Production of outer surface protein A by *Borrelia burgdorferi* during transmission from infected mammals to feeding ticks is insufficient to trigger OspA seroconversion

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Abstract
The Lyme disease spirochete, *Borrelia burgdorferi*, produces two outer surface lipoproteins, OspA and OspB, that are essential for colonization of tick vectors. Both proteins are highly expressed during transmission from infected mammals to feeding ticks and during colonization of tick midguts, but are repressed when bacteria are transmitted from ticks to mammals. Humans and other infected mammals generally do not produce antibodies against either protein, although some Lyme disease patients do seroconvert and produce antibodies against OspA for unknown reasons. We hypothesized that, if such patients had been fed upon by additional ticks, bacteria moving from the patients’ bodies to the feeding ticks would have produced OspA and OspB proteins, which then led to immune system recognition and antibody production. This hypothesis was tested by analyzing immune responses of infected mice following feedings by additional *Ixodes scapularis* ticks. However, results of the present studies demonstrate that expression of OspA and OspB by *B. burgdorferi* during transmission from infected mammals to feeding ticks does not trigger seroconversion.

Introduction
The causative agent of Lyme disease, *Borrelia burgdorferi*, is maintained in nature through an infectious cycle between vertebrate reservoir hosts and infected *Ixodes* spp. ticks. *Borrelia burgdorferi* regulates the expression of a number of proteins important for each stage of the life cycle in order to persist in these drastically different environments. Two of the most intensively studied proteins of *B. burgdorferi* are outer surface proteins A and B (OspA and OspB), encoded by the *ospAB* operon (Howe et al., 1986). These surface-exposed lipoproteins are abundantly expressed by bacteria in the tick midgut. However, production is repressed during tick feeding as bacteria migrate to the ticks’ salivary glands and are transmitted to the vertebrate host, and very few bacteria produce either protein at the time of deposition in the host (Schwan et al., 1995; de Silva et al., 1996; Schwan & Piesman, 2000). Early inhibition of OspA and OspB is essential for vertebrate infection, as *B. burgdorferi* mutants that constitutively express OspA and OspB are rapidly killed by host immune responses (Strother et al., 2007). In contrast, bacteria deficient in OspA or OspB appear to be fully infectious to mice (Yang et al., 2004). Both OspA and OspB are necessary for colonization of the tick midgut (Pal et al., 2000, 2004). OspA can bind a glycoprotein named TROSPA (tick receptor for OspA) found on the lining of the tick midgut (Pal et al., 2000, 2004). Together, these data suggest roles for OspA and OspB only during the tick portion of the *B. burgdorferi* infection cycle.

Consistent with this hypothesis, laboratory animals experimentally infected via tick bite do not produce antibodies against either OspA or OspB (Roehrig et al., 1992; Golde et al., 1994; Brunet et al., 1995; Nowalk et al., 2006 and this work). Wild mice also do not appear to produce OspA- or OspB-directed antibodies (Bunikis et al., 2004). Similarly, the majority of human Lyme disease patients do not produce OspA- and OspB-directed antibodies. However, some humans with prolonged *B. burgdorferi* infections do seroconvert to OspA and/or OspB (Kalish et al., 1993; Batsford et al., 1998; Akin et al., 1999; Nowalk et al., 2006). Development of OspA-directed antibodies has been associated with severe arthritis and neurological manifestations,
possibly due to autoimmune reactions (Gross et al., 1998; Akin et al., 1999; Alaedini & Latov, 2005). The reason some patients produce OspA- or OspB-directed antibodies during chronic Lyme disease is unknown.

The present study was designed to address this apparent contradiction: if OspA and OspB are tick-specific proteins, how is it possible for infected patients to seroconvert? A possible explanation for OspA and OspB seroconversion was hinted at by studies that investigated acquisition of B. burgdorferi by ticks feeding on infected mammals. Such bacteria produce high levels of both OspA and OspB during the time they leave mammalian bodies and enter feeding ticks (Schwan et al., 1995; de Silva et al., 1996; Schwan & Piesman, 2000). We hypothesized that if infected humans or other mammals were fed upon by additional ticks, B. burgdorferi that produce OspA and OspB as the bacteria move from the host into the feeding ticks could be detected by the patient’s immune system, thereby triggering seroconversion. To address this hypothesis, mice were infected by tick bite, and then 1 month later, were fed upon a second time by uninfected ticks. One month after that reinestation, the mice were examined for changes in serum antibody content, including antibodies recognizing OspA and OspB.

**Materials and methods**

**Bacteria and cultivation**

The infectious, clonal B. burgdorferi strain B31-MI-16 (Miller et al., 2003) was utilized for all infection and serological studies. Bacteria were cultured in Barbour–Stoenner–Kelly II (BSK-II) medium (Barbour, 1984) at 34 °C.

**Tick and mouse infections**

Ixodes scapularis egg masses were obtained from the Department of Entomology at Oklahoma State University (Stillwater, OK), and held in a humidified chamber. After hatching, cohorts of 200 larval ticks were each fed on female BALB/c mice (Harlan Sprague Dawley, Indianapolis, IN) that had been previously infected with B. burgdorferi strain B31-MI-16 (Miller et al., 2003). Larval ticks were allowed to feed to repletion and naturally detach from the mouse. Fed larvae were returned to the humidified chamber to molt into nymphs. After ecdisis, 20 nymphs each were fed to repletion on 12 naïve BALB/c mice. Over 50% of each mouse’s nymphs completed blood feeding and dropped off naturally. Seven days later, a 1 mm² ear biopsy from each mouse was individually inoculated into BSK-II medium. Antibiotics that do not affect B. burgdorferi (phosphomycin and rifampicin) and antifungal agents (amphotericin B) (Sigma, St Louis, MO) were included to reduce contamination. Mouse infections were confirmed 10 days later by examination of each biopsy culture using dark-field microscopy. One month postinfection, six of the nymph-infected mice were reinfested with uninfected larval ticks (200 ticks per mouse). This number of animals per group is sufficient for statistically significant analysis of infection parameters (Richardson & Overbaugh, 2005). Greater than 50% of the larvae placed on each mouse fed to repletion and detached naturally. Cohorts of engorged ticks from each mouse were dissected immediately after detachment for immunofluorescence analysis. Serum samples were collected from all 12 BALB/c mice before the initial nymph feeding. Samples were also collected from all 12 mice 1 month postinfection (immediately before the feeding of larvae on half the mice), and then again after 2 months of infection.

**Tick dissection and indirect immunofluorescence analysis**

Following reinfestations, midguts from two engorged ticks per mouse were dissected into 10 μL phosphate-buffered saline (PBS) on glass slides and allowed to air dry overnight. Slides were then fixed and permeabilized in acetone for 15 min and allowed to air dry. Slides were blocked overnight in PBS containing 0.2% bovine serum albumin (BSA) at 4 °C. Slides were then incubated with the monoclonal antibodies H5332, reactive with OspA (Barbour et al., 1983), or H4610, reactive with OspB (Rosa et al., 1992), for 1 h at room temperature. Slides were washed in PBS-0.2% BSA and incubated for 1 h at room temperature with rabbit polyclonal antiserum that was raised against B. burgdorferi total membrane proteins (Miller et al., 2003). Slides were washed and incubated simultaneously with 1:1000 dilutions of Alexa-Fluor 488-labeled goat anti-mouse IgG and Alexa-Fluor 594-labeled goat anti-rabbit IgG (Molecular Probes, Eugene, OR) for 45 min at room temperature. Slides were then washed, dried, and mounted in ProLong Antifade mounting medium (Molecular Probes). Slides were analyzed with a ×40 objective lens using a BX51 epifluorescence microscope (Olympus, Melville, NY) and a Retiga 200R Fast 1394 imaging system (Q-Imaging, Burnaby, BC, Canada). At least 25 fields were examined per dissected tick, with at least one spirochete visible per field.

**Recombinant OspA and OspB**

Borrelia burgdorferi strain B31-MI-16 purified DNA was used as the template for PCR amplification of ospA and ospB. The oligonucleotide primers MW-OspA F (5′-CAC CAA GCA AAA TGT TAG CAG CCT TGA CGA-3′) and MW-OspA R (5′-TTA TTA TTT TAA AGC GTT TTT TTC ATC AAG-3′) were used for amplification of ospA, and MW-OspB F (5′-CAC CGC ACA AAA AGG TGC TGA G-3′) and MW-OspB R (5′-TTA TTA TAA AGC GTT TTT TAA GCT CTG-3′) were used to amplify ospB. Each PCR product was separately cloned into pET200 Champion TOPO
Borrelia burgdorferi OspA seroconversion

Enzyme-linked immunosorbent analyses (ELISA)

Wells of 96-well Maxisorp Nunc-Immuno plates (Nalge Nunc International, Naperville, IL) were coated overnight at 4 °C with 10 μg mL⁻¹ of either recombinant OspA or recombinant OspB in 100 μL of 50 mM carbonate buffer (1.60 g L⁻¹ Na₂CO₃, 2.93 g L⁻¹ NaHCO₃), pH 9.6. Plates were washed in PBS–0.05% Tween 20 (PBS–T) and blocked for 2 h at room temperature in PBS–T containing 2% BSA. After three washes, plates were incubated for 2 h at room temperature with 100 μL of 1:100-diluted mouse sera in duplicate. Following three PBS–T washes, wells were incubated with a 1:5000 dilution of sheep anti-mouse immunoglobulin horseradish peroxidase conjugate (Amersham) for 1 h at room temperature. Plates were washed again with PBS–T, 100 μL of 3,3',5,5'-tetramethylbenzidine (Pierce) was then added to each well, and color was allowed to develop for 30 min at room temperature. The reaction was stopped by adding 50 μL 2 N H₂SO₄ per well. A₄₅₀ nm was then read with a VersaMax tunable microplate reader and SOFTMAX PRO software (Molecular Devices, Sunnyvale, CA). OspA and OspB ELISAs were each performed twice with each serum sample being analyzed in duplicate in each ELISA. Wells were coated with BSA to serve as a negative control. Anti-OspA (H5332) or anti-OspB (H4610) monoclonal antibodies were used as positive controls to ensure detection of recombinant OspA or OspB-coated wells, respectively.

Immunoblot analysis

Borrelia burgdorferi strain B31-MI-16 whole-cell lysate or purified recombinant OspA or OspB were separated by SDS-PAGE, electrotransferred to nitrocellulose membranes, and blocked with 5% nonfat dry milk in Tris-buffered saline–Tween 20 (TBS–T) [20 mM Tris (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween 20] overnight at 4 °C. Membranes were washed after blocking, then simultaneously incubated for 1 h at room temperature with each mouse serum sample, diluted 1:100 in TBS–T, using a MiniProtean II Multiscreen apparatus (Bio-Rad, Hercules, CA). Membranes were also incubated with monoclonal antibodies against OspA and OspB, as controls to identify where those proteins migrated in each gel. Membranes were then washed and incubated with sheep anti-mouse immunoglobulin horseradish peroxidase conjugate (Amersham) and chemiluminescence (Pierce).

Statistical analysis

To determine statistical significance, mouse serum antibody titers were compared using a repeated measures model. A P-value of ≤ 0.05 was considered significant.

Results

Borrelia burgdorferi expressed OspA and OspB as they were acquired by larval ticks feeding on infected mice

Previous studies demonstrated that B. burgdorferi produce OspA and OspB as they migrate from infected mammalian hosts into feeding ticks (Schwan et al., 1995; de Silva et al., 1996; Schwan & Piesman, 2000; Neelakanta et al., 2007). To confirm that the bacteria used in the present study also followed that pattern, mice previously infected by tick bite were reinfested with naïve, larval ticks. Prior cultivation of ear punch biopsies had demonstrated that all tested mice were infected with B. burgdorferi. Fed larvae were dissected, and their midguts were examined by immunofluorescence for the presence of B. burgdorferi and bacterial expression of both OspA and OspB. Borrelia burgdorferi were detected in the midguts of ticks that fed on all six of the reinfested, infected mice, further confirming that all the animals were indeed infected. Greater than 90% of B. burgdorferi detected in tick midguts demonstrated detectable production of OspA and OspB (Fig. 1 and data not shown), as described previously (Schwan et al., 1995; de Silva et al., 1996; Schwan & Piesman, 2000; Neelakanta et al., 2007).

Serum analyses of OspA- and OspB-directed antibodies

For each reinfested mouse, serum samples were collected before infection, 1 month after infection (but before the second tick feeding), and 2 months after infection (1 month after the second tick feeding). ELISA demonstrated that none of the six reinfested mice developed significantly increased levels of antibodies that recognized OspA or OspB after the second tick feeding (Fig. 2). Immunoblot analyses

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using recombinant proteins with the same serum samples similarly demonstrated no increases in antibodies recognizing either OspA or OspB (data not shown). Additionally, there were not any significant differences in antibody levels when ELISA results of mice that were reinfested with ticks were compared with animals that were not reinfested with larvae (Fig. 2). Positive controls using anti-OspA or anti-OspB monoclonal antibodies produced significant signals, with \(A_{450\text{nm}}\) values between 0.8 and 1.0 (data not shown), confirming that we had used the appropriate recombinant proteins and borrelial lysates.

One mouse had above-background levels of antibodies that bound OspB at all points, including before the time of \(B.\ burgdorferi\) infection (Fig. 2). These antibodies were not of a level sufficient for detection by immunoblot (data not shown and Fig. 3). This observation indicates that natural antibodies produced by that particular mouse coincidentally recognized OspB. Such natural antibodies that can bind OspA and/or OspB have been reported previously (Dorward et al., 1992; Belperron & Bockenstedt, 2001). Importantly, this animal did not develop increased levels of antibodies that recognized OspB at any time during infection. Also, of note, this animal never produced antibodies that bound OspA (Fig. 2).

**Antibody profile against \(B.\ burgdorferi\) of infected mice after second tick feeding**

In addition to examining the antibody responses to OspA and OspB, possible changes in antibody levels against other \(B.\ burgdorferi\) proteins were examined by the use of immunoblot analyses with \(B.\ burgdorferi\) whole-cell lysates. As noted above, all animals were confirmed to be infected with \(B.\ burgdorferi\) by outgrowth of \(B.\ burgdorferi\) skin biopsies of each mouse. Robust humoral responses were evident in both sets of infected mice, demonstrated by prominent immunoblot bands that migrate at the same size as the well-characterized \(B.\ burgdorferi\) antigens flagellin at c. 41 kDa and BmpA (P39) at c. 39 kDa (Fig. 3). One animal of each set exhibited only mild antibody responses, the reasons for which are unknown, as infection of those animals was demonstrated by both biopsy cultivation and tick xenodiagnosis. Profiles of \(B.\ burgdorferi\)-directed serum antibodies were indistinguishable between mice that had been reinfested with tick larvae and those that had not. This suggests that other \(B.\ burgdorferi\) proteins produced during mammal-to-tick transmission likewise do not provoke production of host serum antibodies.

**Discussion**

We hypothesized that OspA and OspB seroconversion might be caused by production of the outer surface proteins by \(B.\ burgdorferi\) during transmission from infected mammals to feeding ticks. However, our testing of this hypothesis indicated that the OspA or OspB seroconversion must be due to another cause. What, then, is the trigger for OspAB seroconversion during some cases of Lyme disease? There is some evidence that OspA and OspB may be expressed at some locations in the host body, independent of tick feeding, but the reasons for that are unknown (Coyle et al., 1995; Schutzer et al., 1997; Liang et al., 2004). Host inflammatory processes during mammalian infection have been proposed to induce the production of OspA by \(B.\ burgdorferi\) (Crowley & Huber, 2003; Scheelhoff et al., 2007), although the observation that most infected humans do not seroconvert indicates that the trigger(s) for OspA production must be more complex than just inflammation. We also note that severe inflammation is observed in many animal models of Lyme disease, but OspA or OspB seroconversion has not been reported in such animals. Another, not necessarily exclusionary, explanation is that human antibodies that recognize OspA or OspB are natural antibodies unrelated to infection, as was seen with one mouse in the present studies.

In conclusion, even though \(B.\ burgdorferi\) produces both OspA and OspB during transmission from infected mammals to feeding ticks, such protein expression is not sufficient to prompt seroconversion. Continued research on the OspA and OspB proteins, and host immune system
responses, will lead to a more complete understanding of *B. burgdorferi* infection processes as well as the treatment of late-stage disease manifestations of Lyme disease.

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