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Evaluation of RevA, a Fibronectin-Binding Protein of *Borrelia burgdorferi*, as a Potential Vaccine Candidate for Lyme Disease

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Previous studies indicated that the Lyme disease spirochete *Borrelia burgdorferi* expresses the RevA outer surface protein during mammalian infection. As an adhesin that promotes bacterial interaction with fibronectin, RevA appears to be a good target for preventive therapies. RevA proteins are highly conserved across all Lyme borreliae, and antibodies against RevA protein are cross-reactive among RevA proteins from diverse strains. Mice infected with *B. burgdorferi* mounted a rapid IgM response to RevA, followed by a strong IgG response that generally remained elevated for more than 12 months, suggesting continued exposure of RevA protein to the immune system. RevA antibodies were bactericidal *in vitro*. To evaluate the RevA antigen as a potential vaccine, mice were vaccinated with recombinant RevA and challenged with *B. burgdorferi* by inoculation with a needle or by a tick bite. Cultured tissues from all treatment groups were positive for *B. burgdorferi*. Vaccinated animals also appeared to have similar levels of *B. burgdorferi* DNA compared to nonvaccinated controls. Despite its antigenicity, surface expression, and the production of bactericidal antibodies against it, RevA does not protect against *Borrelia burgdorferi* infection in a mouse model. However, passive immunization with anti-RevA antibodies did prevent infection, suggesting the possible utility of RevA-based immunotherapeutics or vaccine.

Borrelia burgdorferi is the causative agent of Lyme disease, the most common arthropod-borne infection in the United States (1). Early diagnosis and treatment are key to preventing the debilitating long-term sequelae such as musculoskeletal, cardiovascular, and neurological damage (2). A preventative vaccine was approved for human use in 1998, but production was discontinued in early 2002 (3). The incidence of this disease has been steadily increasing since it was first described in the late 1970s, and all evidence indicates that Lyme disease will continue to be a widespread public health problem.

B. burgdorferi can infect immunocompetent humans and other vertebrates for extensive periods of time, even for the animal's lifetime (4, 5, 6). The Lyme disease spirochete is an extracellular organism, but a complete picture of how it manages to avoid clearance from its hosts is lacking. Antigenic variation at the *vls* locus, which occurs only *in vivo*, is continuous throughout infection (7, 8). The outer surface protein VslE appears to be crucial for persistence in the mammalian host, as bacteria lacking VslE are completely cleared (9, 10). Antibody appears to be important for clearance of *B. burgdorferi*, as the variable regions of VslE are accessible to antibodies (11). Other aspects of *B. burgdorferi*, including its tropism for immunologically isolated sites, may also contribute to its persistence *in vivo*.

Extracellular matrix (ECM) has been suggested to provide a protective niche for the spirochete (12). *B. burgdorferi* is frequently found associated with connective tissues (12, 13, 14, 15) and is often detected in and isolated from infected cartilaginous or membranous tissues, such as skin and joints. This suggests specific interactions between the pathogen and host skin tissues (5, 16, 17, 18). *In vitro*, *B. burgdorferi* shows affinity for host extracellular matrix components, such as fibronectin (12, 19, 20, 21). Bacteria deficient in one of the fibronectin-binding proteins, BBK32, exhibit reduced virulence *in vivo* (22, 23). Together, these data indicate that *B. burgdorferi* interacts with its host's ECM and suggest that those interactions are critical in both *B. burgdorferi* pathogenesis and persistence in mammals. Recently, we discovered that an antigenic 17-kDa outer surface lipoprotein, RevA, binds to fibronectin (19). We hypothesize that borrelia-ECM interactions, especially those mediated by RevA fibronectin-binding protein, are crucial for mammalian infection and persistence in the host.

The gene encoding RevA (so named because it is transcribed in the reverse direction from its neighboring genes) is located on a circular prophage (cp32). RevA has no significant homology to any proteins outside *Borrelia* species, yet it is highly conserved within the Lyme disease borrelial genospecies. The *revA* genes are widely distributed among Lyme disease spirochetes, and the predicted amino acid sequences of RevA proteins are highly conserved (19). Many strains of *B. burgdorferi* carry two copies of the *revA* gene; for example, the type strain B31 has two copies, and the well-characterized isolate 297 also has two copies of *revA*. In contrast, *B. burgdorferi* strain N40 and *Borrelia garinii* strain PBi each carry only one *revA* locus (19).

Serological studies indicate that humans and laboratory animals are frequently exposed to RevA during *B. burgdorferi* infection (24, 25). Using quantitative real-time PCR, it was confirmed that *revA* is indeed transcribed during mammalian infection, but not during colonization of vector ticks (19). Sera from patients in the initial stages of Lyme disease contained antibodies against RevA, demonstrating that this protein is expressed early in human infection (26).

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In the current study, we propose that RevA is the target of protective antibodies and that RevA expression remains elevated throughout mammalian infection. To test our hypotheses, we examined mammalian response to RevA expression throughout the natural course of infection. In addition, we vaccinated mice with recombinant RevA antigen and challenged them with *B. burgdorferi.*

MATERIALS AND METHODS

Bacteria. *B. burgdorferi* strain B31 MI-16 is an infectious clone of the sequenced type strain (27, 28) which contains all parental plasmids (29). Bacteria were grown at 34°C to cell densities of approximately 1×10^7 bacteria/ml in modified Barbour-Stoenner-Kelly (BSK-II) medium supplemented with 6% rabbit serum (30). Total DNA (genomic and plasmids) was isolated using a DNeasy blood and tissue kit (Qiagen, Valencia, CA). Plasmid content was monitored by multiplex PCR by the method of Bunikis et al. (31).

Recombinant proteins. Recombinant proteins contained amino-terminal polyhistidine tags, with the RevA segment beginning with that protein's first amino acid following the cysteine lipidation site. The *revA* gene was PCR amplified from total genomic DNA of *B. burgdorferi* strain B31 MI-16 using oligonucleotides 5′-TGTAAAGCATATGTAGAAGAAAAG-3′ and 5′-TTAATTAGTGCCCTCTTCGAGGAA-3′. Amplicons were cloned into pET200 (Invitrogen, Carlsbad, CA). The resultant plasmid inserts were entirely sequenced on both strands to ensure that no undesired mutations had occurred during PCR or cloning procedures. Recombinant proteins were expressed in *Escherichia coli* strain Rosetta (DE3) pLysS (Novagen, Madison, WI) upon induction with isopropyl thiogalactopyranoside. Induced *E. coli* cultures were harvested and lysed by sonication or treatment with a French press, and debris was cleared by centrifugation. Recombinant proteins were purified from cleared lysates by using MagneHis nickel-conjugated magnetic beads (Promega, Madison, WI). All recombinant proteins were dialyzed at 4°C overnight against phosphate-buffered saline (PBS) using 3,500-molecular-weight-cutoff (MWCO) Slide-A-Lyzer cassettes (Pierce, Rockford, IL). Protein purity was assessed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis followed by staining with Coomassie brilliant blue (see Fig. 1A; also data not shown). Protein concentrations were determined by bicinchoninic acid protein assays (Pierce). Synthetic 20-amino-acid RevA peptides were produced commercially from GenScript (Piscataway, NJ); sequences are detailed in Fig. S2 in the supplemental material.

Immunoblot. Polyclonal antiserum directed against RevA was produced by inoculation of purified recombinant protein into a New Zealand White rabbit at AnimalPharm (Healdsburg, CA), using one round of their standard protocol. Antiserum was adsorbed against sonicated *Escherichia coli* Rosetta (DE3) pLysS (Novagen) and then affinity purified using HiTrap protein A columns (GE Healthcare) according to the manufacturer's instructions. The specificity of the purified antibody for RevA was tested by immunoblotting against recombinant RevA proteins, *B. burgdorferi* lysates, and control proteins (bovine serum albumin [BSA] and human plasma fibronectin). Briefly, proteins were separated by SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. Membranes were blocked overnight at 4°C with 5% (wt/vol) BSA in Tris-buffered saline-Tween 20 (TBS-T) (20 mM Tris [pH 7.5], 150 mM NaCl, 0.05% [vol/vol] Tween 20). The membranes were next washed with TBS-T and incubated for 2 h at room temperature with purified anti-RevA antibody diluted 1:500 in TBS-T. After the membranes were washed extensively with TBS-T, they were incubated for 1 h at room temperature with horseradish peroxidase-conjugated donkey anti-rabbit immunoglobulin G (IgG) antibody (GE Healthcare) diluted 1:5,000 in TBS-T. After a final series of washes with TBS-T, bound antibodies were detected by using SuperSignal West Pico enhanced chemiluminescence substrate (Pierce).

Infection of mice and ticks. Female C3H/HEN or BALB/c mice (4 to 6 weeks old) were infected by subcutaneous injection of 1×10^6 *B. burgdorferi* B31 MI-16 bacteria from a mid-exponential-phase culture grown at 34°C. These mice then served to infect *Ixodes scapularis* larvae as follows. Egg masses laid by pathogen-free *I. scapularis* ticks were obtained from the Department of Entomology, Oklahoma State University—Stillwater and held in a humidified chamber until they hatched. For *B. burgdorferi* acquisition studies, approximately 200 naive larvae were placed on each of the above-described *B. burgdorferi*-infected mice. After 96 h, the ticks had fully engorged and naturally dropped off the mice. These ticks were returned to the humidified chamber and were allowed to molt to the nymphal stage. Approximately 3 weeks after ecdysis, the ticks were fed upon vaccinated female C3H/HEN or BALB/c mice. Infection of mice was confirmed by analysis of serum samples by enzyme-linked immunosorbent assay (ELISA) for antibodies directed against *B. burgdorferi* whole-cell lysate (see below). Mice infected through feeding by infected nymphs were killed 2 weeks after completion of tick feeding, and their ear pinnae, hearts, bladders, spleens, and tibiotarsal joints were collected and either frozen for DNA extraction and quantitative PCR (qPCR) or cultured in BSK-II medium plus 6% rabbit serum and 50 μg/ml rifampin.

Immunization. For vaccination protocol 1, C3H/HEN mice were injected with 12.5 μg recombinant RevA in PBS 1:1 with adjuvant (Alhydrogel; Invivogen, San Diego, CA). Mice received 2 boosts at 3-week intervals. Three weeks after the final boost, mice were infected with 1×10^5 *B. burgdorferi* B31 MI-16 via subcutaneous injection or infected via tick bite (20 infected nymphs per mouse). For vaccination protocol 2, C3H/HEN mice were injected with 12.5 μg recombinant RevA in PBS (1:1) with adjuvant (complete Freund's adjuvant; Sigma). Mice received 2 boosts (incomplete Freund's adjuvant) at 10-day intervals. Ten days after the final boost, mice were infected with 1×10^5 *B. burgdorferi* B31 MI-16 via subcutaneous injection. For passive immunization, C3H/HEN mice were injected with approximately 200 μg anti-RevA IgG rabbit sera or rabbit preimmune sera (AnimalPharm). Twenty-four hours after injection, mice were infected with 1×10^4 *B. burgdorferi* B31 MI-16 via subcutaneous injection. Two weeks postinfection, mice were sacrificed and exsanguinated. Joints, ears, bladders, and hearts were cultured for 2 weeks in BSK-II medium plus 6% rabbit serum. The presence or absence of *B. burgdorferi* was confirmed via dark-field microscopy in 10 random fields per culture.

Enzyme-linked immunosorbent assay (ELISA). Mouse blood was drawn from the saphenous vein and collected in heparin-coated tubes. Blood samples were centrifuged ($6{,}000 \times g$) to remove red blood cells, and serum samples were stored at −20°C. To measure mouse IgM or IgG against *B. burgdorferi*, the wells on 96-well plates were coated overnight with 100 μl/well of 10-μg/ml *B. burgdorferi* lysate (mid-log-phase *B. burgdorferi* pelleted and washed three times in PBS) in carbonate coating buffer (0.32 g Na_2CO_3 and 0.586 g $NaHCO_3$ [both per 200 ml] [pH 9.6]) at 4°C. To measure mouse antibody response against RevA, the wells were coated with 10 μg/ml recombinant RevA in carbonate coating buffer. Room temperature plates were washed three times with PBS containing 0.05% Tween 20 (by volume) (PBS-T). The wells were blocked for 2 h at room temperature with PBS containing 10% fetal bovine serum and then washed three times with PBS-T. At the time of the assay, a 1:100 dilution of serum was placed on the plate and incubated for 2 h at 37°C. The wells were washed three times with PBS-T and then incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated goat antiserum against mouse IgM (Pierce) or IgG (GE Healthcare, Piscataway, NJ) diluted 1:5,000 in PBS. Color development was performed using a tetramethylbenzidine (TMB) substrate (Thermo Fisher Scientific, Waltham, MA) for 15 min and stopped with the addition of an equal volume of 2 N sulfuric acid. The plates were read on an Epoch plate reader at 450 nm (BioTek, Winooski, VT). For epitope mapping, recombinant RevA or RevA peptides were solubilized according to the manufacturer's instructions and were coated on a 96-well plate overnight (10 μg/ml in carbonate coating buffer). After the wells were blocked and washed three times with PBS-T, pooled 2- to 4-week-infected mouse serum at 1:200 dilution was

added for 1 h at 37°C, followed by washes, incubation with HRP-conjugated anti-mouse IgG, and detection as described above.

Bactericidal assay. *B. burgdorferi* (5×10^6/ml) in BSK-II medium was treated with 1:25 dilution of rabbit anti-RevA antiserum (produced commercially by AnimalPharm [19]), sera from vaccinated mice, or preimmune sera for 24 h. Fifty microliters from each tube was transferred to 450 μl fresh BSK-II medium to examine the ability *of B. burgdorferi* to replicate after antiserum exposure. One hundred twenty hours after transfer, the bacteria were enumerated by dark-field microscopy; the number of motile bacteria in 10 random fields was determined. Cultures were then examined after an additional week in culture by dark-field microscopy.

Analysis of *B. burgdorferi* DNA levels. Total DNA was extracted from tissue samples by using a DNeasy kit according to the manufacturer's instructions (Qiagen). Frozen mouse tissue samples (20 mg) were first minced with sterile single-use razor blades on a DNA/DNase-free glass surface and resuspended in buffer ATL (Qiagen) with proteinase K for overnight digestion at 56°C as recommended by the manufacturer. qPCR was performed by using a Bio-Rad MyIQ2 thermal cycler and Bio-Rad SYBR green supermix. All DNA samples were analyzed in triplicate. Each run included a sample that lacked template to test for DNA contamination of reagents. Oligonucleotide primers used for amplification are *B. burgdorferi recA* nTM17F (F stands for forward) (5′-GTGGATCTATTGTAT TAGATGAGGCTCTCG-3′), *B. burgdorferi recA* nTM17R (R stands for reverse) (5′-GCCAAAGTTCTGCAACATTAACACCTAAAG-3′) (32), mouse nidogen F 5′-CCAGCCACAGAATACCATCC-3′, and mouse nidogen R 5′-GGACATACTCTGCTGCCATC-3′. The reaction conditions were as follows: (i) a 10-min initial denaturation step at 95°C; (ii) 40 cycles, with 1 cycle consisting of 15 s at 95°C and 1 min at 55°C (for *recA*) or 60°C (for nidogen); (iii) 1 min at 95°C and 1 min at 60°C for 1 min; and (iv) melting-curve analysis starting at 60°C plus 0.5°C with a hold at each temperature for 10 s. Tenfold serial dilutions of *B. burgdorferi* genomic DNA or mouse genomic DNA were included in every assay for each primer set. This enabled the generation of standard curves from which the amount of DNA present in each sample could be calculated, which was done using the Bio-Rad MyIQ2 software. The same software package was also used for melting-curve analyses. To verify amplicon sizes and purities, all products were separated by agarose gel electrophoresis, and DNA was visualized with ethidium bromide. Average values obtained from triplicate runs of each DNA sample for *B. burgdorferi recA* copies were calculated relative to the average triplicate value for the mouse nidogen housekeeping gene from the same DNA preparation. Statistical analyses of data were performed using Student's *t* test and assuming unequal variances.

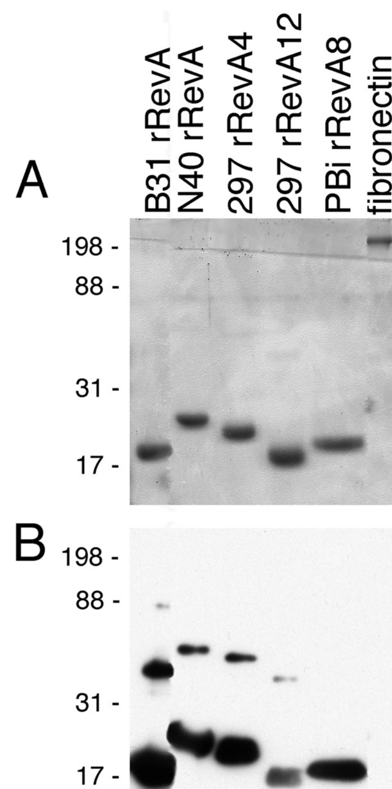

FIG 1 Antisera against *B. burgdorferi* B31 RevA recognizes Rev proteins across the Lyme borreliae. (A) Coomassie brilliant blue-stained 12.5% acrylamide gel of recombinant Rev (rRev) proteins. The positions of molecular weight markers are indicated to the left of the gel. B31 is the type strain of *B. burgdorferi* (28). N40 is an *B. burgdorferi* strain isolated from a tick (47). Strain 297, originally isolated from cerebrospinal fluid from a Lyme disease patient with meningitis (48), has two separate copies of RevA (19). PBi is a European isolate of *B. garinii*. (B) Western blot of the gel in panel A with affinity-purified antibody to RevA from strain B31. Mass spectrometric analysis (University of Kentucky Center for Structural Biology Protein Core Facility) indicated that the higher-molecular-weight bands present in most lanes are also RevA, suggesting that RevA may form homomultimers. Note that the affinity-purified antibodies to RevA from strain B31 do not cross-react with human plasma fibronectin.

RESULTS

Antiserum against *Borrelia burgdorferi* B31 RevA recognizes RevA proteins from other strains. Previously, we tested 7 recombinant RevA proteins representing 7 distinct *revA* alleles from 3 different strains and 3 distinct species of Lyme disease borrelia (*B. burgdorferi*, *Borrelia garinii*, and *Borrelia spielmanii*), and all bound fibronectin (19; our unpublished results). Some outer surface proteins of *B. burgdorferi* are poor vaccine candidates due to their sequence variability or strain-to-strain heterogeneity. An alignment of known RevA sequences demonstrates extensive amino acid identity (see Fig. S1 in the supplemental material). Therefore, we examined whether antiserum against the *B. burgdorferi* type strain B31 RevA would recognize other RevA proteins. Antiserum directed against the *B. burgdorferi* type strain B31 RevA allele is cross-reactive against all tested RevA proteins (Fig. 1). These data suggest that antibodies against the B31 RevA protein will recognize RevA proteins across Lyme borreliae.

RevA antibody production during long-term infection. Preliminary serological studies from infected humans and mice indicate the frequent presence of anti-RevA antibodies. To determine the characteristics of the antibody response over time, female BALB/c mice were infected with *B. burgdorferi* B31 via tick bite and monitored over 1 year. Serum samples were collected at 4- to 6-week intervals and tested for the presence of antibodies against RevA. IgM levels increased upon infection and remained steady throughout the course of infection, never reaching a titer higher than 100 (Fig. 2A). IgG levels varied from animal to animal but once elevated tended to remain high (Fig. 2B and C).

RevA antibodies are bactericidal. Lyme disease borreliae are relatively resistant to killing by complement present in mammalian serum in the absence of specific antiborrelia antibodies (33, 34, 35, 36, 37). To determine whether anti-RevA antibodies were bactericidal, *B. burgdorferi* bacteria were incubated in the standard growth medium (BSK-II medium plus 6% non-heat-inactivated rabbit serum) for 24 h in the presence of rabbit polyclonal anti-RevA antiserum (19), preimmune serum from the same animal, or an equivalent volume of BSK-II medium. Bacteria were subcultured into fresh medium to determine whether *B. burgdorferi* could replicate after antibody exposure. As shown in Fig. 3, treat-

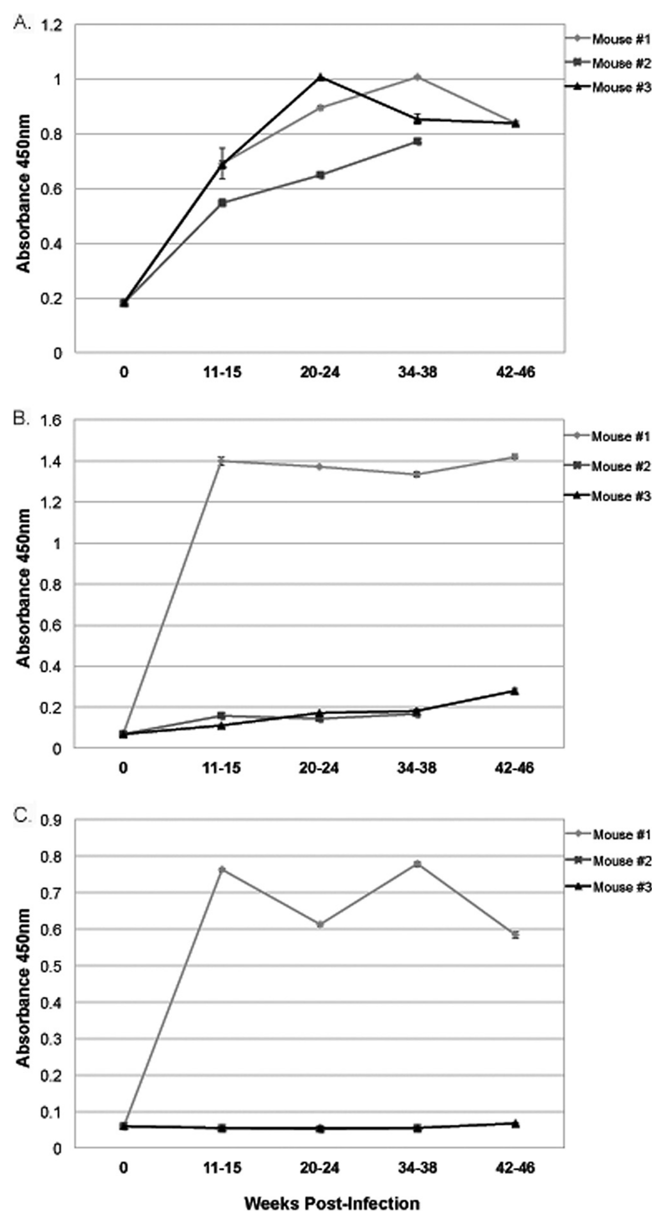

FIG 2 Antibodies to RevA in infected mouse serum. BALB/c mice were infected via tick bite with *B. burgdorferi*. RevA-specific IgG and IgM from three individual mice were measured by ELISA. (A) IgM diluted 1:100. Data represent the mean absorbance and standard errors from 3 replicates per time point. (B and C) IgG diluted 1:100 (B) and 1:1,000 (C). Data in all three panels represent the mean absorbance ± standard errors (error bars) from 3 replicates per time point.

ment with anti-RevA antibodies significantly impaired the ability of *B. burgdorferi* to replicate, while treatment with preimmune serum had no effect.

Assessment of the efficacy of RevA as a vaccine. Female C3H/HEN mice were vaccinated with recombinant RevA protein and challenged 3 weeks after the final boost with *B. burgdorferi* by inoculation with a needle. Serum samples from all mice were examined for the presence of anti-RevA and anti-*B. burgdorferi* antibodies prior to vaccination to ensure that the animals had no prior exposure to the pathogen (data not shown). After vaccination, serum was tested for the presence of RevA-specific IgG or IgM antibodies (Fig. 4). Both mice vaccinated with RevA alone and mice vaccinated with RevA plus adjuvant showed a robust IgG response to RevA, but no measurable difference among groups in the levels of RevA-specific IgM.

Next we tested the ability of RevA vaccination to protect mice

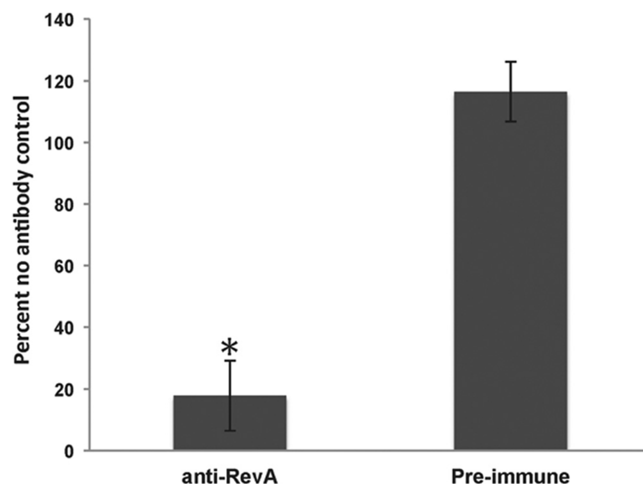

FIG 3 RevA antibodies are bactericidal. *B. burgdorferi* (5×10^6/ml) in BSK-II medium was treated with a 1:25 dilution of anti-RevA antiserum (19) or preimmune sera for 24 h. Fifty microliters from each tube was transferred to 450 μl fresh BSK-II medium plus 6% rabbit serum. Motile bacteria were enumerated in 10 random fields after 5 days by dark-field microscopy. Data are normalized to the percentage for the no-antibody control and represent the means plus standard errors from 3 independent counts for each condition. *, $P < 0.001$ by Student's *t* test assuming unequal variances.

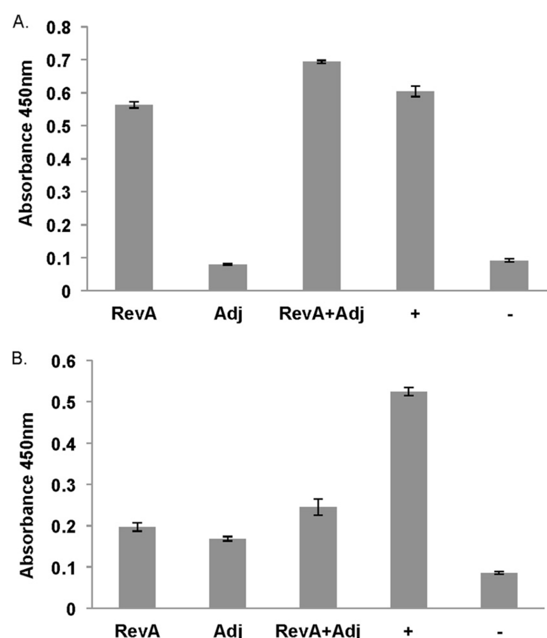

FIG 4 RevA antibody prior to *B. burgdorferi* challenge. After RevA immunization and prior to *B. burgdorferi* challenge, IgG (A) and IgM antibodies against RevA (B) in serum samples was measured by ELISA. Data represent the means ± standard errors (error bars) from 1 experiment with eight mice. RevA, RevA only; Adj, adjuvant alone; +, positive control (confirmed infected mouse); −, negative control (naive mouse).

TABLE 1 Immunization with RevA in Alhydrogel

Method of inoculation	Treatment group	Culture positive[a]
Needle	Adjuvant only	9/9
	RevA	8/8
	RevA + adjuvant	8/8
Tick	Adjuvant only	7/7
	RevA	7/7
	RevA + adjuvant	7/7

[a] At least one tissue type cultured was positive for the presence of live *B. burgdorferi.* For mice inoculated with a needle, the tissues cultured included heart, ear, bladder, and tibiotarsal joint. For mice inoculated by tick bites, the tissues cultured included ear and tibiotarsal joint.

against *B. burgdorferi* infection. C3H/HEN mice were inoculated subcutaneously with *B. burgdorferi*. Two weeks later, the mice were sacrificed, and their organs were cultured for the presence of live spirochetes. As shown in Table 1 (inoculation with a needle), mice vaccinated with RevA in the presence of adjuvant were not protected from subsequent infection with *B. burgdorferi*.

We also examined whether there was a difference in tissue load or dissemination between vaccinated and control groups by qPCR. We observed no differences in the number of positive samples or amount of *B. burgdorferi* DNA in spleen, heart, or joint samples (Table 2). We were unable to detect any *B. burgdorferi* DNA in the ears of challenged mice (data not shown).

We challenged mice with a large bolus of *B. burgdorferi* by inoculation with a needle, a situation that does not accurately reflect natural infection (transfer of a few organisms by tick bite). A second group of vaccinated mice was challenged by tick bite. Mice were infected via tick bite from *B. burgdorferi*-carrying *I. scalpularis* nymphs. Three weeks postchallenge, the animals were sacrificed and their tissues were cultured for the presence of *B. burgdorferi*. All mice produced anti-RevA antibodies postchallenge, regardless of whether they were vaccinated with RevA or adjuvant alone, suggesting that all mice were indeed infected (Fig. 5). Tissue samples were cultured, and as shown in Table 1 (tick bites), all mice were culture positive regardless of vaccination status.

Alum is a relatively weak adjuvant, so to better assess the immunoprotective activity of RevA, we also performed immunization with a stronger adjuvant. Mice were immunized with RevA in complete Freund's adjuvant, followed by two boosts in incomplete Freund's adjuvant. As shown in Table 3, mice immunized with RevA by this protocol were also not protected from subsequent infection with the Lyme disease spirochete.

Next, we revisited the question of the bactericidal nature of anti-RevA antibodies. To determine whether anti-RevA antibodies produced in our vaccinated mice were bactericidal, *B. burgdorferi* bacteria were incubated in the standard growth medium (BSK-II medium plus 6% non-heat-inactivated rabbit serum) for 24 h in the presence of serum from the vaccinated mice, preimmune serum from the same animal, or an equivalent volume of BSK-II medium. Bacteria were subcultured into fresh medium to determine whether *B. burgdorferi* could replicate after antibody exposure. At 5 days posttransfer, there was no difference between *B. burgdorferi* growth between bacteria incubated in the presence of preimmune serum or serum from vaccinated mice (Fig. 6).

Finally, we assessed passive immunization with the anti-RevA

TABLE 2 *B. burgdorferi* tissue loads measured by qPCR[a]

Parameter	Spleen			Heart			Joint		
	Adj	RevA	RevA + Adj	Adj	RevA	RevA + Adj	Adj	RevA	RevA + Adj
Copy no.[b]	ND	$4 \times 10^{-6} \pm 1 \times 10^{-6}$	2.5×10^{-7}	$3 \times 10^{-11} \pm 2 \times 10^{-11}$	$10 \times 10^{-11} \pm 3 \times 10^{-11}$	$5 \times 10^{-11} \pm 2 \times 10^{-11}$	ND	$1 \times 10^{-5} \pm 7 \times 10^{-11}$	$4 \times 10^{-5} \pm 3 \times 10^{-12}$
No. of positive samples/no. of tissues sampled	0/8	4/8	1/8	4/8	4/8	7/8	0/8	3/8	3/8

[a] For each tissue type, the value is shown for mice treated with adjuvant only (Adj), RevA alone, and RevA plus Adj. ND, not detected.
[b] Average *B. burgdorferi* copy number per pg of mouse DNA ± standard error of the mean for *B. burgdorferi*-positive specimens only.

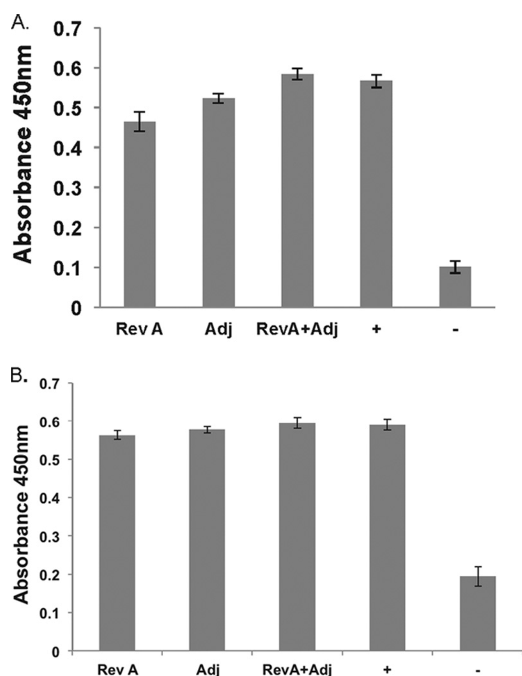

FIG 5 RevA antibodies in mouse serum postinfection. C3H/HEN mice were infected via tick bite with *B. burgdorferi*. Three weeks after the final boost, mice were bled from the saphenous vein, serum samples were collected, and RevA-specific IgG (A) and IgM (B) were measured by ELISA. Data represent the means ± standard errors from 6 wells per mouse ($n = 7$) per condition. RevA, RevA only; Adj, adjuvant alone; +, positive control (confirmed infected mouse); −, negative control (naive mouse).

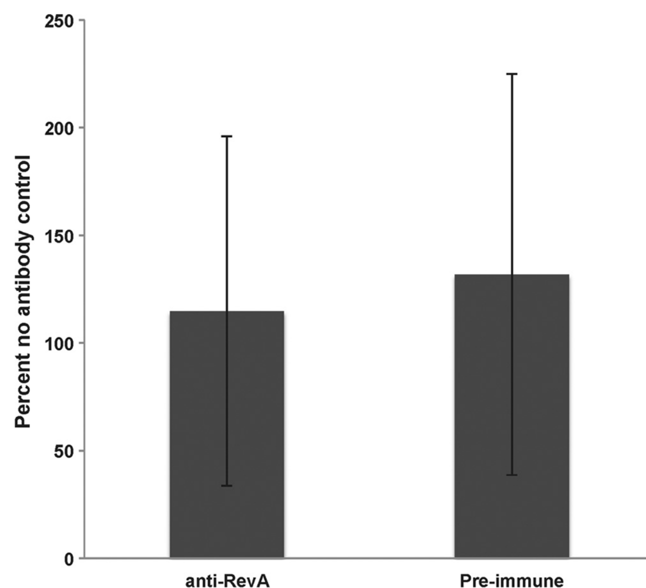

FIG 6 Serum from vaccinated mice is not bactericidal. *B. burgdorferi* (5×10^6/ml) in BSK-II medium was treated with a 1:25 dilution of antiserum from vaccinated mice or preimmune sera for 24 h. Fifty microliters from each tube was transferred to 450 μl fresh BSK-II medium plus 6% rabbit serum. Bacteria were enumerated after 5 days by dark-field microscopy with a Petroff-Hausser chamber. Data are normalized to the percentage for the no-antibody control and represent the means and standard errors from 3 independent counts for each condition.

polyclonal serum produced in rabbits, which proved bactericidal *in vitro* (Fig. 3). Groups of six mice were immunized with either anti-RevA antiserum or rabbit preimmune serum. Two weeks after challenge, the ears, hearts, and tibiotarsal joints of the mice were cultured and examined for *B. burgdorferi*. Of the six mice immunized with preimmune serum, five were positive for infection. In contrast, of the six mice receiving anti-RevA antibodies, only one mouse became infected (Table 4). These results suggest that *in vivo*, anti-RevA antibodies are protective.

DISCUSSION

RevA is a surface-exposed protein of *B. burgdorferi* and is an early antigen of Lyme disease. Its expression is upregulated upon mammalian infection, and RevA-specific antibodies are frequently detected in experimentally infected animals and Lyme disease patients (19, 26, 38, 39, 40). RevA has also been shown to bind host fibronectin; the ability to adhere to host extracellular matrix and cells is a critical virulence factor for many bacteria (41). As a surface-exposed, highly antigenic adhesin, RevA is a prime target for the host immune system. The current study demonstrates that antibodies against RevA are made continuously throughout long-term, natural *B. burgdorferi* infection. Infection with *B. burgdorferi* results in a rapid IgM response, followed by a variable IgG response. IgM levels appeared to remain low, but steady, throughout infection (Fig. 2A). However, when the levels were examined individually, serum IgM levels often spiked, a pattern suggestive of restimulation of the immune system. A similar pattern has been seen for the Erp and OspC outer surface proteins of *B. burgdorferi* (29). The episodic nature of many Lyme disease symptoms, such as arthritis, may be linked to the reemergence of *B. burgdorferi* from tissue and the subsequent reactivation of inflammatory responses by the host immune system (6).

Specific antibodies produced against RevA in rabbits are bactericidal *in vitro*, while RevA antibodies from vaccinated mice had no effect on *B. burgdorferi* growth in culture. This could reflect

TABLE 3 Immunization with RevA in Freund's adjuvant[a]

Immunization	No. of positive samples/total no. of samples cultured				ELISA IgG[b]
	Heart	Bladder	Ear	Joint	
RevA	4/6	6/6	6/6	6/6	6/6
RevA + Adj	5/6	5/6	6/6	6/6	6/6
Adj	6/6	5/5	6/6	6/6	6/6

[a] Mice were immunized with RevA alone (control), RevA plus adjuvant (Adj), and Adj alone (control).
[b] ELISA for *B. burgdorferi*-positive IgG. The numbers of positive serum samples/total numbers of samples tested are shown.

TABLE 4 Passive immunization with anti-RevA antibodies[a]

Immunization	No. of positive samples ($n = 6$)			ELISA IgG ($n = 6$)[b]
	Heart	Joint	Ear	
Anti-RevA	1	1	1	1
Preimmune serum	5	5	5	5

[a] Mice were immunized with anti-RevA polyclonal rabbit bactericidal antibody or with preimmune serum from a rabbit.
[b] ELISA for *B. burgdorferi*-positive IgG. The numbers of positive serum samples are shown.

species specificity of complement—*B. burgdorferi* is cultured in rabbit serum—but this idea was not borne out, as passive immunization of rabbit anti-RevA antibodies was protective *in vivo*. Immunization with RevA in Alhydrogel or Freund's adjuvant, however, was not protective *in vivo*, as vaccinated mice were infected despite a robust anti-RevA response. There are numerous differences between cultured *B. burgdorferi* and bacteria growing *in vivo* (42, 43) that may account for our results. The amount and accessibility of the RevA protein on the surfaces of organisms *in vivo*, for instance, may be altered compared to those of cultured bacteria. The interaction of antibodies against RevA with *B. burgdorferi* organisms *in vivo* may also be limited by both the paucibacilliary nature of *B. burgdorferi* infection and the ability of the organism to disseminate widely throughout its host. Another factor is suggested by a recent study by Hastey et al. (44); *B. burgdorferi* may evade B cell immunity by interfering with the quality of the antibody response. *B. burgdorferi* infection results in a strong but ineffective serum antibody response due to a lack of accumulation of long-lived plasma cells (44).

Animals injected with RevA or RevA plus Alhydrogel adjuvant had roughly equivalent anti-RevA IgG responses (Fig. 5). We expected a more vigorous response to RevA in the presence of this adjuvant. The purified recombinant RevA was free of protein contaminants (Fig. 1A). Similar results were seen when we immunized animals with a more potent adjuvant (complete Freund's adjuvant; data not shown). Despite the ability of the host to mount a strong antibody response to RevA, in the presence or absence of adjuvant, we were unable to stimulate a protective response *in vivo*.

Our results suggest that while rabbit anti-RevA antibodies are bactericidal *in vitro*, vaccination with RevA fails to protect mice from subsequent infection by *B. burgdorferi*, either through inoculation with a needle or the natural mode of infection, tick bite. In contrast, passive immunization with bactericidal anti-RevA antibodies prevented infection, suggesting that RevA is indeed highly expressed in the early stages of infection. Coupled with the fact that RevA is a highly expressed, surface-exposed protein that elicits a long-lasting antibody response, our data suggest that RevA may still be a useful target for rational vaccine development. For example, quantitative response to the lipoprotein OspA is not indicative of protection; instead, protective immunity correlates with a specific epitope (45). Using linear, overlapping peptides, we were unable to detect an immunodominant epitope (see Fig. S2 in the supplemental material). This suggests that for RevA, conformational, rather than linear, epitopes are important. Further research to identify protective epitopes, combined with a more exhaustive survey of RevA sequence variation, may aid in the design of an effective vaccine (46).

Finally, our studies emphasize the fact that *B. burgdorferi*, like many spirochetes, causes persistent, life-long infections in immunocompetent hosts. How spirochetes continually evade the host immune response and resist clearance is a conundrum that warrants further investigation.

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REFERENCES

1. **Centers for Disease Control and Prevention.** 2011. Summary of notifiable diseases—United States, 2009. Morb. Mortal. Wkly. Rep. **58**:1–100.
2. **Stanek G, Wormser GP, Gray J, Strle F.** 2012. Lyme borreliosis. Lancet **379**:461–473.
3. **Nigrovic LE, Thompson KM.** 2007. The Lyme vaccine: a cautionary tale. Epidemiol. Infections **135**:1–8.
4. **de Souza MS, Smith AL, Beck DS, Terwilliger GA, Fikrig E, Barthold SW.** 1993. Long-term study of cell-mediated responses to *Borrelia burgdorferi* in the laboratory mouse. Infect. Immun. **61**:1814–1822.
5. **Stanek G, Strle F.** 2003. Lyme borreliosis. Lancet **362**:1639–1647.
6. **Steere AC.** 2001. Lyme disease. N. Engl. J. Med. **345**:115–125.
7. **Zhang J-R, Norris SJ.** 1998. Genetic variation of the *Borrelia burgdorferi* gene *vlsE* involves cassette-specific, segmental gene conversion. Infect. Immun. **66**:3698–3704.
8. **Zhang J-R, Norris SJ.** 1998. Kinetics and in vivo induction of genetic variation of *vlsE* in *Borrelia burgdorferi.* Infect. Immun. **66**:3689–3697.
9. **Bankhead T, Chaconas G.** 2007. The role of VlsE antigenic variation in the Lyme disease spirochete: persistence through a mechanism that differs from other pathogens. Mol. Microbiol. **65**:1547–1558.
10. **Palmer GH, Bankhead T, Lukehart SA.** 2009. 'Nothing is permanent but change'- antigenic variation in persistent bacterial pathogens. Cell. Microbiol. **11**:1697–1705.
11. **Eicken C, Sharma V, Klabunde T, Lawrenz MB, Hardham JM, Norris SJ, Sacchettini JC.** 2002. Crystal structure of Lyme disease variable surface antigen VlsE of *Borrelia burgdorferi.* J. Biol. Chem. **277**:21691–21696.
12. **Cabello FC, Godfrey HP, Newman SA.** 2007. Hidden in plain sight: *Borrelia burgdorferi* and the extracellular matrix. Trends Microbiol. **15**: 350–354.
13. **Coburn J, Medrano M, Cugini C.** 2002. *Borrelia burgdorferi* and its tropisms for adhesion molecules in the joint. Curr. Opin. Rheumatol. **14**:394–398.
14. **Franz JK, Fritze O, Rittig M, Keysser G, Priem S, Zacher J, Burmester GR, Krause A.** 2001. Insights from a novel three-dimensional in vitro model of Lyme arthritis: standardized analysis of cellular and molecular interactions between *Borrelia burgdorferi* and synovial explants and fibroblasts. Arthritis Rheum. **44**:151–162.
15. **Häupl T, Hahn G, Rittig M, Krause A, Schoerner C, Schonherr U, Kalden JR, Burmester GR.** 1993. Persistence of *Borrelia burgdorferi* in ligamentous tissue from a patient with chronic Lyme borreliosis. Arthritis Rheum. **36**:1621–1626.
16. **Asbrink E, Hovmark A.** 1988. Early and late cutaneous manifestations in *Ixodes*-borne borreliosis (erythema migrans borreliosis, Lyme borreliosis), p 4–15. *In* Benach JL, Bosler EM (ed), Lyme disease and related disorders. New York Academy of Sciences, New York, NY.
17. **Berger BW.** 1989. Dermatologic manifestations of Lyme disease. Rev. Infect. Dis. **11**(Suppl 6):S1475–S1481.
18. **Ohlenbusch A, Matuschka FR, Richter D, Christen HJ, Thomssen R, Spielman A, Eiffert H.** 1996. Etiology of the acrodermatitis chronica atrophicans lesion in Lyme disease. J. Infect. Dis. **174**:421–423.
19. **Brissette CA, Bykowski T, Cooley AE, Bowman A, Stevenson B.** 2009. *Borrelia burgdorferi* RevA antigen binds host fibronectin. Infect. Immun. **77**:2802–2812.
20. **Coburn J.** 2001. Adhesion mechanisms of the Lyme disease spirochete, *Borrelia burgdorferi.* Curr. Drug Targets Infect. Disord. **1**:171–179.
21. **Grab DJ, Givens C, Kennedy R.** 1998. Fibronectin-binding activity in *Borrelia burgdorferi.* Biochim. Biophys. Acta **1407**:135–145.
22. **Seshu J, Esteve-Gassent MD, Labandeira-Rey M, Kim JH, Trzeciakowski JP, Hook M, Skare JT.** 2006. Inactivation of the fibronectin-binding adhesin gene *bbk32* significantly attenuates the infectivity potential of *Borrelia burgdorferi.* Mol. Microbiol. **59**:1591–1601.
23. **Li X, Liu X, Beck DS, Kantor FS, Fikrig E.** 2006. *Borrelia burgdorferi* lacking BBK32, a fibronectin-binding protein, retains full pathogenicity. Infect. Immun. **74**:3305–3313.
24. **Nowalk AJ, Gilmore RD, Jr, Carroll JA.** 2006. Serologic proteome analysis of *Borrelia burgdorferi* membrane-associated proteins. Infect. Immun. **74**:3864–3873.
25. **Gilmore RD, Jr, Mbow ML, Stevenson B.** 2001. Analysis of *Borrelia burgdorferi* gene expression during life cycle phases of the tick vector *Ixodes scapularis.* Microbes Infect. **3**:799–808.
26. **Brissette CA, Rossmann E, Bowman A, Cooley AE, Riley SP, Hunfeld KP, Bechtel M, Kraiczy P, Stevenson B.** 2010. The borrelial fibronectin-

binding protein RevA is an early antigen of human Lyme disease. Clin. Vaccine Immunol. **17**:274–280.

27. **Casjens S, Palmer N, van Vugt R, Huang WM, Stevenson B, Rosa P, Lathigra R, Sutton G, Peterson J, Dodson RJ, Haft D, Hickey E, Gwinn M, White O, Fraser C.** 2000. A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs of an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi.* Mol. Microbiol. **35**:490–516.
28. **Fraser CM, Casjens S, Huang WM, Sutton GG, Clayton R, Lathigra R, White O, Ketchum KA, Dodson R, Hickey EK, Gwinn M, Dougherty B, Tomb J-F, Fleischmann RD, Richardson D, Peterson J, Kerlavage AR, Quackenbush J, Salzberg S, Hanson M, van Vugt R, Palmer N, Adams MD, Gocayne J, Weidmann J, Utterback T, Watthey L, McDonald L, Artiach P, Bowman C, Garland S, Fujii C, Cotton MD, Horst K, Roberts K, Hatch B, Smith HO, Venter JC.** 1997. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi.* Nature **390**:580–586.
29. **Miller JC, von Lackum K, Babb K, McAlister JD, Stevenson B.** 2003. Temporal analysis of *Borrelia burgdorferi* Erp protein expression throughout the mammal-tick infectious cycle. Infect. Immun. **71**:6943–6952.
30. **Zückert WR.** 2007. Laboratory maintenance of *Borrelia burgdorferi*. Curr. Protoc. Microbiol. **Chapter 12**:Unit 12C.1. doi:10.1002/9780471729259.mc12c01s4.
31. **Bunikis I, Kutschan-Bunikis S, Bonde M, Bergstrom S.** 2011. Multiplex PCR as a tool for validating plasmid content of *Borrelia burgdorferi.* J. Microbiol. Methods **86**:243–247.
32. **Ornstein K, Barbour AG.** 2006. A reverse transcriptase-polymerase chain reaction assay of *Borrelia burgdorferi* 16S rRNA for highly sensitive quantification of pathogen load in a vector. Vector Borne Zoonotic Dis. **6**:103–112.
33. **Breitner-Ruddock S, Würzner R, Schulze J, Brade V.** 1997. Heterogeneity in the complement-dependent bacteriolysis within the species of *Borrelia burgdorferi.* Med. Microbiol. Immunol. **185**:253–260.
34. **Brissette CA, Cooley AE, Burns LH, Riley SP, Verma A, Woodman ME, Bykowski T, Stevenson B.** 2008. Lyme borreliosis spirochete Erp proteins, their known host ligands, and potential roles in mammalian infection. Int. J. Med. Microbiol. **298**(Suppl 1):257–267.
35. **Herzberger P, Siegel C, Skerka C, Fingerle V, Schulte-Spechtel U, van Dam A, Wilske B, Brade V, Zipfel PF, Wallich R, Kraiczy P.** 2007. Human pathogenic *Borrelia spielmanii* sp. nov. resists complement-mediated killing by direct binding of immune regulators factor H and factor H-like protein 1. Infect. Immun. **75**:4817–4825.
36. **Kurtenbach K, Sewell H-S, Ogden NH, Randolph SE, Nuttall PA.** 1998. Serum complement sensitivity as a key factor in Lyme disease ecology. Infect. Immun. **66**:1248–1251.
37. **van Dam AP, Oei A, Jaspars R, Fijen C, Wilske B, Spanjaard L, Dankert J.** 1997. Complement-mediated serum sensitivity among spirochetes that cause Lyme disease. Infect. Immun. **65**:1228–1236.
38. **Carroll JA, El-Hage N, Miller JC, Babb K, Stevenson B.** 2001. *Borrelia burgdorferi* RevA antigen is a surface-exposed outer membrane protein whose expression is regulated in response to environmental temperature and pH. Infect. Immun. **69**:5286–5293.
39. **Gilmore RD, Jr, Mbow ML.** 1998. A monoclonal antibody generated by antigen inoculation via tick bite is reactive to the *Borrelia burgdorferi* Rev protein, a member of the 2.9 gene family locus. Infect. Immun. **66**:980–986.
40. **Mbow ML, Gilmore RD, Jr, Stevenson B, Golde WT, Piesman J, Johnson BJB.** 2002. Borrelia burgdorferi-specific monoclonal antibodies derived from mice primed with Lyme disease spirochete-infected *Ixodes scapularis* ticks. Hybrid Hybridomics **21**:179–182.
41. **Henderson B, Nair S, Pallas J, Williams MA.** 2011. Fibronectin: a multidomain host adhesin targeted by bacterial fibronectin-binding proteins. FEMS Microbiol. Rev. **35**:147–200.
42. **Brooks CS, Hefty PS, Jolliff SE, Akins DR.** 2003. Global analysis of *Borrelia burgdorferi* genes regulated by mammalian host-specific signals. Infect. Immun. **71**:3371–3383.
43. **Caimano MJ.** 2005. Cultivation of *Borrelia burgdorferi* in dialysis membrane chambers in rat peritonea. Curr. Protoc. Microbiol. **Chapter 12**: Unit 12C.3. doi:10.1002/9780471729259.mc12c03s00.
44. **Hastey CJ, Elsner RA, Barthold SW, Baumgarth N.** 2012. Delays and diversions mark the development of B cell responses to *Borrelia burgdorferi* infection. J. Immunol. **188**:5612–5622.
45. **Kramer MD, Schaible UE, Wallich R, Moter SE, Petzoldt D, Simon MM.** 1990. Characterization of *Borrelia burgdorferi* associated antigens by monoclonal antibodies. Immunobiology **181**:357–366.
46. **Luft BJ, Dunn JJ, Lawson CL.** 2002. Approaches toward the directed design of a vaccine against *Borrelia burgdorferi.* J. Infect. Dis. **185**(Suppl 1):S46–S51.
47. **Barthold SW, Moody KD, Terwilliger GA, Duray PH, Jacoby RO, Steere AC.** 1988. Experimental Lyme arthritis in rats infected with *Borrelia burgdorferi.* J. Infect. Dis. **157**:842–846.
48. **Steere AC, Grodzicki RL, Kornblatt AN, Craft JE, Barbour AG, Burgdorfer W, Schmid GP, Johnson E, Malawista SE.** 1983. The spirochetal etiology of Lyme disease. N. Engl. J. Med. **308**:733–740.