Evaluation of the *Borrelia burgdorferi* BBA64 Protein as a Protective Immunogen in Mice

Kevin S. Brandt, Toni G. Patton, Anna S. Allard, Melissa J. Caimano, Justin D. Radolf, and Robert D. Gilmore

The *Borrelia burgdorferi* bba64 gene product is a surface-localized lipoprotein synthesized within mammalian and tick hosts and is involved in vector transmission of disease. These properties suggest that BBA64 may be a vaccine candidate against Lyme borreliosis. In this study, protective immunity against *B. burgdorferi* challenge was assessed in mice immunized with the BBA64 protein. Mice developed a high-titer antibody response following immunization with soluble recombinant BBA64 but were not protected when challenged by needle inoculation of culture-grown spirochetes. Likewise, mice passively immunized with an anti-BBA64 monoclonal antibody were not protected against needle-inoculated organisms. BBA64-immunized mice were subjected to *B. burgdorferi* challenge by the natural route of a tick bite, but these trials did not demonstrate significant protective immunity in either outbred or inbred strains of mice. Lipidated recombinant BBA64 produced in *Escherichia coli* was assessed for possible improved elicitation of a protective immune response. Although inoculation with this antigen produced a high-titer antibody response, the lipidated BBA64 also was unsuccessful in protecting mice from *B. burgdorferi* challenge by tick bites. Anti-BBA64 antibodies raised in rats eradicated the organisms, as evidenced by *in vitro* borreliacidal assays, thus demonstrating the potential for BBA64 to be effective as a protective immunogen. However, passive immunization with the same monospecific rat anti-BBA64 polyclonal serum failed to provide protection against tick bite-administered challenge. These results reveal the challenges faced in not only identifying *B. burgdorferi* proteins with potential protective capability but also in producing recombinant antigens conducive to preventive therapies against Lyme borreliosis.

Lyme borreliosis has emerged over the last 35 years, affecting thousands of individuals in North America and Eurasia annually, and is a significant public health concern worldwide. When diagnosed properly, antibiotic administration is an effective treatment for a large majority of patients with this tick-borne disease. However, some patients go undiagnosed or exhibit symptoms after the course of antibiotic treatment, e.g., post-Lyme disease syndrome and antibiotic refractory arthritis, indicating a need for improved therapeutic treatments and/or vaccines (1). A recent study by the Centers for Disease Control and Prevention indicated that a substantial number of cases go unreported, underscoring the magnitude of annual infections in the United States (http://www.cdc.gov/lyme/faq/index.html#cases).

The causative agent of Lyme borreliosis is *Borrelia burgdorferi*, a spirochete that is transmitted through an enzootic cycle to reservoir animals and humans by *Ixodes* sp. tick bites. In the tick gut, in response to a tick's acquisition of a blood meal, *B. burgdorferi* differentially expresses genes encoding surface lipoproteins in preparation for its trafficking through the tick and eventual transfer to the newly infected host. Several borrelial genes upregulated at this stage have been identified and are regarded as putative essential components for borrelial survival (2–5). Although few functions have been described for these gene products, some have been postulated as potential vaccine targets, mainly because of their surface localization and production during a critical juncture of the spirochete's biological cycle in nature.

There has been no commercial vaccine for Lyme disease since the withdrawal of the LYMErix vaccine in 2002 (6). The LYMErix vaccine was based on the *B. burgdorferi* outer surface protein A (OspA) antigen, whereby host antibodies against OspA targeted *B. burgdorferi* within the tick, thus preventing borrelial transmission to the tick-bitten individual (7). Because OspA is not normally produced during infection of the mammalian or human host, the vaccine must be prophylactically immunized so that circulating anti-OspA antibodies in the bloodstream can neutralize the spirochetes in the tick after host attachment (8, 9). This characteristic was perceived to be a limitation of the efficacy of the vaccine, as booster immunizations would be necessary to achieve and maintain a sufficient titer for prophylaxis. However, the OspA vaccine was reasonably effective when properly administered, but it was discontinued for a variety of reasons (6).

Alongside OspA, another *B. burgdorferi* surface antigen shown to stimulate an effective protective immune response is OspC (10–12). Unlike OspA, OspC is synthesized by *B. burgdorferi* in the tick gut in response to the uptake of host blood (13). OspC is essential for establishing host infection, and antibodies against OspC are among the first to be detected in human and mammalian infections (14–17). A perceived limitation to an OspC vaccine involves the protein heterogeneity among *B. burgdorferi* isolates, whereby cross-protection against different strains may not be afforded or...
optimal (18–20). However, the effectiveness of OspC as a protective immunogen in experimental animals has led to a strategy for identifying additional vaccine candidates from *B. burgdorferi* proteins that play essential roles in pathogen transfer from ticks and the subsequent establishment of infection in mammalian hosts. Based on this concept, we previously identified and characterized the *B. burgdorferi* bba64 gene product as a critical component in mouse infection via a tick transmission mechanism (21, 22).

BBA64 is a surface-localized lipoprotein, known as P35 protein prior to the sequencing of the *B. burgdorferi* genome (23–25). The *bba64* gene is expressed in both feeding ticks and during murine infection after the dissemination and colonization of target tissues (22, 26, 27). *bba64* orthologs are present in *B. burgdorferi sensu stricto* and are also found in *Borrelia garinii* and *Borrelia afzelii*, which are Lyme disease-causing strains in Europe (28). BBA64 is induced in culture by changes in temperature and pH, and the gene is regulated by the RpoS regulatory cascade, which is responsible for controlling gene expression in response to environmental stimuli encountered by *B. burgdorferi* when infecting ticks and mammalian hosts (29–32). Antibodies against BBA64 have been detected in experimentally infected mice and Lyme disease patient blood serum samples, indicating that the synthesis of the protein occurs during infection (24, 33). Additionally, anti-BBA64 antibodies exhibit bactericidal activity in vitro and can attenuate borrelial attachment to cells in tissue culture (25, 34). Based on the information that BBA64 is surface exposed, produced in the host, and is essential for mammalian infection via tick transmission, we hypothesized that BBA64 is a protective antigen against *B. burgdorferi* infection. We report here that BBA64 immunization elicited a strong antibody response in mice but did not successfully prevent infection postchallenge.

**MATERIALS AND METHODS**

**Bacterial strains, ticks, and mice.** The *B. burgdorferi* clonal infectious strain B31-A3 was used in all mouse/tick challenge experiments (35). An assessment of a full complement of plasmids (except for cp9, which is missing in the B31-A3 strain) was demonstrated during all phases of culture reisolation from ticks and mice, based on a multiplex PCR described by Bunikis et al. (36). *B. burgdorferi* was cultivated in BSK-II complete medium in sealed tubes at 34°C.

Female 6- to 8-week-old outbred CD-1 mice were purchased from Charles River Laboratories (Wilmington, MA). Female inbred C3H/HeJ mice were purchased from Jackson Laboratories (Bar Harbor, ME). Infected *Ixodes scapularis* tick colonies were generated by feeding unfed *I. scapularis* larvae on CD-1 outbred mice previously infected via needle inoculation with 1 × 10⁶ organisms, as described previously (21). The mice were anesthetized (described as below for nymphal feeds) prior to the placement of approximately 150 to 300 larvae per mouse. Replicate larvae were collected daily, stored at 21°C in a saturated humidity chamber, and allowed to molt to the nymphal stage (about 6 to 8 weeks). In all experiments, replicate larvae, molted flat nymphs, and replete nymphs were assessed for *B. burgdorferi* infection by surface sterilization, maceration, and culturing in BSK-II medium, as described previously (21).

Nymphal tick feeds for infectious challenge of immunized mice were performed as follows: mice were anesthetized by intraperitoneal injection with a mixture of ketamine (80 to 100 mg/kg of body weight) and xylazine (10 mg/kg). *B. burgdorferi*-infected nymphs (*n* = 10 for BBA64-immunized mice and *n* = 5 for OspC-immunized mice) were placed dorsally on mice between the scapulae and allowed to feed to repletion (approximately 4 days). Challenges with cultured *B. burgdorferi* were performed by subcutaneous needle inoculation of 10⁶ organisms into isoflurane-anesthetized mice. The mice were assayed for infection at 21 days postchallenge by serology (immunoblotting against whole-cell *B. burgdorferi* lysates) and culture of ear biopsy samples in BSK-II medium supplemented with antibiotics and fungizone, as described previously (37). The experimental protocols involving mice were approved by the Institutional Animal Care and Use Committee at the Division of Vector-Borne Diseases, CDC, Fort Collins, CO.

**Preparation of recombinant proteins.** The *bba64* and *ospC* genes were cloned for recombinant protein expression using the Expresso T7 cloning and expression system (Lucigen Corporation, Middleton, WI). The genes were amplified from *B. burgdorferi* B31-A3 genomic DNA with primers designed to ligate into the linearized plasmid pETite N-His or C-His Kan vector. Primers were designed to amplify either the full-length coding regions, including the signal peptide, or truncated coding regions beginning with the first codon after the cysteine residue. The primers for full-length BBA64 in C-His vector were forward primer GAAGGAGATA TACATATGAAAAATAATATTTATGCA and reverse primer GTGA TGGTGGTGATGATGCTGATGTTGAGCAAGAATT; the primers for truncated BBA64 in N-His vector were forward primer CATCATCA CCACCATCACTCTGGAAGTCAAAGACAGC, and the reverse was the same primer as the full-length reverse; and the primers for truncated OspC in N-His vector were forward primer CATCATCACACCATCAC AATATTCAGGAAAAAGATGG and reverse primer GTGCGCGCCG GCTCTATTAGGGTTTGGACCTCTGCG.

pETite vector constructs were transformed into and propagated in *Escherichia coli* 10G (Lucigen), and purified plasmids isolated from the 10G cells were transformed into BL21 (DE3) Hi-Control E. coli cells (Lucigen) for protein expression. *bba64* and *ospC* cloned inserts isolated from transformant colonies were verified by DNA sequencing.

Soluble recombinant BBA64 (rBBA64) and rOspC with N-terminal 6×His tag were affinity purified according to the manufacturer’s recommended protocol for the purification of native protein using the nickel nitritocitratic acid (Ni-NTA) Fast Start kit system (Qiagen, Valencia, CA). The washing steps were modified to remove endotoxin by adding Triton X-100 to the kit wash buffer at 0.1% for the 1st wash, followed by 10-column volumes of washing without Triton X-100 prior to elution. The eluates were dialyzed against phosphate-buffered saline (PBS) for storage at −20°C.

Lipidated rBBA64 was constructed in the plasmid with C-terminal 6×His tag so as not to disrupt the amino-terminal signal lipidation peptide. The isopropyl-β-D-thiogalactopyranoside (IPTG) induction phase of culture was increased to 12 to 16 h. Bacterial pellets were resuspended in native lysis buffer for 30 min, followed by the addition of Triton X-114 to a 0.3% final concentration, with incubation continuing at room temperature for 30 min with occasional stirring. The samples were shifted to 37°C for 15 to 30 min before centrifugation at 15,000 × g for 30 min at 4°C, followed by collection of the supernatant, including Triton phase. The soluble-plus-Triton material was loaded onto the Ni-NTA columns, followed by washing and elution, as described for native purification.

**Determination of lipid modification of rBBA64.** rBBA64 with C-terminal 6×His was tested for lipidation using Click-it labeling technology (Life Technologies, Grand Island, NY) with labeled palmitic acid, azide (15-azidopentadecanoic acid) added to the culture medium during the induction of recombinant protein. Affinity purification was performed with the Dynabeads His tag isolation kit (Life Technologies), followed by dialysis into 50 mM Tris (pH 8.0). Streptavidin-conjugated alkaline phosphatase was used to visualize the biotin-tagged triazole complex as per the manufacturer’s instructions according to the standard protein blotting to membrane procedure. Control inductions without IPTG or without labeled palmitic acid, azide were performed in parallel to the labeled reactions.

**Immunization of mice with recombinant proteins and assessment of titers.** Isoflurane-anesthetized female 6- to 8-week old mice (CD-1) or inbred (C3H/HeJ) mice were immunized subcutaneously with rBBA64 (35 to 60 μg) or rOspC (25 μg) solubilized in Imject (1:1) (Thermo Scientific), followed by two booster injections 3 weeks apart. Primary immunization with lipidated rBBA64 (10 μg) in Imject adjuvant was followed....
by one boost 3 weeks later. The mice were bled 8 to 14 days following the final boost, and an enzyme-linked immunosorbent assay (ELISA) was performed on the serum samples against recombinant protein to assess the antibody titers. rBBA64 in sodium carbonate-bicarbonate buffer (pH 9.6) (100 μl/well at a concentration of 1 ng/ml) was coated overnight at 4°C onto Immulon 2 HB (Thermo Labsystems, Rochester, NY) 96-well plates. Following a 30-min block in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) and 1% normal rabbit serum, duplicate serial dilutions of mouse serum samples were added to the wells and incubated for 1 h at room temperature. The plate wells were washed three times in TBS-T, followed by the addition of alkaline phosphatase-conjugated goat anti-mouse IgG (KPL, Gaithersburg, MD) (1:1,500) and incubated for 1 h at room temperature. The TBS-T washes were followed by addition of 0.1 ml p-nitrophenyl phosphate (pNPP) substrate (Sigma-Aldrich, St. Louis, MO) for color development, which was stopped by adding an equal volume of 5 N NaOH. The plates were read at an optical density at 405 nm (OD405) on a Bio-Tek EL 808 plate reader with Gen5 1.11 software (Winooski, VT). Preimmunized mouse serum or PBS-immunized mouse serum was used to generate the background absorbance values.

**Immunization of rats with recombinant BBA64.** Female Sprague-Dawley rats (150 to 174 g) were purchased from Harlan Laboratories (Madison, WI). Purified rBBA64 (80 to 100 μg) in 1× PBS was mixed 1:1 with complete Freund’s adjuvant and injected intraperitoneally into two Sprague-Dawley rats. The animals were given booster injections of the same amount of the same antigen preparation mixed 1:1 with incomplete Freund’s adjuvant at 21 and 35 days postpriming. The animals were sacrificed by exsanguination approximately 2 weeks after the second booster.

BBA64 antisera was tested and confirmed to be specific for BBA64 by immunoblot analysis using lysates prepared from wild-type and BBA64 isolates. The immunizations were conducted in accordance with the Guide for the Care and Use of Laboratory Animals, Eighth Edition (38) and in accordance with the protocols reviewed and approved by the University of Connecticut Health Center Institutional Animal Care and Use Committee.

**Passive immunization of mice.** The monoclonal antibodies used for passive immunization were anti-BBA64 (34), anti-OspC (39), and anti-Bartonella vinsonii (34). Two hundred to three hundred micrograms of IgG-purified monoclonal antibody resuspended in 1× PBS was injected subcutaneously in a 100-μl volume into isoflurane-anesthetized mice 24 h prior to needle challenge or at the initiation of tick placement. Anti-rBBA64 polyclonal serum generated in rats (150 μl) was inoculated subcutaneously into mice at the initiation of B. burgdorferi infectious challenge via tick bite. The rat anti-BBA64 polyclonal serum (1:1,000) detected recombinant BBA64 on immunoblots at <25 ng.

**In vitro borreliacidal assay.** The serum samples were tested for B. burgdorferi killing activity by combining serial dilutions with an equal volume of B. burgdorferi in 96-well tissue culture plates. Doubling dilutions of non-heparin-activated serum, starting at 1:5, were prepared in 25 μl BSF-II medium per well in duplicate, followed by the addition of 2 × 10^9 to 5 × 10^9 B. burgdorferi cells in 25 μl BSF-II in each well. The plates were sealed and incubated in an incubator at 37°C and 5% CO₂. The wells were checked for viable B. burgdorferi following 24 h and 42 h of incubation using dark field microscopy. When appropriate, spirochetes were counted in several fields (>10) of ×400 magnification.

**RESULTS**

**Production of recombinant BBA64.** The bba64 coding sequence minus the signal peptide, i.e., from the amino acid after the cysteine lipidation signal peptide II processing site (Fig. 1A), was cloned into an E. coli pET expression vector with a 6×His tag present on the N terminus. Following induction with IPTG, the cells were lysed, and predicted mature recombinant BBA64 (rBBA64) was purified from the soluble fraction (Fig. 1B). In this system, rBBA64 was produced in abundant quantities from the soluble fraction and was therefore presumed to be in a properly folded (i.e., native) conformation. The protein produced from this construct will be referred to as soluble rBBA64.

A construct that included the BBA64 signal peptide was made to generate lipidated recombinant protein. Synthesis of the full-length lipidated rBBA64 in E. coli was reasonably efficient, as determined by protein staining and immunoblotting (Fig. 1C and D). Two assays were performed to determine whether rBBA64 was lipidated in E. coli. The first was a labeling reaction for palmitoylated lipoproteins with palmitic acid, azide (15-azido-4-decanonic acid) added to the E. coli culture during induction. Lipidated recombinant protein was detected colorimetrically following purification, Click-iT chemistry reaction, SDS-PAGE, and transfer to polyvinylidene difluoride (PVDF) membranes (Fig. 1E). The affinity-purified detergent-soluble fraction rBBA64 produced from the full-length coding sequence was also analyzed by mass spectrometry and Edman degradation. N-terminally degraded forms of full-length rBBA64 were detected, but the presence of a lipid moiety was not confirmed from the dominant bands analyzed by these assays (data not shown). However, the palmitate-labeled blot clearly showed the production of a lipidated fraction in the expression lysate, which was used for mouse immunizations.

**Immunization and B. burgdorferi challenge of mice.** The mice were given an initial inoculation of the soluble rBBA64, followed by two booster injections. Prior to challenge, the mice were bled and assayed for seroconversion. The ELISA results showed that all mice seroconverted and produced high titers of antibody (Fig. 2A). The mouse sera also were reactive against rBBA64 in immunoblots (Fig. 2B).

Initially, the mice were challenged by needle with an infectious dose (1 × 10^5) of in vitro-cultured B. burgdorferi. All soluble-rBBA64-immunized mice became infected (Table 1, experiment 1). We then challenged a second group of soluble-rBBA64-immunized mice by tick inoculation. In this trial, 3/4 mice were protected from infection (Table 1, experiment 2). However, when we repeated the experiment with a larger numbers of both inbred and outbred mouse strains, 10/10 CD-1 inbred and 10/11 C57H inbred mice became infected postchallenge (Table 1, experiments 3 and 4). All nonimmunized control mice also became infected. Using quantitative PCR (qPCR), we found no significant differences in spirochete burdens in the tissues from immunized and control groups 26 days postchallenge (data not shown).

OspC is a known protective antigen. Therefore, we constructed a soluble OspC expression clone using the same pET vector to produce recombinant protein with an N-terminal 6×His tag as a control. Immunization with this recombinant OspC proved to be protective, and we ascertained that the presence of the 6×His domain on the amino terminus of the protein did not diminish the protective capacity of the protein (Table 1, experiment 5). These data and recent BBA64 crystal structure data indicating that the N terminus forms an unstructured region that acts as a tether between the structured region and the cell (40) support the notion that the N-His tag on rBBA64 is not likely to have adversely affected its potential to provide protective immunity.

We immunized mice with lipidated rBBA64 to determine if lipidation conferred an advantage in eliciting a protective response. Lipidated rBBA64 produced significant antibody titers with one boost (with adjuvant), in contrast to soluble rBBA64, which required 2 booster inoculations to equal and surpass the titers provided by the lipidated antigen (Fig. 2A). This observation
indicated that lipid moiety enhances the immunogenicity of BBA64, similar to what has been seen with recombinant lipidated OspA vaccines. However, immunization with the lipidated antigen also failed to protect against tick-borne *B. burgdorferi* challenge, as 9/10 mice in that challenge became infected (Table 1, experiment 6).

Passive immunizations were performed by injecting either anti-BBA64 monoclonal antibody or rat monospecific anti-

FIG 1 (A) Amino-terminal coding sequence of *bba64* gene. Nucleotides in bold type represent the signal sequence, with the lipiddation signal peptidase site underlined. The arrow denotes the start of the protein sequence cloned to express BBA64 minus the signal peptide. (B) SDS-PAGE stained with GelCode Blue (Thermo Fisher, Rockford, IL) (lanes 1 to 3) of soluble rBBA64 protein expression in *E. coli*. Lane 1, IPTG-induced culture lyssate soluble fraction; lane 2, flowthrough fraction from His tag affinity Ni column; lane 3, elution fraction from column; lane 4, immunoblot (IB) of elution fraction with anti-BBA64. (C) SDS-PAGE stained with GelCode Blue of full-length lipidated rBBA64 protein expression in *E. coli*. (D) Immunoblot of lipidated rBBA64 using anti-BBA64. (E) Click-iT blot for detection of lipidated proteins. (+/+), IPTG-induced *E. coli* culture expressing BBA64 plus labeled palmitic acid; (−/+), noninduced *E. coli* culture containing BBA64 expression plasmid plus labeled palmitic acid; (+/−), IPTG-induced *E. coli* culture expressing BBA64 minus labeled palmitic acid; (ap), affinity-purified lipidated rBBA64 minus labeled palmitic acid. (B to E) Numbers on left denote molecular mass in kilodaltons. MW, molecular weight standards; IB, immunoblot.

FIG 2 (A) ELISA of mouse anti-rBBA64 IgG antibodies 8 to 14 days after final rBBA64 boost and prior to tick challenge. Bars represent the mean (with standard error) OD$_{405}$ of pooled serum samples at 1:25,600. Shown are 3 groups of soluble rBBA64-immunized mice (n = 10, 4, and 5, left to right), lipidated rBBA64-immunized mice (n = 10), and representative PBS-adjuvant-only-immunized mice (n = 5). Mice were boosted twice with soluble rBBA64 or once with lipidated rBBA64. (B) Representative immunoblot showing BBA64-specific reactivity of immunized mouse serum against *B. burgdorferi* whole-cell lysate (left). The anti-BBA64 monoclonal antibody served as a reference blotted against the *B. burgdorferi* whole-cell lysate (right).
TABLE 1 Active immunizations with BBA64 and OspC recombinant antigens

| Expt no. | Antigen                  | Challenge administration route | Mouse strain | No. of immunized mice infected/total no. of mice challenged | No. of control mice infected/total no. of mice challenged |
|----------|--------------------------|-------------------------------|--------------|------------------------------------------------------------|----------------------------------------------------------|
| 1        | rBBA64 soluble           | Needle                        | CD-1         | 4/4                                                        | 5/5                                                       |
|          | PBS/adjuvant             | Needle                        | CD-1         |                                                            |                                                          |
| 2        | rBBA64 soluble           | Tick                          | CD-1         | 1/4                                                        | 2/2                                                       |
|          | PBS/adjuvant             | Tick                          | CD-1         |                                                            |                                                          |
| 3        | rBBA64 soluble           | Tick                          | CD-1         | 10/10                                                      | 3/3                                                       |
|          | PBS/adjuvant             | Tick                          | CD-1         |                                                            |                                                          |
| 4        | rBBA64 soluble           | Tick                          | C3H          | 10/11                                                      | 3/3                                                       |
|          | PBS/adjuvant             | Tick                          | C3H          |                                                            |                                                          |
| 5        | rOspC (N-His)            | Tick                          | CD-1         | 1/5                                                        | 2/2                                                       |
|          | PBS/adjuvant             | Tick                          | CD-1         |                                                            |                                                          |
| 6        | rBBA64 lipided           | Tick                          | CD-1         | 9/10                                                      | 4/4                                                       |
|          | PBS/adjuvant             | Tick                          | CD-1         |                                                            |                                                          |

BBA64 polyclonal antiserum into mice 24 h prior to infectious challenge. Mice passively immunized with the anti-BBA64 antibodies were not protected from culture-grown needle-inoculated or tick-borne challenges (Table 2). As a control, a known protective anti-OspC monoclonal antibody was used to passively immunize mice, and these mice did not become infected following tick bite- or needle-administered challenge (Table 2). A Bartonella-specific monoclonal antibody used as a negative control was also nonprotective.

In vitro killing of *B. burgdorferi* by serum antibodies. Borrelialidial assays were performed to ascertain whether BBA64 antibodies have borrelialidial activity *in vitro*. Prechallenge anti-BBA64 mouse serum had no effect on *B. burgdorferi* viability or growth following 42 h of incubation at a 1:5 dilution. However, rat anti-BBA64 serum at a 1:5 dilution showed demonstrable killing of *B. burgdorferi* at 42 h of incubation. Control normal rat serum had no bactericidal effect in this assay.

DISCUSSION

We considered the *B. burgdorferi* BBA64 protein to be a suitable vaccine candidate against Lyme borreliosis based on four properties, (i) bacterial surface localization, (ii) synthesis in ticks and in the mammalian host, (iii) bactericidal abilities of anti-BBA64 antibodies, and (iv) the inability of *B. burgdorferi* strains with a disabled bba64 gene to infect mice by tick-borne transmission. We hypothesized that BBA64-specific antibodies from an immunized host neutralize the BBA64 function within feeding ticks, resulting in a failure to infect mice. However, this study found that BBA64 immunization with either lipidated or nonlipidated forms was not protective against *B. burgdorferi* challenge inoculation by needle or tick administration.

The soluble BBA64 construct was generated from the coding sequence minus the signal peptide so as to minimize the potential export signals that could have resulted in lower yields of recombinant protein in *E. coli*. We also generated a full-length BBA64 construct that consisted of the coding sequence plus the N-terminal signal sequence to produce lipidated recombinant protein. The production of rBBA64 in *E. coli*, in both forms, resulted in the purification of sufficient quantities of protein, although purification of the lipidated form, which included soluble and detergent phases to avoid loss of desired material, was less efficient. The presence of lipid on rBBA64 determined by the labeled palmitate assay combined with the antibody titers achieved after a single boost with this material led us to believe that a sufficient fraction of purified protein contained a lipid moiety so as to be biologically relevant for immunization purposes.

We avoided lysing conditions utilizing harsh denaturants. Soluble protein is more likely to retain a native structure, which may be important if a protective epitope is conformational rather than linear. We reported such a physical characteristic when purifying recombinant OspC, whereby soluble fractions were protective, but proteins isolated from insoluble inclusion bodies, while antigenic, did not provide immune protection (41).

The immunization of mice resulted in high antibody titers; however, following challenge by either needle or tick bite administration, there was no demonstrated protective capability. The lack of observed protection using needle-inoculated culture-grown organisms was not surprising given that BBA64 production is low when *B. burgdorferi* is grown in culture medium, with most protein being synthesized in the stationary phase (23). Moreover, it has been demonstrated by several groups that cultured *B. burgdorferi* is not the most appropriate vehicle for infectious challenges, as it does not exhibit the same protein expression patterns.
as organisms in ticks that adapt their surface structures to the tick feeding environment. However, we found that challenge by the tick bite route also resulted in the infection of BBA64-immunized mice. Although our initial experiment showed intriguing and encouraging results, with 3 of 4 mice exhibiting protection, subsequent experiments using more mice did not elicit parallel results. This finding was consistent regardless of whether the animals used were outbred or inbred mouse strains. Additionally, quantitation showed no differences in the spirochete burdens in tissues from immunized and nonimmunized control animals. This result indicated that BBA64 immunization did not prevent the establishment of infection and also had no effect on dissemination and tissue colonization.

We extended the experiments to immunizations with lipidated recombinant antigen, with the idea that lipiddation may stimulate a more effective immune response, specifically acting as an enhanced adjuvant formulation. The lipiddation of OspA has been shown to improve elicitation of the protective response in mice (42, 43) and was used in the formulations for the human vaccine trials (8, 9). However, as seen with the soluble nonlipiddated BBA64, lipiddated antigen was not a protective immunogen. It should be noted that this version of rBBA64 possessed a C-terminal 6×His tag to avoid potential interference with posttranslational processing. We cannot rule out conformational changes resulting from this tag, which may have altered potentially protective epitopes present in native BBA64.

Anti-BBA64 polyclonal serum samples obtained prechallenge from immunized mice were not effective in killing B. burgdorferi cells in an in vitro borrelial assay. Although the organisms were not derived from ticks, the failure of the mouse anti-BBA64 antibodies to kill or even inhibit their growth was an indication of the unsuccessful nature of the active immunization. However, the anti-BBA64 antibodies generated in rats were borrelialcid, albeit at a dilution of 1:5, and corroborated the results observed by the researchers, who demonstrated that anti-BBA64 antibodies made in rats were borrelialcid for >95% of the input organisms (25).

Our results demonstrated that passive immunization with either the mouse- or rat-derived anti-BBA64 antiserum failed to provide protection against tick-borne B. burgdorferi challenge. Observing the in vitro killing property of the rat antiserum, it was disconcerting to find that passive immunization with this sample did not provide an in vivo correlate.

The factors behind the failure of the BBA64 immunization to elicit a protective immune response are unknown. At first glance, BBA64 appears similar in activity to that observed for the protective antigen, OspC. Both are upregulated in the tick in response to blood meal uptake, and the inactivation of each gene prevents mammalian infection via tick transmission. Therefore, it was reasonable to expect that antibodies against BBA64, a protein critical for borrelial passage from feeding tick to host, would prevent a tick-borne infection. However, upon closer inspection, there is a key difference between the phenotypes of the respective mutant strains. OspC is essential for the establishment of host infection regardless of the delivery route (i.e., needle or tick bite) and functions to resist host innate defenses early after deposition in the skin (15, 44). In contrast, the function of BBA64 may be solely within the tick. Earlier work from our laboratory indicated that the uptake of anti-OspC antibodies by the tick in the blood meal inhibited B. burgdorferi from exiting the tick (45). We proposed that a similar mode of action would occur with BBA64 immunization, but the findings here proved otherwise.

An explanation for the lack of protection may be that anti-BBA64 antibodies do not recognize their borrelial target within the tick. BBA64 may function by interacting with a tick cell receptor in a mechanism designed to transition borrelial migration from the midgut to salivary glands for host deposition. Binding to such a receptors may block the accessibility of the antibody to the borrelial cell surface. Also, anti-BBA64 antibodies raised against the recombinant form of BBA64 in E. coli may not be specific for a critical native conformational site of the protein involved in functional activity. Regardless, the findings of this study show that a hypothesized neutralization of BBA64 activity with antibodies did not result in host protection as that observed when BBA64 synthesis was knocked out by gene inactivation.

In conclusion, identifying gene products utilized by B. burgdorferi to facilitate migration to and from the arthropod vector, and to infect vertebrate hosts, continues to be a goal that may ultimately assist in deciphering the intricate pathways involved in this biological process. From a public health viewpoint, the knowledge gained from these studies may provide targets for disruption of the B. burgdorferi infectious cycle leading to new preventive therapies for Lyme borreliosis. The B. burgdorferi BBA64 protein as utilized in this study was not an adequate protective immunogen. These results demonstrate the challenges confronted in not only discovering the relevant protein factors involved in infectious pathways of tick-borne borreliosis but also in ascertaining whether they can be effective targets to circumvent disease.

ACKNOWLEDGMENTS

We thank Jan Pohl, Olga Stuchlik, and Matthew Reed at the Biotechnology Core Facility Branch at the Centers for Disease Control and Prevention for significant work on the lipidated BBA64, including Edman degradation and mass spectrometry. We thank Phil Stewart and Patti Rosa, Rocky Mountain Laboratories, Hamilton, Montana, for kindly providing strain B31-A3 for our studies. We thank Theresa Russell and Barbara J. Johnson for discussions on the lipidated BBA64 and Jennifer Damics for technical assistance. We also thank members of the DVBD Animal Resources: John Liddell, Sarah Maes, Ashley Walker, Verna O’Brien, and Andrea Peterson.

This work was supported by NIH/NIAID grants AI29735 (to M.J.C. and J.D.R.) and AI85248 (to M.J.C.).

REFERENCES

1. Wormser GP, Dattwyler RJ, Shapiro ED, Halperin JJ, Steere AG, Klempner MS, Krause PJ, Bakken JS, Strle F, Stanek G, Bockenstedt L, Fish D, Dumler JS, Nadelman RB. 2006. The clinical assessment, treatment, and prevention of Lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. Clin. Infect. Dis. 43:1089–1134. http://dx.doi.org/10.1086/508667.
2. Tokarz R, Anderton JM, Katona LI, Benach JL. 2004. Combined effects of blood and temperature shift on Borrelia burgdorferi gene expression as determined by whole genome DNA array. Infect. Immun. 72:5419–5432. http://dx.doi.org/10.1128/IAI.72.9.5419-5432.2004.
3. Kenedy MR, Lenhart TR, Atkins DR. 2012. The role of Borrelia burgdorferi outer surface proteins. FEMS Immunol. Med. Microbiol. 66:1–19. http://dx.doi.org/10.1111/j.1574-695X.2012.00980.x.
4. Kung F, Anguita J, Pal U. 2013. Borrelia burgdorferi and tick proteins supporting pathogen persistence in the vector. Future Microbiol. 8:41–56. http://dx.doi.org/10.2217/fmb.12.121.
5. Kumar M, Yang X, Coleman AS, Pal U. 2010. BBA52 facilitates Borrelia burgdorferi transmission from feeding ticks to murine hosts. J. Infect. Dis. 201:1084–1095. http://dx.doi.org/10.1086/651172.
6. Poland GA. 2011. Vaccines against Lyme disease: what happened and what lessons can we learn? Clin. Infect. Dis. 52 (Suppl 3):S253–S258. http://dx.doi.org/10.1093/cid/ciq116.

7. de Silva AM, Telford SR, III, Brunet LR, Barthold SW, Fikrig E. 1996. Borrelia burgdorferi OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. J. Exp. Med. 183:271–275. http://dx.doi.org/10.1084/jem.183.1.271.

8. Steere AG, Sikand VK, Meurice F, Parenti DL, Fikrig E, Schoen RT, Nowakowski J, Schmid CH, Laukamp S, Buscarino C, Krause DS. 1998. Vaccination against Lyme disease with recombinant Borrelia burgdorferi outer-surface lipoprotein A with adjuvant. Lyme Disease Vaccine Study Group. N. Engl. J. Med. 339:209–215.

9. Sigal LH, Zabradnik JM, Lavin P, Patella SJ, Bryant G, Haselby H, Hilton E, Kunek M, Adler-Klein D, Doherty T, Evans J, Molloy PJ, Seidner AL, Sabetta JR, Simon HJ, Krum JG, Bestor A, Jewett MW, Grimm D, Bueschel D, Byram RD, Jr, Howison RR, Dietrich G, Patton TG, Clifton DR, Brangulis K, Tars K, Petrovskis I, Kazaks A, Ranka R, Baumanis V. 2011. Molecular characterization of a 35-kilodalton protein of Borrelia burgdorferi, an antigen of diagnostic importance in early Lyme disease. J. Clin. Microbiol. 33:86–91.

10. Brooks CS, Vuppala SR, Jett AM, Akins DR. 2006. Identification of Borrelia burgdorferi outer surface proteins. Infect. Immun. 74:296–304. http://dx.doi.org/10.1128/IAI.74.1.296–304.2006.

11. Paterson RL, Nelder CL, Nowalk AJ, Clifton DR, Howison RR, Schmit VL, Nolder C, Hughes JL, Akins D, Schwartz I. 2003. Identification of 11 pH-regulated genes in Borrelia burgdorferi localized to linear plasmids. Infect. Immun. 71:6677–6684. http://dx.doi.org/10.1128/IAI.68.12.6677–6684.2000.

12. Caron CF. 2000. Identification of an outer surface protein on Borrelia burgdorferi. J. Infect. Dis. 182:2137–2142. http://dx.doi.org/10.1086/318068.

13. Brangulis K, Tars K, Petrovskis I, Kazaks A, Ranka R, Baumanis V. 1999. An OspC-specific monoclonal antibody passively protects mice from tick-transmitted infection by Borrelia burgdorferi B31. Infect. Immun. 67:5470–5472.

14. England MA, Nelder CL, Nowalk AJ, Clifton DR, Howison RR, Schmit VL, Nolder C, Hughes JL, Akins D, Schwartz I. 2003. Identification of 11 pH-regulated genes in Borrelia burgdorferi localized to linear plasmids. Infect. Immun. 71:6677–6684. http://dx.doi.org/10.1128/IAI.68.12.6677–6684.2000.

15. Gilmore RD, Jr, Howison RR, Schmit VL, Carroll JA. 2008. Borrelia burgdorferi expression of the bba64, bba65, bba66, and bba73 genes in tissues during persistent infection in mice. Microb. Pathog. 45:355–360. http://dx.doi.org/10.1016/j.micpath.2008.06.006.

16. Nolder CL, Nelder AJ, Clifton DR, Howison RR, Schmit VL, Gilmore RD, Jr, Carroll JA. 2008. Borrelia burgdorferi surface-localized proteins expressed during persistent murine infection are conserved among diverse Borrelia spp. Infect. Immun. 76:2498–2511. http://dx.doi.org/10.1128/IAI.76.6.2498–2511.2008.

17. Brangulis K, Tars K, Petrovskis I, Kazaks A, Ranka R, Baumanis V. 2011. Molecular characterization of a 35-kilodalton protein of Borrelia burgdorferi, an antigen of diagnostic importance in early Lyme disease. J. Clin. Microbiol. 33:86–91.

18. Brooks CS, Vuppala SR, Jett AM, Akins DR. 2006. Identification of Borrelia burgdorferi outer surface proteins. Infect. Immun. 74:296–304. http://dx.doi.org/10.1128/IAI.74.1.296–304.2006.
2013. Structure of an outer surface lipoprotein BBA64 from the Lyme disease agent Borrelia burgdorferi which is critical to ensure infection after a tick bite. Acta Crystallogr. D. Biol. Crystallogr. 69:1099–1107. http://dx.doi.org/10.1107/S0907444913005726.

41. Gilmore RD Jr, Mbow ML. 1999. Conformational nature of the Borrelia burgdorferi B31 outer surface protein C protective epitope. Infect. Immun. 67:5463–5469.

42. Erdile LF, Brandt MA, Warakomski DJ, Westrack GJ, Sadziene A, Barbour AG, Mays JP. 1993. Role of attached lipid in immunogenicity of Borrelia burgdorferi OspA. Infect. Immun. 61:81–90.

43. Weis JJ, Ma Y, Erdile LF. 1994. Biological activities of native and recombinant Borrelia burgdorferi outer surface protein A: dependence on lipid modification. Infect. Immun. 62:4632–4636.

44. Tilly K, Bestor A, Jewett MW, Rosa P. 2007. Rapid clearance of Lyme disease spirochetes lacking OspC from skin. Infect. Immun. 75:1517–1519. http://dx.doi.org/10.1128/IAI.01725-06.

45. Gilmore RD Jr, Piesman J. 2000. Inhibition of Borrelia burgdorferi migration from the midgut to the salivary glands following feeding by ticks on OspC-immunized mice. Infect. Immun. 68:411–414. http://dx.doi.org/10.1128/IAI.68.1.411-414.2000.