second group of mice displayed concurrent infection with more than one *ospC* type as evidenced by the SSCP banding patterns that

FIG. 2. Single strand conformation polymorphism (SSCP) gel showing 11 *ospC* variants recovered by cloning of a single polymerase chain reaction (PCR) amplicon (2002072311) from an individual *P. leucopus* (Mouse 005). Identical banding patterns are in lanes 2 and 5; lanes 6 and 9; and lanes 8 and 12. The molecular marker (M) is used as a guide for identifying banding patterns and not as a size indicator.

FIG. 3. Distribution of *ospC* variants identified in individual *P. leucopus* each month. The letter designations for each variant are valid within each individual only. They do not correspond between individuals.

changed from sample to sample. The changes observed in the SSCP banding patterns in these individuals were hypothesized to be due to fluctuations within complex infections of multiple *ospC* variants or due to the introduction of new infections with different variants. Our results show that different situations can coexist in an individual mouse: some *ospC* variants were detected repeatedly over the span of months, but at varying levels; other variants completely disappeared from subsequent samples; still other variants appeared for the first time several months after the study began, possibly indicating the introduction of a new infection. In order to fully understand the implications of this study, all variants need to be sequenced. Sequence data would confirm

whether or not the differences seen in *ospC* variants in these serial samples are sufficiently divergent (>8%) to be considered different variants and would allow comparison of the variants to the current *ospC* groups.

The immune response to *B. burgdorferi* in *P. leucopus* prevents re-infection with the same *ospC* variant as the original infecting variant but does not prevent infection with spirochetes carrying other *ospC* groups through subsequent exposures (Preac-Mursic et al. 1992, Probert et al. 1997). Multiple variants of *ospC* are known to infect at least 30% of *P. leucopus* in southern Maryland, with 25% of the mice infected with at least three different *ospC* groups simultaneously (Anderson and Norris 2006). Based on SSCP, approximately 31% of *P. leucopus* in our

TABLE 1. *ospC* VARIANTS OBSERVED FOR A SUBSET OF INDIVIDUAL *P. LEUCOPUS* WHEN THE *ospC* PCR PRODUCT WAS CLONED AND ANALYZED BY SSCP

Mouse	Number of samples cloned	Total number of variants identified	Total number of predominant variants*
001	3	34	8
005	5	41	9
034	3	24	7
037	4	21	8

*Predominant variants are those occurring in more than 10% of clones analyzed.

study were infected with multiple variants of *ospC*, confirming the point prevalence findings of Anderson and Norris (2006). Experimentally, infection with two strains of *ospC* can be maintained as subpopulations which persist in the mouse and then re-emerge at a later time (Hofmeister et al. 1999, Derdakova et al. 2004). In our study of naturally infected *P. leucopus*, a variant that was clearly prevalent in an individual and became either less common or undetectable at later points in time suggests that the variant was being maintained below a detectable level, that the variant had been completely eliminated from the mouse, or that the variant had possibly undergone genetic modifications of *ospC*. Intensive study of the *ospC* variants in four mice showed that the most frequent variant in a mouse remained so only during one sampling time point. Although the variant may have been present in earlier or later samples, it was never again the most prevalent

variant in that individual. This indicates that the mouse's immune system may be able to control the infection, thereby preventing it from becoming the most prevalent variant again, even though it may remain in circulation as a minor variant. This would allow other *ospC* variants to become established as the most abundant variants at other time points.

This study demonstrates that enormous variability exists in *ospC* isolates, within a small sample size from a small geographic region. The number of *ospC* variants observed in each mouse at any single time point differed widely, ranging from 7 to 19 at a single time point in an individual mouse. Of the 22 recognized variants of *ospC*, eight have been identified in Maryland and confirmed through sequencing and phylogenetic analysis (Anderson and Norris 2006). In *P. leucopus* in southern Maryland, two *ospC* groups (identified as A and K) occurred in 68% of the samples from a cross-sectional

TABLE 2. *ospC* VARIANT FREQUENCIES FOR A SUBSET OF INDIVIDUAL *P. LEUCOPUS* BY MONTH

Mouse	Month	Number of clones analyzed by SSCP	Number of variants present	Number of predominant variants*	Range of variant frequency (%)
001	May	109	17	2	1-45
	June	98	14	4	1-42
	July	72	9	2	1-72
005	May	80	13	2	1-34
	June	88	8	3	1-49
	July	73	15	2	1-45
	August	82	11	2	1-40
	October	89	7	1	1-87
034	May	82	11	3	1-42
	June	111	19	1	1-53
	August	98	8	3	1-43
037	May	91	8	1	1-80
	June	87	10	2	1-60
	July	85	10	3	1-40
	August	89	9	2	1-71

*Predominant variants are those occurring in more than 10% of clones analyzed.

study while two other groups (identified as C and D) were identified in less than 2% of the samples (Anderson and Norris 2006). An earlier study in Baltimore County, Maryland, identified *ospC* groups A and K in *P. leucopus* (Hofmeister et al. 1999). Due to the large number of *ospC* variants recognized in the current study, variants were not identified by sequencing, and, therefore, cannot be defined as belonging to specific *ospC* groups. However, the number of SSCP variants seen in this study suggest that genetic sequencing of serially collected samples would allow comparison with the findings of these other two studies, and would allow the determination of patterns of predominance of specific *ospC* groups in individuals showing complex infections with *B. burgdorferi*. The subtle differences observed in the SSCP banding patterns in our serial samples may represent point mutations. Sequencing results from a previous study identified 127 point mutations among *ospC* amplicons recovered from *P. leucopus* and *I. scapularis* (Anderson and Norris 2006). If all variants in this study were sequenced, the number of variants per sample may be reduced since variants with less than 2% difference between each other would be included in the same group. Alternatively, the *ospC* variation observed in individuals between time points could also be due to PCR bias for the most prevalent variant in the sample. Bias for certain genotypes has also been demonstrated through the PCR amplification of wild-type and mutant *ospA* at different frequencies (Malawista et al. 2000). Patterns of bias towards amplification of specific variants, however, were not noted which diminishes this possibility in our dataset.

We have corroborated the laboratory-based experiments of Hofmeister et al. (1999) showing that, in some individual mice, a single clonal population of *B. burgdorferi* appears to be maintained in a persistent infection. However, we found that, in other *P. leucopus*, the state of persistent infection was a result of the presence of multiple variants of *B. burgdorferi* and that the relative frequency of each variant was different at subsequent time points. Fluctuations in the frequency of *ospC* variants indicates that either new variants are regularly introduced to the mice while the existing infections are minimized or cleared, creating a changing pattern of pre-

dominance, or that the *B. burgdorferi* genetic profile constantly fluctuates in these mice as a result of dynamic spirochete populations. The maintenance of mixed infections of *B. burgdorferi* in naturally infected *P. leucopus* is hypothesized to be common (Hofmeister et al. 1999).

The high levels of variability present in individual mouse reservoirs may affect the diversity of variants transmitted to humans via ticks. Therefore, since certain *ospC* groups may be more infective to humans, the potential disease risk to humans depends on which *ospC* groups are circulating in the reservoir population. This study demonstrated that not all variants present in this reservoir population can be detected without cloning and that the presence and frequency of variants changes on a temporal scale. Therefore, without the benefit of cloning the samples, the presence of infective variants may go unrecognized.

The extensive *ospC* variation found in individual mice reinforces the need to fully understand the dynamics of *ospC* infections in the reservoirs since the vectors can presumably acquire any of these different *ospC* variants and transmit them to humans. The proposed links between *ospC* genotype and degree of invasiveness in human disease (Seinost et al. 1999) and the recent findings which contradict this association (Alghaferi et al. 2005) underscore the importance of understanding the complexity of the variants of *ospC* that are infecting mice. However, recent studies indicate that plasmids lp25 and lp28-1, rather than plasmid cp26 (which contains *ospC*), are important for infectivity and, in the case of lp28-1, persistence of *B. burgdorferi* in mammals (Purser and Norris 2000, Labandeira-Rey et al. 2001, 2003). Both of these plasmids encode for virulence factors which may be more important and relevant than *ospC* when studying *B. burgdorferi* pathogenesis. Therefore, studies examining these plasmids and the diversity of genes located on them in naturally infected *P. leucopus* will be useful in future genetic studies of *B. burgdorferi*.

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DISCLOSURE STATEMENT

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