

***Borrelia burgdorferi* OspA Is an Arthropod-specific Transmission-blocking Lyme Disease Vaccine**

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Summary

Borrelia burgdorferi, the spirochetal agent of Lyme disease, is transmitted by *Ixodes* ticks. A vaccine based on *B. burgdorferi* outer surface protein (Osp) A protects mice from spirochete infection. Here we report on the expression of OspA on spirochetes inside engorging ticks and relate OspA expression to antispirochetal immunity. Spirochetes in the gut of unfed nymphal ticks were stained by an OspA antibody, whereas in feeding ticks, the majority of spirochetes in the gut and salivary glands did not stain with the antibody. Thus, OspA was not expressed on most spirochetes during transmission from the vector to the vertebrate host. To examine the mechanism of protection afforded by OspA antibody, mice were passively immunized with OspA antibody at different times relative to tick attachment. When OspA antibody was administered to mice before or at the time of tick attachment, spirochetal development events in the vector, such as growth and salivary gland invasion, were blocked and the mice were protected from *B. burgdorferi* infection. When OspA antibody was administered to mice 48 h after tick attachment, spirochetes persisted in the nymphs and the mice were not protected despite the presence of circulating antibodies in the host as well as in the tick blood meal. Thus, OspA immunity appears to be effective only during a narrow window time at the beginning of the blood meal when antibodies bind to OspA-expressing spirochetes in the tick gut and block transmission from the vector to the host.

The spirochetal agent of Lyme disease, *Borrelia burgdorferi* is transmitted by *Ixodes* ticks. In unfed nymphal ticks, spirochetes are found in the gut (1). During tick feeding, the bacteria disseminate to the salivary glands and infect the vertebrate host (2–4). A vaccine based on *B. burgdorferi* outer surface protein (Osp) A which protects mice from infection is currently being tested in human trials (5–7). Here we examine the expression of OspA on spirochetes within engorging nymphs and relate OspA expression to protection afforded by OspA antibody.

Materials and Methods

Mice. Pathogen-free C3H/HeN (C3H) mice were purchased from the National Institutes of Health (Bethesda, MD).

Maintenance and Infection of Ticks. *Ixodes dammini* (also known as *I. scapularis*) were derived from a colony in its second generation from field-collected adults, and were free of inherited infection. Larvae were allowed to engorge on CD-1 mice that had been infected 2 wk previously by the bites of three to five nymphal *I. dammini* containing the N40 strain of *B. burgdorferi*. Engorged larvae were collected, held in mesh-covered plaster of

paris containing vials, and were allowed to molt at 95% relative humidity at 21°C. The infected nymphs were held under the same conditions before their use in experiments, usually within 2 mo of molting.

Antibody Staining of OspA-expressing Spirochetes in Nymphal Ticks. Guts and salivary glands were dissected out of nymphs and prepared for antibody staining as previously described (2). mAb CIII.78, which binds to a COOH-terminal protective epitope on OspA, was used to assess OspA expression while a rabbit polyclonal antibody raised against the JD-1 strain of *B. burgdorferi* was used for visualizing spirochetes. The tissues were imaged on a confocal scanning laser microscope (MRC 600; Bio Rad Laboratories, Microscience Division, Cambridge, MA) equipped with an argon/krypton laser (2). When examining tick organs for spirochetes, the whole organ, including the entire thickness, was evaluated for the presence of spirochetes.

Estimation of Number of Spirochetes Expressing OspA inside Ticks. All internal organs were dissected out of infected nymphs that had either not fed or fed for 70 h on C3H/HeN mice. Organs from 10 nymphs were pooled in PBS and the tissues were ground using a 1-ml ounce homogenizer (Wheaton Instruments, Millville, NJ) to disrupt the tick cells and tissues while causing minimal damage to the much smaller spirochetes. From each homogenate, a total of four individual 10- μ l drops were placed on

silylated glass slides (PGC Scientific, Gaithersburg, MD) and allowed to air dry. Each slide was dipped in acetone for 5 min before staining with an anti-*B. burgdorferi* FITC-conjugated rabbit polyclonal antibody or polyclonal rabbit sera against OspA or OspB. Using a conventional fluorescence microscope, the number of spirochetes stained in 10 microscope fields (40× objective) was counted in each 10-μl drop. Four such drops were counted for each antisera and the mean number of spirochetes stained in a 10-μl drop was calculated for each antibody. The antiserum against whole spirochetes was used to estimate the total number of spirochetes in 10 μl, while the OspA and OspB antisera were used to assess the percentage of the total expressing these Osps.

Evaluation of Mice for *B. Burgdorferi* Infection and Disease. 2 wk after the detachment of ticks, the mice were killed and selected tissues (blood, spleen, urinary bladder, ear punch, and skin near tick attachment site) were aseptically collected and cultured in Barbour-Stoenner-Kelley II media and examined for spirochetes. For experiments involving the assessment of disease, knees, tibiotarsi, and hearts were formalin fixed, paraffin embedded, sectioned, and examined for inflammation. The sections were examined blindly and an animal was considered to have arthritis or carditis when at least one joint or the heart showed evidence of inflammation.

Results and Discussion

Although OspA is an abundant protein on *B. burgdorferi* grown in culture, vertebrate hosts infected by tick bite rarely seroconvert to OspA (8). Furthermore, once spirochetes have adapted to the host, they are not vulnerable to *B. burgdorferi* or OspA antisera (9, 10). OspA may therefore be differentially expressed during the life cycle of *B. burgdorferi* in the tick and vertebrate host. Accordingly, we used confocal fluorescence microscopy to examine OspA expression on *B. burgdorferi* within unfed and feeding ticks.

I. dammini were infected with a clonal population of *B. burgdorferi* strain N40. Guts and salivary glands were dissected from unfed nymphs and from ticks removed from mice after 60 h of feeding. Spirochetes were restricted to the gut of unfed nymphs, and were labeled with an OspA mAb and *B. burgdorferi* antiserum, indicating that OspA was expressed before tick attachment (Fig. 1, *A* and *B*). During tick feeding, the nymphs had many spirochetes in concentrated arrays in the gut, and smaller numbers in the salivary glands, which stained with *B. burgdorferi* antiserum (Fig. 1, *C* and *E*) but did not react with the OspA mAb (Fig. 1, *D* and *F*). A few OspA-labeled spirochetes were observed in the guts of engorged nymphs (data not shown). Thus, OspA was readily expressed by *B. burgdorferi* before tick feeding, but the protein was not present in the majority of spirochetes during engorgement.

An estimation of the proportion of *B. burgdorferi* expressing OspA in engorging nymphs was made by directly counting spirochetes that bound different antisera. Equal aliquots of tick homogenates were spotted onto glass slides and stained with *B. burgdorferi*, OspA, or OspB antisera. The *B. burgdorferi* antiserum reflected the total number of spirochetes, whereas the OspA or OspB antisera assessed the percentage of spirochetes expressing these Osps (Fig. 2). In unfed nymphs, all spirochetes were stained with the

OspA or OspB antisera (Fig. 2). In contrast, only 30 and 31% of *B. burgdorferi* from engorging ticks bound the OspA or OspB antisera respectively (Fig. 2). The lack of both OspA and OspB staining on the majority of spirochetes during tick feeding suggests that expression of these proteins may be regulated at the level of transcription because the genes encoding these two antigens are in the same operon (11).

The absence of OspA on the surface of spirochetes during transmission prompted us to investigate the mechanism by which the OspA vaccine protected mice. OspA antiserum was injected into C3H/HeN mice 24 h before placing four to seven infected nymphs on each mouse. 60 h after attachment, the partially engorged ticks were removed from the mice and examined for *B. burgdorferi*. 2 wk later, the mice were evaluated for infection. Nymphs that fed on mice treated with the control (glutathione transferase [GT]) antiserum were heavily infected and spirochetes were found in the salivary glands (Table 1). In contrast, only two of seven nymphs that had fed on mice injected with OspA antiserum contained a few *B. burgdorferi* in the guts. None of these ticks had spirochetes in the salivary glands (Table 1). Moreover, mice treated with OspA antiserum were protected from *B. burgdorferi* infection (Table 1). Therefore, in the presence of OspA antiserum, spirochetes in the tick gut were destroyed, and replication and salivary gland invasion, critical parts of the transmission process, were blocked. OspA antiserum primarily protected mice by blocking transmission within the vector.

Table 1. Effect of OspA Antibody on Transmission of *B. Burgdorferi* by Nymphal Ticks

Ticks* (antisera used for murine immunization)	Infected tick organs‡		Infection of mice§
	Midguts	Salivary glands	
Unfed nymphs	3/4	0/4	NA
Fed nymphs (GT)	6/6	6/6	6/6
Fed nymphs (GT-OspA)	2/7	0/7	0/6

* *B. burgdorferi* N40-infected *I. dammini* nymphs were divided into three groups. The first group was set aside as an unfed control group. The second group was placed on six C3H/HeN mice (four to seven nymphs per mouse) which, 24 h before the placement of ticks, had received a control rabbit antiserum raised against GT (*Fed nymphs*, *GT*). The third group of nymphs were placed on C3H/HeN mice (four to seven nymphs per mouse), which had received serum from a rabbit immunized with a OspA-GT fusion protein (*Fed nymphs*, *GT-OspA*). Each mouse received 200 μl (100 μl i.p. and 100 μl s.c.) of the antiserum.

‡ Ticks were removed by forceps 60 h after placement, and midguts and salivary glands were dissected out and examined for spirochetes by staining with a FITC-conjugated rabbit polyclonal antibody raised against *B. burgdorferi* and performing confocal microscopy.

§ The mice were killed 2 wk after tick removal and checked for infection by attempting to culture spirochetes from blood, spleen, urinary bladder, and ear skin near the tick attachment site.

|| Very few spirochetes were observed in these two infected midguts. NA, not applicable.

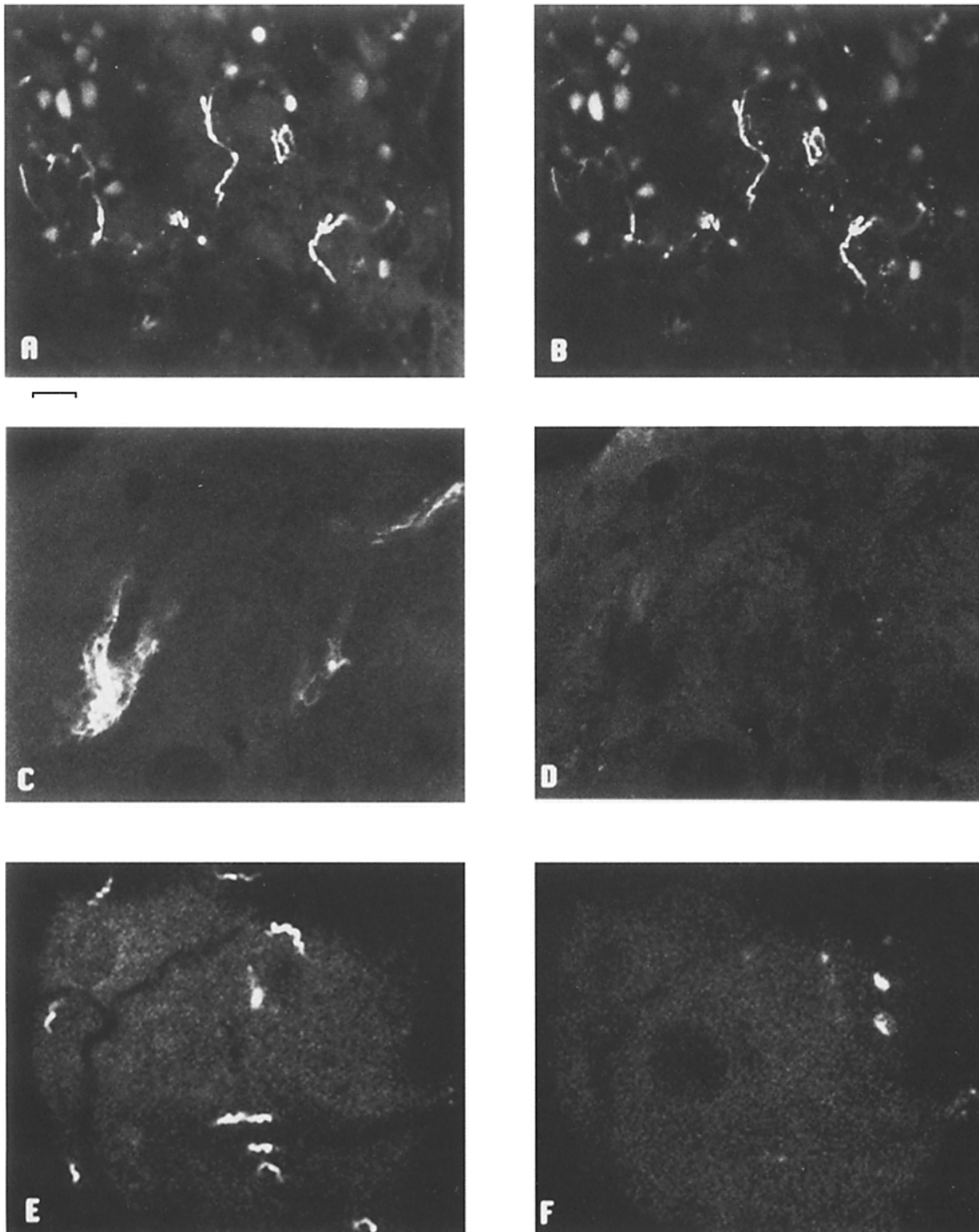


Figure 1. Confocal fluorescence images of *B. burgdorferi* in tick organs stained for OspA expression. (A and B) The same field of a gut from an uninfected nymph. (C and D) The same field of a gut from an infected nymph that had fed for 60 h. (E and F) are the same field of a salivary acinus from an infected nymph that had fed for 60 h. A, C, and E display the FITC signal from a rabbit serum raised against whole spirochetes, while B, D, and F display the Texas red signal from an mAb that binds to OspA. OspA antibody staining was readily detected on spirochetes before tick feeding. During engorgement, the majority failed to stain with the OspA antibody. Bar, 12.5 μm .

Because OspA is differentially expressed during tick feeding (Figs. 1 and 2), we assessed *B. burgdorferi* transmission when OspA antiserum was administered at various times during tick engorgement. Infected nymphs were placed on mice and at various times relative to attachment

(-24, 0, 24, 48, and 72 h) OspA antiserum was passively administered to the animals. The nymphs fed to repletion, detached from the mice, and 10 d later, they were examined by immunofluorescence microscopy for spirochetes. *B. burgdorferi* were rarely observed in the nymphs recovered

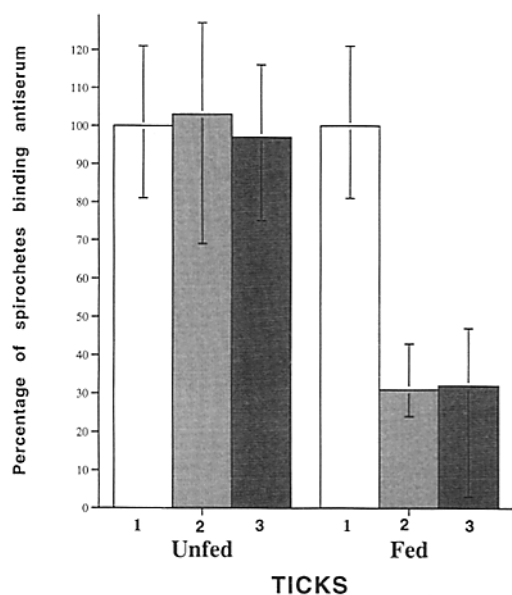


Figure 2. OspA and OspB are both not expressed on spirochetes within feeding nymphs. To estimate the number of spirochetes that have OspA and B on the surface before and during tick feeding, the organs were dissected out of infected nymphs that had not fed (10 nymphs) or that had fed for 70 h (10 nymphs). Homogenates were prepared from the dissected organs and stained with a polyclonal antibody raised against whole spirochetes (1), or polyclonal sera raised against OspA (2) or OspB (3) to estimate the percentage of spirochetes expressing OspA and OspB.

from mice given OspA antiserum 24 h before or during tick placement (Table 2). When OspA antiserum was administered to mice at the 24-, 48- and 72-h time points, spirochetes remained in all nymphs, indicating that the disappearance of OspA begins between 0 and 24 h after attachment. Infected nymphs at the 24-h time point had low-grade infections (Table 2), suggesting that these ticks had a mixture of *B. burgdorferi*, including organisms with and without OspA. Selective destruction of the spirochetes

expressing OspA may therefore have led to a reduction in the overall number of *B. burgdorferi*. At later time points (48 and 72 h), as more spirochetes failed to express OspA, the nymphs remained heavily infected (Table 2).

B. burgdorferi were not cultured from mice that were treated with OspA antiserum at -24 and 0 h relative to tick attachment (Table 2). Mice were protected even when antiserum was administered 24 h after attachment, despite a low level of spirochete infection within the nymphs (Table 2). The absence of host infection at the 24-h time point may be caused, in part, by the large decrease in the population of spirochetes in these nymphs (Table 2). In contrast to the 24-h time point, when OspA antiserum was administered 48 and 72 h after tick attachment, all mice became infected and developed disease (Table 2), suggesting that the spirochetes entering mice did not express OspA and were unaffected by circulating OspA antibody.

After nymphal tick attachment, spirochete transmission requires ~48 h, and during this time period, *B. burgdorferi* multiply and cross the gut epithelial barrier into the hemolymph, disseminate to the salivary glands, and infect the host via tick saliva (2, 3, 12). Our results document that during tick engorgement, OspA, which is abundantly expressed on spirochetes in unfed ticks, is no longer expressed on the majority of *B. burgdorferi* in the vector. The loss of OspA probably begins ~24 h after tick attachment, since spirochetes resistant to OspA antiserum were first detected at this time. The absence of OspA appears to continue even after the spirochetes enter the mammalian host because mice were infected when OspA antiserum was administered 48 and 72 h after tick attachment (Table 2). A recent report demonstrated that OspC is absent from the spirochetes in unfed ticks, and is present in feeding ticks and infected mice (13). During tick engorgement, we observed that spirochetes in the gut and salivary glands that do not express OspA react with OspC antiserum (data not shown).

Table 2. Effect of OspA Antibody Administered to Mice at Different Times after Tick Attachment

Antibody* (time administered relative to tick placement)	Infected nymphs† (severity of infection‡)	Infection of mice§	
		Culture	Disease
Anti-GT serum (-24 h)	5/7 (3, heavy; 2, moderate)	4/4	3/3
Anti-GT-OspA serum (-24 h)	0/6	0/4	0/4
Anti-GT-OspA serum (0 h)	1/7 (1, low)	0/4	0/4
Anti-GT-OspA serum (24 h)	6/6 (6, low)	0/4	0/4
Anti-GT-OspA serum (48 h)	9/9 (7, heavy; 2, moderate)	4/4	4/4
Anti-GT-OspA serum (72 h)	7/7 (2, heavy; 2, moderate; 3, low)	4/4	4/4

* Four mice were used in each group and four *B. burgdorferi* N40-infected *I. dammini* nymphs were placed on each mouse. The groups of mice were infused with 200 µl (100 µl i.p. and 100 µl s.c.) of serum from a rabbit immunized with a rt OspA-GT fusion protein (GT-OspA) at the indicated times before or after tick attachment. A control rabbit serum raised against GT was used at the -24 h time point.

† 10 d after detachment, ticks were examined by indirect immunofluorescence microscopy for spirochetes.

§ The mice were killed 2 wk after tick detachment for culture (blood, spleen, urinary bladder, ear, and tick attachment site) and histopathology (evaluation of the joints and hearts for arthritis and/or carditis).

|| To distinguish nymphs with low, moderate and heavy infections the number of spirochetes in 10 (100× objective) microscope fields were counted. Nymphs with <20 *B. burgdorferi* were characterized as having low, 21-50 as moderate, and 51-200 as heavy infection, respectively.

Thus, as spirochetes move from the vector, many appear to be "changing their coat" by, at least in part, differential expression of OspA and OspC. During engorgement, temperature shifts and biochemical changes in the tick may directly or indirectly influence Osp expression (13, 14).

The loss of OspA expression by spirochetes during transmission has revealed the mechanism by which the OspA vaccine protects vertebrate hosts: antibody binds to the OspA-expressing *B. burgdorferi* in the tick gut and prevents their replication and subsequent dissemination to the salivary glands and, ultimately, to the vertebrate host. This is

the first example of a vaccine undergoing human clinical trials that protects by blocking transmission from the vector. Approximately 24 h after tick attachment, some of the spirochetes in the vector become resistant to vaccine-induced OspA immunity, probably because they no longer express OspA on their surface. By 48 h after tick engorgement, *B. burgdorferi* that had entered mice were fully resistant to OspA antiserum. Thus, the OspA vaccine is likely to be effective only during a narrow window of 24 h after tick attachment, when OspA antibody comes into contact with OspA-expressing spirochetes in the tick gut.

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