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Arthropod- and Host-Specific *Borrelia burgdorferi bbk32* Expression and the Inhibition of Spirochete Transmission[1]

Erol Fikrig,* Wen Feng,† Stephen W. Barthold,§ Sam R. Telford III,¶ and Richard A. Flavell[2†‡]

Antisera to BBK32 (a *Borrelia burgdorferi* fibronectin-binding protein) and BBK50, two Ags synthesized during infection, protect mice from experimental syringe-borne Lyme borreliosis. Therefore, *B. burgdorferi bbk32* and *bbk50* expression within *Ixodes scapularis* ticks and the murine host, and the effect of BBK32 and BBK50 antisera on spirochetes throughout the vector-host life cycle were investigated. *bbk32* and *bbk50* mRNA and protein were first detected within engorged ticks, demonstrating regulated expression within the vector. Then *bbk32* expression increased in mice at the cutaneous site of inoculation. During disseminated murine infection, *bbk32* and *bbk50* were expressed in several murine tissues, and mRNA levels were greatest in the heart and spleen at 30 days. BBK32 antisera protected mice from tick-borne *B. burgdorferi* infection and spirochete numbers were reduced by 90% within nymphs that engorged on immunized mice. Moreover, 75% of these ticks did not retain spirochetes upon molting, and subsequent *B. burgdorferi* transmission by adult ticks was impaired. Larval acquisition of *B. burgdorferi* by *I. scapularis* was also inhibited by BBK32 antisera. These data demonstrate that *bbk32* and *bbk50* are expressed during tick engorgement and that BBK32 antisera can interfere with spirochete transmission at various stages of the vector-host life cycle. These studies provide insight into mechanisms of immunity to Lyme borreliosis and other vector-borne diseases. *The Journal of Immunology,* 2000, 164: 5344–5351.

Borrelia burgdorferi, the causative agent of Lyme disease, expresses different genes during its life cycle (1–3). In the gut of *Ixodes scapularis* ticks, spirochetes abundantly synthesize outer surface protein (Osp)[3] A (4–6). When ticks engorge, *B. burgdorferi* begin to express *ospC* and down-regulate *ospA* (4, 5, 7). Several genes are then subsequently expressed during mammalian infection, including *ospE/F* paralogues (*erps*), *bbk32* and *bbk50*, among others (8–17). *bbk32* encodes a 47-kDa fibronectin-binding protein: this gene was initially named *p35* based on partial sequence data that were available before the publication of the *B. burgdorferi* genome, and subsequently designated as *p47* or *bbk32* (11, 13, 18). Similarly, the *p37* gene corresponds to the *bbk50* genome sequence, and unrelated 37-kDa proteins have been described (11, 18, 19). To avoid confusion, the *bbk32* and *bbk50* designations for the genes previously referred to as *p35* and *p37* will be used throughout this manuscript (18).

B. burgdorferi N40 preferentially synthesize BBK32 and BBK50 during murine and human Lyme disease. These proteins were first simultaneously identified by differentially probing a *B. burgdorferi* N40 expression library with sera from *B. burgdorferi*-infected mice and sera from mice hyperimmunized with killed spirochetes (11, 16). RT-PCR and immunofluorescence studies then directly showed that *bbk32* and *bbk50* were expressed in vivo but not in vitro (11, 20). BBK32 and BBK50 Abs were also found in the sera of *B. burgdorferi*-infected mice and individuals with Lyme disease, further indicating that these Ags were synthesized during infection of the vertebrate host (11, 21). Studies by Probert and Johnson (13) then showed that BBK32 is a fibronectin-binding protein and plays an important role in the attachment of spirochetes to the extracellular matrix. These studies also confirmed that BBK32 was not synthesized by *B. burgdorferi* N40 during in vitro cultivation (13). It was also demonstrated, however, that five of six *Borrelia* species and four of eight *B. burgdorferi* sensu lato isolates synthesized BBK32 or other fibronectin-binding proteins when cultured in BSK II medium (13, 22).

Humoral responses to BBK32 and BBK50 can influence the course of Lyme borreliosis. Murine arthritis and carditis develops at 2–4 wk and regresses during the subsequent 1–2 mo. The resolution of disease correlated with the genesis of high-titer BBK32 and BBK50 Abs (11). The ability of BBK32 and BBK50 Abs to afford immunity was therefore directly tested. Active immunization with BBK32 and BBK50, or passive immunization with BBK32 and BBK50 antisera, partially protected mice from challenge with a syringe inocula of spirochetes (11). Partial or complete immunity following vaccination has been observed with several other *B. burgdorferi* proteins, most notably OspA, OspB, OspC, OspF, and decorin-binding protein (Dbp) A (23–29). OspA has been the most extensively studied immunogen, has successfully completed phase III clinical trials, and has been approved by the Federal Drug Administration for use as a vaccine against Lyme disease (30, 31).

The administration of BBK32 and BBK50 Abs to mice that had been infected with spirochetes for 24 h also eradicated *B. burgdorferi* infection (11). Postinfection clearance of *B. burgdorferi* has not been observed with OspA Abs and has only been described

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[3] Abbreviations used in this paper: Osp, outer surface protein; Dbp, decorin-binding protein; GT, glutathione transferase; IFA, indirect immunofluorescence.

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in experimental syringe-challenge studies with Abs to OspC and DbpA (29, 32–34). To extend these observations to human infection, the Ag-specific humoral responses in a cohort of patients with untreated Lyme disease were correlated with progression from early- to late-stage disease (21). High levels of BBK32 Abs were associated with early-stage disease that resolved, whereas the lack of significant BBK32 Abs resulted in an increased likelihood of the development of late-stage Lyme arthritis. These studies suggest that BBK32 and BBK50 Abs, and in particular BBK32 Abs, can affect the course of Lyme disease in mice or humans.

An investigation of *bbk32* and *bbk50* expression throughout the spirochete life cycle should provide a more realistic understanding of the importance of these proteins. Research on the selective expression of *bbk32* and *bbk50* in vivo has relied upon comparing spirochetes cultured in BSK II medium with *B. burgdorferi* in mice following experimental challenge with inocula of in vitro-cultivated organisms. Obviously, spirochetes are not normally exposed to BSK II medium, but rather persist in a tick-mouse-tick cycle (1). More specifically, spirochetes reside in particular tissues in the vector or host at precise intervals during infection. *B. burgdorferi* are located in the gut of flat *I. scapularis* and migrate to the salivary glands of feeding ticks before moving to a reservoir host. Spirochetes are then deposited into the host skin. After several days, *B. burgdorferi* disseminate via the bloodstream to distant tissues. Spirochetes must then return from the host to the ticks that feed on *B. burgdorferi*-infected mice because the efficiency of transovarial transmission is low (35). Temporal and tissue-specific *B. burgdorferi* gene expression should consider these diverse niches.

Materials and Methods

Spirochetes

A clonal isolate of *B. burgdorferi* N40 that is infectious and pathogenic in mice was used throughout (36). Spirochetes were cultivated in BSK II medium at 33°C (37).

Animals

Three-week-old female C3H/HeNCr (C3H) and C.B-17-*scid* mice were obtained from the Frederick Cancer Research Center (Frederick, MD) and housed in filter-frame cages. Mice were given food and water ad libitum and euthanized with CO_2.

Two hundred- to 500-g female Hartley guinea pigs were housed in individual hanging cages at Yale University animal facilities.

Ticks

Mated adult female *I. scapularis* were collected in the field. The egg mass was then laid in the laboratory. Hatched larvae were fed on uninfected C3H mice to produce pathogen-free nymphs. Alternatively, to generate *B. burgdorferi*-infected nymphal ticks, larvae were allowed to engorge to repletion upon *B. burgdorferi* N40-infected mice and then molt to the nymphal stage. *B. burgdorferi* infection rates within the nymphs were >90%. All tick rearing was performed in an incubator at 26°C with 85% relative humidity and a 12-h:12-h light:dark photo period regimen.

Infection and immunization

For infection, 4- to 6-wk-old C3H mice were intradermally challenged with 10^4 *B. burgdorferi* N40 and sacrificed at 14, 30, and 60 days. In an experiment, groups of 10–20 mice were inoculated with *B. burgdorferi* and 4–5 mice were sacrificed at each time point. All studies were repeated, and in each experiment, tissue samples from each mouse were collected and stored individually.

For the postinfection clearance studies, mice were first infected with 10^4 spirochetes by intradermal syringe inoculation and administered 200 μl of rabbit BBK32 and BBK50 antisera on days 1, 2, 4, or 8 (11). Control mice were administered glutathione transferase (GT) antisera in an identical manner (11). The BBK32 and BBK50 antisera had been previously produced (11). BBK32 antisera were prepared by immunizing animals with the p35 portion of the BBK32 protein and have been shown to be immunogenic in the murine model (11). The full-length BBK32 protein from *B. burgdorferi* N40 has also been produced in our laboratory, but is not highly antigenic in its current form (data not shown) and was therefore not used for these studies. Mice were killed at 14 days. At necropsy, blood, spleen, urinary bladder, and skin specimens were collected aseptically, cultured in BSK II medium, and examined by dark-field microscopy for spirochetes. The tibiotarsal joints and the hearts were Formalin-fixed, paraffin-embedded, sectioned, and examined microscopically for inflammation (36). Arthritis was graded by blinded analysis on a scale from 0, no inflammation, to 3, severe disease (36).

For the larval acquisition studies, groups of four to five C.B.17-*scid* mice were infected with 10^4 *B. burgdorferi* by intradermal challenge. At 20 days, mice were passively immunized with 200 μl of BBK32 and BBK50 antisera or a control GT antiserum. Twenty-four hours after passive immunization, mice were anesthetized with ketamine/xylazine and 50 larvae were placed on each mouse. The larvae fed to repletion and were examined for spirochetes. Individual larvae were tested by immunofluorescence (see below), using *B. burgdorferi*-antisera, for the presence of spirochetes.

For the tick-borne transmission studies, groups of four to five mice were first administered 200 μl of BBK32 and BBK50 antisera. Twenty-four hours later, 4 or 20 *I. scapularis* nymphs, infected with *B. burgdorferi* N40, were allowed to feed to repletion on each of the mice. Engorged ticks usually fell from the animals within 4 days and were collected in water baths below the cages. Two weeks later, all mice were sacrificed and examined for infection and disease, as previously stated.

In the guinea pig infection studies, a 5- to 10-cm section of the animals' posterior mid thorax was shaved and then three to four adult ticks were applied per guinea pig and allowed to attach. The adult ticks were obtained from groups of nymphal ticks that had fed upon mice immunized with BBK32, BBK50, or GT antisera and then allowed to undergo the molting process. Guinea pigs were sacrificed 2 wk after tick challenge. Sera were collected and tested for Abs to *B. burgdorferi* by immunoblot (38).

PCR

PCR amplification of *B. burgdorferi* DNA was performed for 30 cycles with denaturing, annealing, and extension temperatures of 94°C for 1 min, 55°C for 1 min, and 72°C for 2 min. 5′ and 3′ primers were: 5′-CAAT CAGGTAACGGC-3′ and 5′-TCTATTTTGGAAAGC-3′ for *flaB*, 5′-GC GGTGAAAGTGGTGAATTGG-3′ and 5′-CTTTAGCAAAGTTGTCAA GGCGTG-3′ for *bbk32*, and 5′-GACAACAACAGAAGTGGTCGCAC-3′ and 5′-TAGCATCGGAA TGAGCTGTACC-3′ for *bbk50. flaB*, which has one copy on the *B. burgdorferi* chromosome, was used to quantify the organism load (18).

For RT-PCR, total RNA was isolated from the spleens, skin, tibiotarsal joints, bladders, and hearts of *B. burgdorferi*-infected mice by acid guanidinium thiocyanate phenol-chloroform extraction (16). RNA was treated with RNase-free DNase (Promega, Madison WI) for 3 h at 37°C. A total of 2 μg of RNA was then reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Stratagene, La Jolla, CA). PCR was then conducted for 30 cycles.

For quantitative PCR, a competition assay was performed based on established techniques (12, 39). Competitor constructs were created in which a 540-bp fragment of unrelated DNA was flanked by the *flaB, bbk32*, or *bbk50* primers. The unrelated DNA was a fragment of the gene encoding the 44-kDa Ag of the agent of human granulocytic ehrlichiosis, amplified using the *Ehrlichia* primers 5′-TTTGCAGGACGAAGAAGAATGG-3′ and 5′-CCAACGCAAGCATAAGGAACAAC-3′, which have previously been used in constructs for competitive PCR (40). Varying concentrations of competitor were added to the RT-PCR reactions, and the concentration at which the amplified competitor product and the gene of interest were similar in intensity was used to calculate the quantity of mRNA or DNA.

Immunofluorescence of B. burgdorferi *within ticks*

Groups of *B. burgdorferi* N40-infected ticks, including unfed ticks and ticks that fed to repletion on mice, were checked for spirochetes, and the presence of specific *B. burgdorferi* Ags, within the vector by indirect immunofluorescence (IFA). Each tick was dissected to separate the internal organs from the cuticle and lightly homogenized in 100 μl of PBS. Then triplicate 10-μl aliquots were spotted on silylated glass slides. The slides were air dried and fixed with 4% paraformaldehyde. The specimens were incubated in rabbit BBK32, BBK50, OspC, or OspA antisera (1:100 dilution) for 1 h, washed, followed by incubation in anti-rabbit IgG coupled with FITC at a 1:500 dilution for another hour, and viewed under a Zeiss Axioskop fluorescence microscope (Oberkochen, Germany) (11, 27, 41). *B. burgdorferi* antisera or FlaB antisera, which recognizes *B. burgdorferi* within both unfed and engorged ticks, was used as positive controls (4, 40).

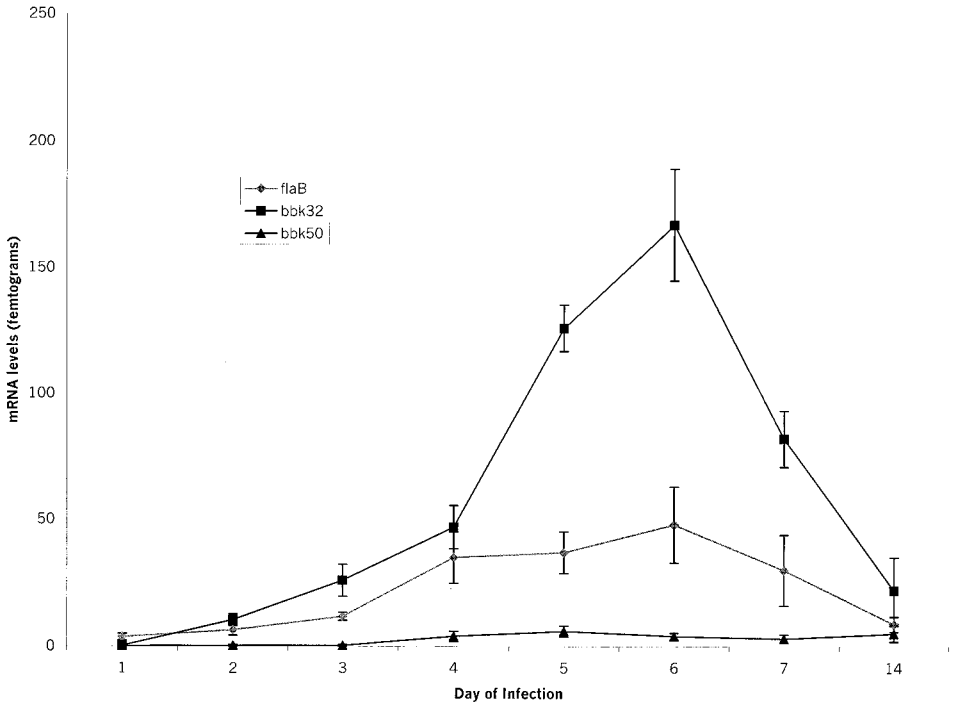

FIGURE 1. *bbk32*, *bbk50,* and *flab* mRNA levels at the initial cutaneous site following challenge with 10^4 *B. burgdorferi* via intradermal syringe inoculation. mRNA levels were measured by quantitative RT-PCR and expressed as femtograms per skin specimen. Each skin sample weighed 20 mg.

GT antisera was used as the negative control. Ten high-power fields (×400) were examined, and the total number of spirochetes was calculated.

Results

B. burgdorferi bbk32 *and* bbk50 *expression during murine infection*

bbk32 and *bbk50* have been shown to be induced during murine infection and in several patients with erythema migrans or Lyme arthritis (11, 20). We have now quantitatively examined the temporal and tissue-specific expression of *B. burgdorferi bbk32* and *bbk50* during experimental murine Lyme borreliosis. Following intradermal challenge, spirochetes are deposited in the skin and then begin disseminating (4–10 days) to the joints, heart, spleen, and bladder (36, 42). Arthritis and carditis then become prominent (2–4 wk) and regress over 1–2 mo (36, 42). At later intervals, mice remain infected but disease is no longer apparent (36, 42). *bbk32* and *bbk50* mRNA levels were therefore first measured at the cutaneous site of inoculation from days 1 to 14 (Fig. 1) and then at different disseminated sites on days 14, 30, and 60, intervals that reflect the various stages of disease (Table I). *flaB* mRNA, which is constitutively expressed, was used for comparison (12). Representative examples of the *flaB*, *bbk32*, and *bbk50* quantitative RT-PCR are shown in Fig. 2.

At the local skin site, following the intradermal inoculation of 10^4 spirochetes, *flaB* mRNA was detected (3.8 ± 1.3 femtograms/tissue specimen) on day 1 (Fig. 1). At this time point, quantitative DNA-PCR revealed that $2 \times 10^4 \pm 0.5 \times 10^4$ spirochetes were present at the site of inoculation, representing the original inocula and perhaps some initial spirochete growth. By day 6, *flaB* mRNA levels increased 10-fold (37 ± 10 femtograms/tissue specimen) from the initial values before declining on day 14. Quantitative DNA-PCR demonstrated that the spirochete number increased up to $3 \times 10^5 \pm 0.6 \times 10^5$ on day 6 before declining, reflecting the changes in *flaB* mRNA detection, and further demonstrating that *flaB* mRNA levels can be used to follow spirochete burden in infected mice (12). *bbk32* ands *bbk50* mRNA levels were not significant on day 1, consistent with previous studies that *B. burgdorferi* N40 does not immediately express these genes following

Table I. B. burgdorferi bbk32, bbk50, *and* flaB *mRNA levels in specific tissues during experimental murine Lyme borreliosis*[a]

Day of Infection	mRNA Levels (femtograms/tissue specimen)				
	Skin	Joints	Heart	Spleen	Bladder
Day 14					
bbk32	2.3 ± 0.4	2.1 ± 0.3	2.4 ± 0.3	1.1 ± 0.4	2.3 ± 0.2
bbk50	0.9 ± 0.2	0.8 ± 0.3	1.0 ± 0.2	1.5 ± 0.4	1.3 ± 0.5
flaB	7.2 ± 2.8	36 ± 12	38 ± 14	36 ± 12	6.8 ± 2.1
Day 30					
bbk32	2.3 ± 0.3	0.7 ± 0.3	3.4 ± 0.3	17.3 ± 3.1	0.2 ± 0.1
bbk50	0.4 ± 0.1	0.8 ± 0.3	1.4 ± 0.2	1.8 ± 0.1	0.2 ± 0.1
flaB	27 ± 5	51 ± 10	54 ± 8	54 ± 10	18 ± 3
Day 60					
bbk32	0.06 ± 0.03	0.03 ± 0.02	0.02 ± 0.01	0.6 ± 0.1	0.07 ± 0.04
bbk50	0.01 ± 0.005	0.013 ± 0.002	0.02 ± 0.02	0.04 ± 0.02	0.01 ± 0.004
flaB	1.1 ± 0.2	5.4 ± 0.4	4.2 ± 0.8	7.2 ± 2.4	2.4 ± 0.8

[a] The means and SD of three experiments are presented. Each tissue specimen was 10 mg.

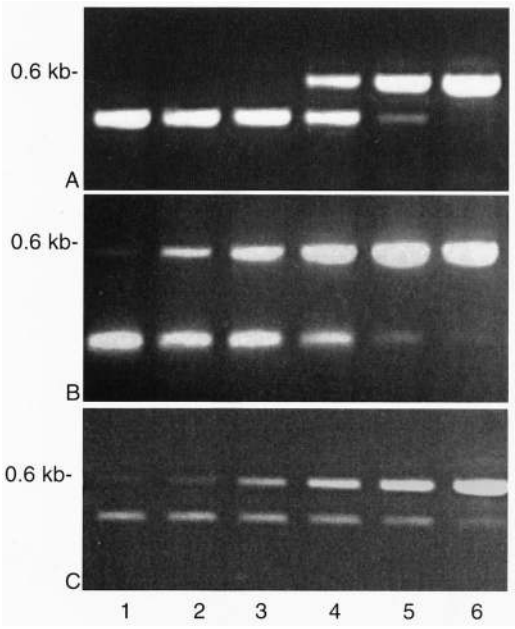

FIGURE 2. Representative competitive RT-PCR assays to quantify *flaB* (*A*), *bbk50* (*B*), and *bbk32* (*C*) mRNA levels during murine infection with *B. burgdorferi*. In all panels, the upper band represents the competitor construct, which was added in increasing concentrations from *lanes 1* to *6*. Amount of competitor: *lane 1*, 3.2×10^{-10} μg; *lane 2*, 1.1×10^{-9} μg; *lane 3*, 8×10^{-9} μg; *lane 4*, 4×10^{-8} μg; *lane 5*, 2×10^{-7} μg; and *lane 6*, 2×10^{-6} μg. The lower band represents the amplified *B. burgdorferi* product.

intradermal syringe challenge. Low levels of *bbk50* were detected at the local site of inoculation on days 4–14 and reached levels of up to 6.0 ± 2 femtograms/tissue specimen on day 5. As the number of total spirochetes declines at the local site of inoculation on day 14, more *bbk50* mRNA is expressed per spirochete at this later interval than at earlier time points. *bbk32* mRNA levels greatly increased from days 3 to 7 and then declined. The change in *bbk32* expression is consistent with the study of Probert and Johnson (13) demonstrating the BBK32 is a fibronectin-binding protein and may therefore required during the initial stage of infection.

Gene expression in the skin, joints, heart, spleen, and bladder was then assessed during disseminated murine infection (Table I). Skin specimens were obtained from the murine mid-posterior thorax, away from the site of inoculation. *flaB* mRNA was detected in all of the tissues and at all intervals, confirming that spirochetes were present at these locations and that *B. burgdorferi* mRNA had been successfully isolated. *flaB* mRNA levels increased from days 14 to 30 and then decreased at 60 days, consistent with studies suggesting that the number of spirochetes decrease in mice as the host immune response to *B. burgdorferi* expands. At 14, 30, and 60 days, *flaB* mRNA levels were generally greater in the joints, heart, and spleen than in the skin and bladder, suggesting that the spirochete burden is higher in these tissues. *bbk32* and *bbk50* mRNA levels were lower than *flaB* mRNA levels at each location. At 14 days, *bbk32* and *bbk50* mRNA levels were generally similar in the tissues examined. At 30 days, *bbk32* mRNA levels in the skin, heart, and spleen were greater than the *bbk50* mRNA levels, particularly in the spleen. *bbk32* and *bbk50* mRNA levels in the joints and bladder at 30 days were not different. At 60 days, *bbk32* and *bbk50* mRNA levels declined as the number of spirochetes decreased in all tissues; however, spirochetes in the spleen continued to produce more *bbk32* than *bbk50* mRNA.

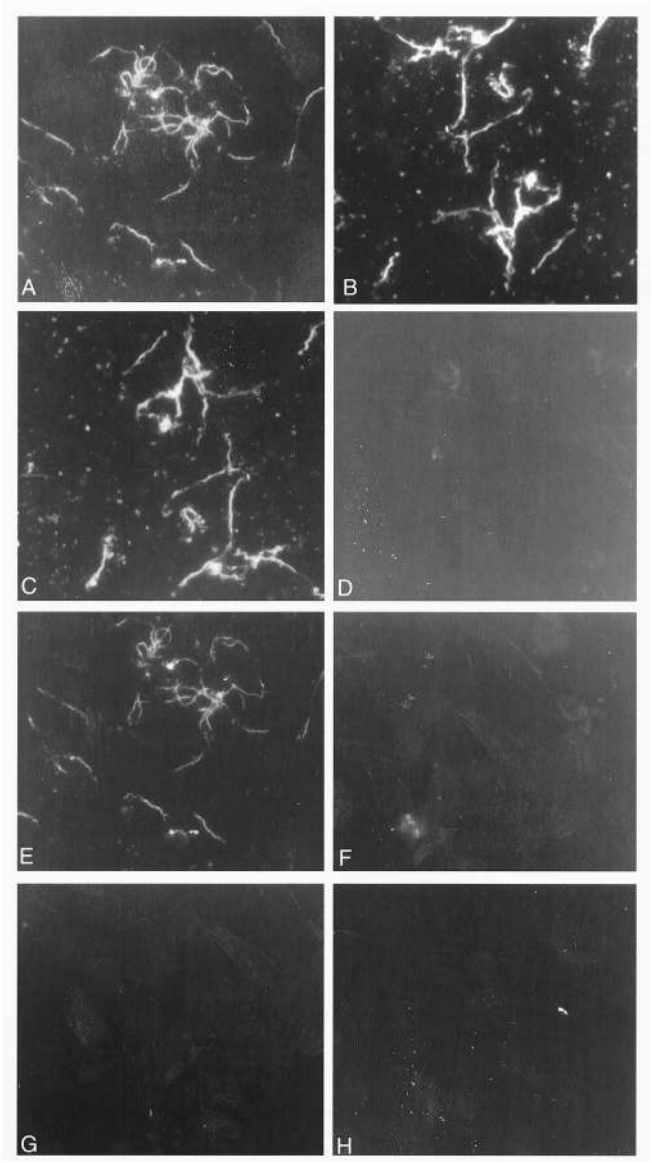

FIGURE 3. Immunofluorescence of spirochetes within engorged (*A*, *C*, *E*, and *G*) or flat (*B*, *D*, *F*, and *H*) ticks probed with specific antisera. *A* and *B*, *B. burgdorferi*-antisera. *C* and *D*, BBK32 antisera. *E* and *F*, BBK50 antisera. *G* and *H*, GT antisera (control).

B. burgdorferi bbk32 *and* bbk50 *expression in ticks*

Previous research demonstrated that *B. burgdorferi* N40 expressed *bbk32* and *bbk50* during mammalian infection but not when cultured in vitro (11). *B. burgdorferi* are, however, maintained in a natural vector-host-vector cycle and not usually exposed to laboratory medium. *bbk32* and *bbk50* expression in ticks was therefore investigated to further understand when these genes are normally expressed. Individual spirochetes within ticks are readily discernible by IFA, unlike *B. burgdorferi* within murine tissues. Therefore IFA rather than RT-PCR was primarily used to examine spirochetes within the vector. BBK32 and BBK50 Ag on spirochetes within flat and engorged *I. scapularis* were examined by immunofluorescence using BBK32 or BBK50 antisera. BBK32 and BBK50 were not evident on spirochetes within unfed ticks but were apparent on *B. burgdorferi* in engorged ticks (Fig. 3). The number of BBK32- and BBK50-producing spirochetes was determined by probing *B. burgdorferi* within flat and fed ticks with selected antisera. Fields containing ~1000 spirochetes were first

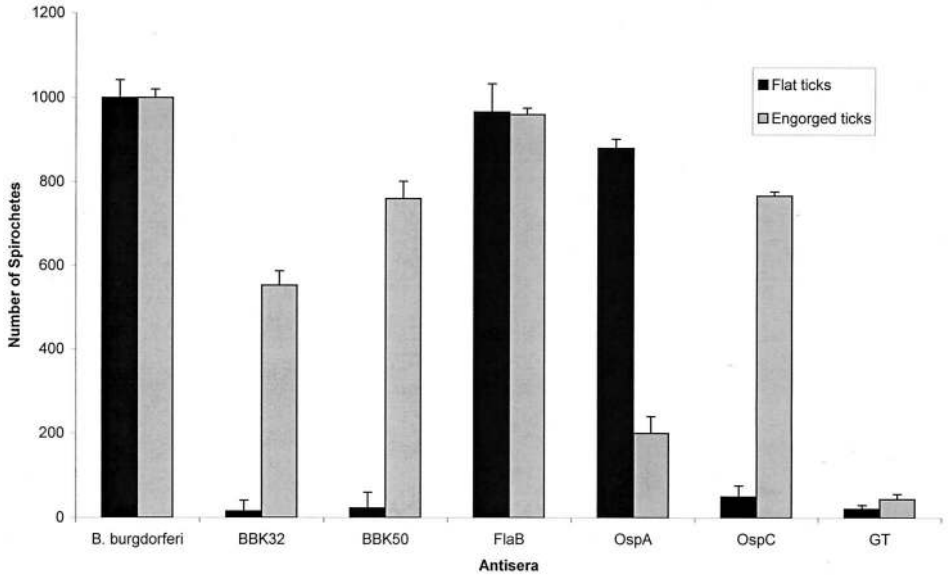

FIGURE 4. Number of spirochetes within flat or engorged ticks that can be detected with selected antisera. Antisera used: *B. burgdorferi*, BBK32, BBK50, FlaB, OspA, OspC, and GT (control). The means ± SD of three experiments are shown.

examined by dark-field microscopy. As the number of *B. burgdorferi* within ticks increases exponentially during engorgement, a larger number of fields from flat tick extracts were required to identify 1000 organisms. Following engorgement, 57 ± 3% of *B. burgdorferi* synthesized BBK32 and 78 ± 4% produced BBK50 (Fig. 4). As expected, *B. burgdorferi*- or FlaB antisera (controls) readily identified spirochetes in flat and engorged ticks. OspA-producing spirochetes were only abundant in the flat ticks, and OspC-synthesizing organisms increased in number during feeding, consistent with previous reports (4, 5, 7). RT-PCR substantiated the IFA results (Fig. 5). *bbk32* and *bbk50* mRNA were detected in engorged, but not flat ticks, whereas *flaB* mRNA (control) was evident in both groups of ticks. These studies demonstrated that *bbk32* and *bbk50* are expressed on *B. burgdorferi* within feeding *I. scapularis* ticks.

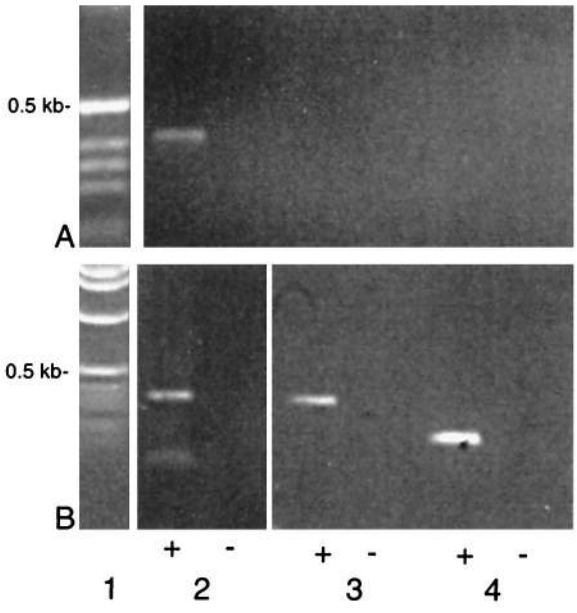

FIGURE 5. RT-PCR to detect *B. burgdorferi bbk32*, *bbk50,* or *flaB* mRNA within flat (*A*) or engorged (*B*) ticks. *Lane 1*, Molecular mass marker; *lane 2*, *flaB*; *lane 3*, *bbk32;* and *lane 4*, *bbk50*. +, with reverse transcriptase; −, without reverse transcriptase.

Immunization with BBK32 antisera is protective against tick-borne B. burgdorferi *infection: BBK32 and BBK50 antisera do not, however, clear spirochetes from persistently infected animals*

BBK32 and BBK50 antisera have previously been shown to protect mice from experimental challenge with an intradermal inocula of spirochetes and also to clear *B. burgdorferi* from mice infected with spirochetes for 24 h (11). We have now performed studies to examine whether the postinfection immunity phenomenon extends to later intervals after infection (Table II). Results were similar when 10^2 rather than 10^4 *B. burgdorferi* were used as the challenge inocula (data not shown). Partial eradication was observed when BBK32 and BBK50 antisera were administered up to 2 days after infection. At 4 and 8 days after infection, when spirochetes had begun to disseminate from the skin to distant organs, *B. burgdorferi* clearance was no longer evident. In addition, passive immunization with BBK32 and BBK50 antisera at these intervals did not influence the severity of disease in the *B. burgdorferi*-infected mice. Arthritis was graded on a scale from 0 to 3, with 1, 2 and 3 representing mild, moderate, and severe inflammation, respectively. Tibiotarsal joints from BBK32- and BBK50-immunized mice had mild to moderate arthritis (1.7 mean ± 0.6 SD, similar to control mice (1.5 mean ± 0.5 SD).

Our results have now demonstrated that *B. burgdorferi* express *bbk32* and *bbk50* within ticks during engorgement before transmission to the host. We therefore also studied whether BBK32 and BBK50 Abs could protect mice against tick-borne infection (Table II). Passive transfer of murine BBK32 antisera to naive mice partially prevented *B. burgdorferi* infection transmitted by tick bite. Murine BBK50 antisera did not provide immunity and the combination of murine BBK32 and BBK50 antisera did not afford greater protection than murine BBK32 antisera alone. Although the passive transfer of murine BBK32 antisera was partially protective against *B. burgdorferi* infection when 4 ticks engorged on mice, protection was not obtained when 20 ticks were placed on each animal.

Influence of BBK32 and BBK50 antisera on B. burgdorferi *within ticks*

Spirochetes synthesize BBK32 and BBK50 within ticks during engorgement and BBK32 antisera provides partial protection

Table II. *Passive immunization with BBK32 and BBK50 antisera against murine Lyme borreliosis*[a]

Antisera	*B. burgdorferi* Challenge	Murine Infection
BBK32 and BBK50	Syringe-borne	
1 day postinfection	10^4	1/8
2 days postinfection		5/9
4 days postinfection		7/8
8 days postinfection		7/7
GT (control)		
1 day postinfection		8/9
2 days postinfection		7/7
4 days postinfection		6/6
8 days postinfection		8/9
	Tick-borne	
BKK32 and BBK50	4 ticks	3/10
BBK32		2/10
BBK50		6/9
GT (control)		10/10
BKK32 and BBK50	20 ticks	9/10
BBK32		9/10
BBK50		10/10
GT (control)		10/10

[a] In the syringe-borne studies, mice were challenged with 10^4 *B. burgdorferi* and then administered antisera at selected intervals after infection. In the tick-borne studies, mice were first treated with antisera and then engorged upon by 4 or 20 *B. burgdorferi*-infected ticks at 24 h. Mice were sacrificed at 14 days and the spleen, skin, and bladder were cultured for spirochetes and the joints and hearts were histopathologically examined for disease. An animal that was culture positive or had disease was considered infected.

Table III. *Spirochete within ticks that fed on mice passively immunized with BBK32 or BBK50 antisera*[a]

Ticks	Antisera (given to mice that the nymphal ticks fed on)	No. of Spirochetes Within Ticks
Engorged nymphs	BBK32	72 ± 18
	BBK50	443 ± 146
	BBK32/BBK50	51 ± 13
	GT control	929 ± 321
Adults[b]	BBK32	4 ± 1
	BBK50	22 ± 7
	BBK32/BBK50	2 ± 1
	GT control	43 ± 13

[a] Groups of three animals were administered specific antisera and 24 h later four to five *B. burgdorferi*-infected nymphal ticks were placed on the mice. All ticks fed to repletion and were collected. In each group, 10 engorged nymphs were dissected and examined for spirochetes by immunofluorescence. Ten high-power fields were examined for each tick and the total number of spirochetes in these fields was determined. A group of the engorged nymphs was stored and allowed to molt to the adult stage. Following the molt, 10 adults in each group were dissected and examined for spirochetes in an identical manner.

[b] Some engorged nymphs were allowed to molt.

against tick-borne infection. Therefore, the effects of BBK32 and BBK50 antisera on spirochetes within engorging ticks were examined. The number of spirochetes within ticks that fed on mice administered BBK32 or BBK50 antisera were assessed by IFA (Table III). Spirochetes were abundant in nymphal ticks that fed on mice given GT antisera (control). *B. burgdorferi* within ticks that engorged on mice administered BBK50 antisera also had substantial numbers of spirochetes. Ticks that fed on mice given BBK32 antisera had markedly fewer spirochetes (90% less) and the combination of BBK32 and BBK50 antisera did not result in a greater reduction in spirochete number.

The effect of BBK32 antisera on the survival of spirochetes following the molt was then examined. Nymphs that had engorged on immunized mice were allowed to molt to the adult stage over several months. Adult ticks were then examined for spirochetes using IFA. *B. burgdorferi* were readily detected within adult ticks that had been exposed to GT or BBK50 antisera during nymphal engorgement. All 12 GT-exposed ticks had spirochetes. Seven of 11 ticks exposed to BBK50 antisera had spirochetes. Only 2 of 11 ticks exposed to both BBK32 and BBK50 antisera and 3 of 12 ticks exposed to BBK32 antisera had *B. burgdorferi*. In addition, adult ticks that had been previously exposed to BBK32 or BBK32 and BBK50 antisera had markedly fewer spirochetes than controls (Table III). The number of spirochetes in adult control ticks was less than that in engorged control nymphs because *B. burgdorferi* increase in number during tick feeding.

We then performed studies to determine whether this reduction in *B. burgdorferi* due to BBK32 antisera could prevent spirochete transmission from ticks to a mammalian host. Guinea pigs were used for these experiments because adult ticks do not engorge on mice and guinea pigs can be infected with *B. burgdorferi* (38, 43). Four or five adult ticks that had been exposed to GT, BBK32, or BBK50 antisera during nymphal engorgement were allowed to feed to repletion on guinea pigs. The guinea pigs were then assessed for exposure to *B. burgdorferi* infection by immunoblot (38). Guinea pigs fed upon by GT (three of four animals) or BBK50 (three of three animals) antisera-exposed ticks, but not ticks exposed to BBK32 (zero of four animals) or BBK32 and BBK50 (zero of three animals) antisera had evidence of *B. burgdorferi* infection. These data demonstrate that BBK32 antisera inhibited spirochete transmission by adult ticks.

BBK32 and BBK50 antisera inhibits larval acquisition

BBK32 Abs play an important role in *B. burgdorferi* survival within ticks. Therefore, the effects of BBK32 and BBK50 antisera on the larval acquisition of spirochetes were examined because these Ags are synthesized within engorging ticks and during mammalian infection. SCID mice were infected with *B. burgdorferi* and at 14 days the animals were administered BBK32 and BBK50 antisera. Larvae were allowed to engorge on the mice and spirochete transmission from the host to larvae was examined by IFA (Fig. 6). Larvae that engorged on control animals had large numbers of spirochetes. In contrast, ticks that fed on BBK32- and BBK50-immunized mice had reduced numbers of *B. burgdorferi*.

Discussion

The expression of *bbk32* and *bbk50* during the spirochete life cycle in the *I. scapularis* tick and the murine host was extensively examined. Our studies demonstrate that *bbk32* and *bbk50* are expressed by *B. burgdorferi* N40 within ticks during the process of engorgement. Since these genes are located on different plasmids, it is most likely that expression is temporally associated, but not genetically linked. Over the past 5 years, it has been shown that several of the genes that are expressed and are antigenic during infection appear to first be induced within the feeding tick. For example, OspC, an Ag that is readily recognized by Abs during the early stage of infection, is first expressed by spirochetes during tick engorgement (7). In contrast, other in vivo-expressed genes, such as *erpT* and *p21*, are predominantly down-regulated within the vector and then only expressed in the mammalian host (10, 12).

We then performed a detailed analysis of the expression of *bbk32* and *bbk50* during murine Lyme borreliosis. In contrast with *erpT*, an in vivo-expressed gene which appears to be most prominently expressed in extracutaneous tissues, *bbk32* and *bbk50* expression were apparent in diverse tissues, including the skin, joints, heart, spleen, and bladder of infected mice (12). Expression was most abundant on day 30 and then declined. At this later interval

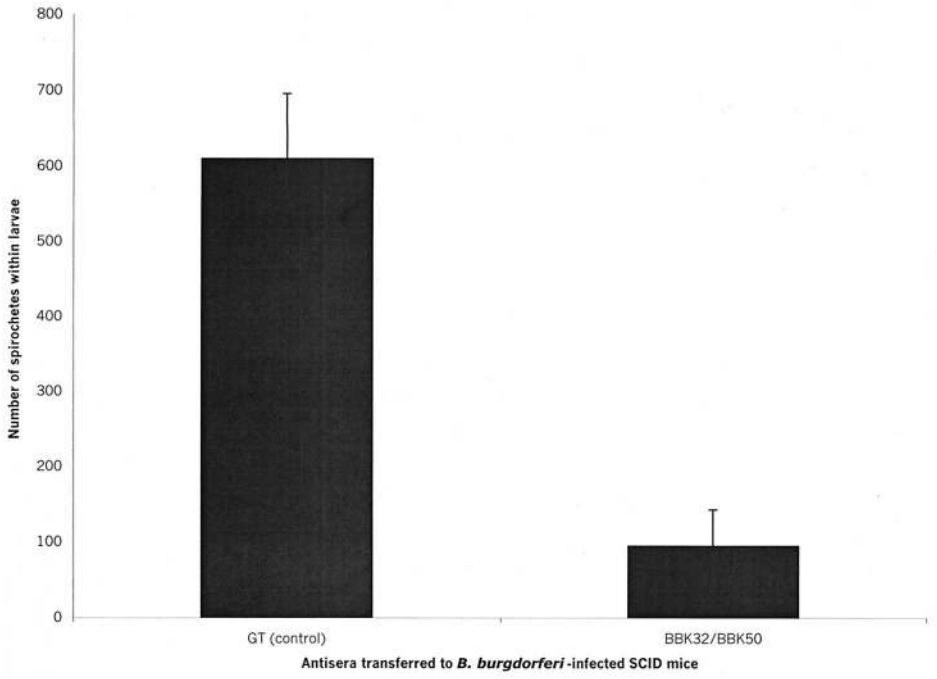

FIGURE 6. Number of spirochetes within larvae that engorged on *B. burgdorferi*-infected SCID mice that had been passively immunized with BBK32 and BBK50 antisera. Groups of five mice were infected with *B. burgdorferi* for 2 wk and then administered antisera. Four larval ticks were then allowed to feed to repletion on the mice, collected, and then dissected and examined for spirochetes by immunofluorescence.

(60 days), mice have developed high titer BBK32 and BBK50 Abs, and the lower levels of expression may represent either the destruction of spirochetes expressing *bbk32* or *bbk50* or the down-regulation of gene expression. Our early time points studies also demonstrate that *bbk32* and *bbk50* are expressed at low levels early after intradermal inoculation and that *bbk32* expression then rapidly increases at a greater rate than *bbk50* at the local site of inoculation.

BBK32 antisera influenced spirochete survival at various intervals in the *B. burgdorferi* life cycle, most prominently in the tick. We demonstrated that the "postinfection clearance" of BBK32 and BBK50 is not apparent after 2 days. One hypothesis to account for this short-lived effect is that the accessibility of these proteins to Ab during the course of infection may influence protective immunity (44). Various Abs have borreliacidal effects at different intervals of infection. OspA Abs are only protective when administered before spirochete challenge, presumably because of the substantial down-regulation of *ospA* expression in vivo. In contrast, BBK32 Abs have an early clearance effect similar to what has been seen with DbpA Abs (29). Since both BBK32 and DbpA mediate spirochete attachment to the extracellular matrix, these Abs may influence *B. burgdorferi* at this location, perhaps before spirochetes have been able to coat themselves with fibronectin or decorin. In contrast, Abs to OspC, when repeatedly administered in high doses, have the capacity to clear spirochetes after several weeks of infection, a phenomenon that has not been demonstrated with other antisera (33, 34). Therefore, Abs to different in vivo-expressed proteins, when used together, and at different intervals may have synergistic effects on preventing spirochete survival and persistence.

BBK32 antisera has a significant impact on the survival of spirochetes within ticks and in the ability of ticks to maintain spirochetes throughout the molt. Spirochete numbers are reduced, but not eradicated, within ticks that engorged on BBK32-immunized mice and adult ticks could then not readily transmit *B. burgdorferi* to guinea pigs. These data show that the Abs interfere with the capacity of ticks to retain organisms and transmit infection. This effect is also evident during larval acquisition, for BBK32 and BBK50 antisera interfered with the ability of larval ticks to acquire spirochetes when feeding on immunized SCID mice. The ability of these antisera to affect spirochetes in the vector may account for enhanced protection against tick-borne challenge rather than needle inoculation.

BBK32 and BBK50 are two *B. burgdorferi* genes that are expressed during tick feeding and then also during systemic murine infection. Abs to these proteins, in particular BBK32, can partially protect mice from tick-borne infection with *B. burgdorferi* and may therefore be used as alternative vaccines that destroy *B. burgdorferi* at different time points. The diversity, and expression, of BBK32 by different *B. burgdorferi* isolates will help determine whether BBK32 will have broader utility as an immunogen. BBK32 antisera also had the capacity to affect spirochete survival within ticks, especially during the molting process. Therefore, another strategy to control *B. burgdorferi* would be to selectively immunize the natural reservoir, i.e., *Peromyscus leucopus*, with BBK32, thereby reducing the larval acquisition of spirochetes, and the ability of engorged nymphs and adults to harbor the pathogen. A detailed understanding of the temporal expression *of B. burgdorferi* genes and the specific location in which Abs can exert pressure on the spirochete may lead to new rational approaches to multivalent vaccines that can effect the spirochete during different stages of the *B. burgdorferi* life cycle.

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