HHS Public Access

Author manuscript

Vaccine. Author manuscript; available in PMC 2020 February 25.

Published in final edited form as:

Vaccine. 2017 September 25; 35(40): 5310–5313. doi:10.1016/j.vaccine.2017.08.054.

Immunization of mice with *Borrelia burgdorferi* lp54 gene encoded recombinant proteins does not provide protection against tick transmitted infectious challenge

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Abstract

The *Borrelia burgdorferi* outer surface membrane proteins BBA65, BBA66, BBA69, BBA70, and BBA73 were tested for their ability to confer protection against *B. burgdorferi* infection challenge. Mice were immunized with recombinant forms of the proteins singly or in combinations. Following initial protein inoculation and booster injections, seroconversion was confirmed prior to *B. burgdorferi* challenge by tick bite. Despite mice having high antibody titers for each antigen, no significant protections against the challenge infections were observed. These results demonstrate that these recombinant proteins were not protective and reflects the challenges confronted to identify effective novel vaccine candidates for Lyme disease.

Keywords

Borrelia burgdorferi; Lyme borreliosis; pfam54 genes

1. Introduction

Lyme disease, or Lyme borreliosis, caused by the bacterium *Borrelia burgdorferi* (sensu lato family) via transmission by tick bites, remains a major public health concern in North America and temperate regions worldwide. In the United States, cases continue to rise despite integrated prevention efforts including personal protective measures, acaricide application, and control of tick populations through management of small mammalian reservoir and deer hosts [1,2]. Although each control and prevention application has merit and can be effective, they have specific limitations. It has been proposed that vaccination would be a more effective strategy to reduce the Lyme disease case burden [3].

A commercially available vaccine was manufactured and sold until 2002 when it was withdrawn for multiple reasons [4] and there is currently no human vaccine for Lyme disease. The commercial vaccine (Lymerix) was composed of the *B. burgdorferi* protein antigen termed OspA (outer surface protein A). OspA is present on *B. burgdorferi* that reside

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Conflict of interest

The authors report no conflict of interest.

in unfed nymphal and adult stage ticks. When the tick engorges on blood from an OspA-vaccinated individual, the OspA-specific antibodies present in the blood eradicate the organisms within the tick, thereby preventing transmission to the human host [5]. Interestingly, when *B. burgdorferi* infects a non-vaccinated host following a tick bite, the OspA antigen is not produced by the organism. Therefore, protection by this vaccine can only occur if the individual has been prophylactically immunized and has sufficient titer of circulating anti-OspA antibodies in the blood at the time of infected tick bite.

Second generation vaccine candidates have focused on *B. burgdorferi* antigens that are synthesized within the tick and/or upon establishment of infection in the host. Several candidate proteins have been identified that fit this criteria and have been evaluated for protective capability following immunization in experimental animals [6]. Most have shown little or no immunizing protection against tick bite challenge with the exception of OspC which has shown the best potential as a protective immunogen [7–10]. However, doubts have arisen that with OspC strain heterogeneity, cross protection from different *B. burgdorferi* strains would be limited. Other strategies for Lyme disease vaccine candidates have been proposed including tick protein antigens (anti-tick vaccine), alternate novel *B. burgdorferi* proteins, and a combination of both [6,11].

The *B. burgdorferi* genome consists of an approximate 900 kilobase chromosome and numerous linear and circular plasmids [12]. Although some plasmids are dispensible for *B. burgdorferi* viability, the 54 kilobase linear plasmid (lp54) is regularly maintained, an indication that genes on this plasmid encode proteins with essential functions. A contiguous series of lp54 genes with annotated designations of BBA64, −65, −66, −69, −70, and −73, have been extensively studied, and they have been referred to as a paralogous gene family [12]. Several of these genes have been predicted by microarray studies to be highly upregulated under conditions of tick blood feeding, mammalian infection, and other environmental conditions such as pH and temperature [13–15]. The outer surface location of the lipoproteins encoded by these genes, their elicitation of host antibody responses during infection, and that antibodies against them are bactericidal, have led to proposals that these proteins are candidates as vaccinogens [16–18]. In this study, we evaluated the protective efficacy afforded by recombinant forms of these proteins via mouse immunizations, either singly or in combinations, followed by infectious tick transmitted *B. burgdorferi* challenge.

2. Materials and methods

2.1. Bacterial strains, ticks, and mice

B. burgdorferi clonal infectious strain B31-A3 was used in all mouse/tick challenge experiments, following cultivation in BSK-II complete media in sealed tubes at 34 °C in a 5% CO_2 incubator.

Generation of infected *I. scapularis* tick colonies and assessment of infection with *B. burgdorferi* were performed as described [18].

2.2. Preparation of recombinant proteins

The *bba64*, *−65*, *−66*, *−69*, *−70*, *−73*, and *ospC* coding sequences minus the signal peptide 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 genomic DNA with primers designed to ligate into the linearized plasmid pETite N-His Kan vector with soluble expressed proteins purified from *E. coli* as described [19].

2.3. Immunization of mice with recombinant proteins and assessment of titer

CD-1 mice were immunized subcutaneously with approximately 15–35 μg (for single antigen) or approximately 2–20 ug each (for multi-antigen) recombinant protein solubilized in Imject (1:1) (Thermo Scientific) followed by two booster injections 3 weeks apart. Mice were bled 14 days following the final boost, and ELISA was performed on serum samples against recombinant protein to assess antibody titer as described [19].

2.4. Tick challenge of immunized mice

Immunized mice were challenged by infected nymphal stage ticks at 16–21 days following the last boost. Mice were anesthetized by intraperitoneal injection with a ketamine (50–100 mg/kg) and xylazine (5–10 mg/kg) mixture prior to placement of ticks (n = 5–8/mouse).

Mice were assayed for infection at 14 days post-challenge by serology (immunoblotting against whole cell *B. burgdorferi* lysates) and culture of ear biopsies in BSK-II supplemented with antibiotics and fungizone as described previously [18]. Ticks collected from mice that were uninfected following the feed were cultured for *B. burgdorferi* to ensure that at least one infected tick had fed on the mouse. Experimental protocols involving mice were approved by the Institutional Animal Care and Use Committee at the Division of Vector Borne Diseases, CDC, Fort Collins, Colorado.

3. Results

3.1. Immunization of mice with recombinant proteins

Soluble recombinant proteins BBA64, BBA65, BBA66, BBA69, BBA70, BBA73, and OspC were purified for mouse immunization (Fig. 1). Groups of mice were immunized either with a single antigen or with a combination cocktail of antigens (Table 1). Following the second boost (3 injections total) and prior to infectious challenge, individual mice were bled and assayed for seroconversion to the specific antigen with antibody titers determined. The majority of mice (i.e. >90%) immunized with a particular antigen had antibody titers of ≥25,600 indicating a robust humoral response. A representative ELISA of serum samples from individual mice immunized with one antigen (BBA70) is shown in Fig. 2.

3.2. *B. burgdorferi* infectious challenge by tick bite

Once seroconversion and antibody titers were established, mice were administered *B. burgdorferi* via tick bite transmission. Protection from challenge was assessed by serology and direct culturing from mouse ear tissues 2 weeks following tick feeding. Significant protection was not observed in mice immunized with the single antigens, BBA66 or BBA65

as 10/12 (p value = 0.73) and 11/11 respectively became infected (Table 1). The antigen combination of BBA69 and BBA70 were similarly non-protective with 11/11 mice infected following challenge. The four antigen combination of BBA64, BBA65, BBA66, and BBA73 conferred protection to 3/11 mice, but this was not statistically significant compared with the non-immunized controls which had one mouse that did not become infected (p value = 0.63) (Table 1). As a control for immunization protection, 7/9 mice inoculated with OspC did not become infected post challenge. Ticks that fed on mice that did not become infected were cultured to determine whether *B. burgdorferi* were present. Culture results of these ticks indicated that at least one infected tick had fed on protected mice.

4. Discussion

Alternative antigens for a second generation Lyme disease vaccine for humans have been a quest for researchers since the OspA-based commercial vaccine was made unavailable by the manufacturer. OspC, despite studies demonstrating its protective capability, is comprised of multiple serotypes that may limit its protective specificity and has not been developed commercially for humans.

We focused on the lp54 gene products as potential vaccine candidates and have previously reported on the evaluation of immunization efficacy of BBA64 in mice [18]. We found that soluble recombinant BBA64, lipidated or non-lipidated, did not provide protective immunity against either needle or tick-borne challenge. Here, we tested additional lp54 gene encoded proteins for protective properties.

We first tested antigens BBA65 and BBA66 individually, and finding a lack of protection, we performed additional experiments with a combination of antigens. Our reasoning was as follows: (i) to simultaneously test multiple antigens in a single experiment; (ii) to determine whether a combination of antigens would act synergistically; and (iii) to minimize the use of experimental animals. Although the multi-antigen cocktail as administered contained less of a particular protein than the single antigen dose trials, this did not appear to be a reason for nonprotection as the antibody titers for each antigen were high, i.e. 1:25,600, prior to challenge. Our findings indicated that none of ≥the antigens provided protection in the form administered.

We utilized the soluble form of the recombinant antigens when purified from *E. coli* to maintain conformation that may be essential for protective epitopes. We previously found this to be a critical point in the formulation of protective recombinant OspC [20]. Although we cannot exclude the possibility that *E. coli*-based recombinant proteins may not have properly folded protective conformational epitopes, the occurrence seems unlikely. Our results also demonstrate that surface localization of proteins is not a sole predictor for protective efficacy.

In conclusion, several investigations have demonstrated that the lp54 encoded gene products in this study are surface exposed, expressed during tick feeding and/or in mammalian hosts, and elicit host antibody responses suggesting their utility as vaccine candidates. The findings presented here are provided to inform that the requisite experiments were performed to

evaluate the efficacy of these antigens as proposed alternative candidates for second generation Lyme disease immunogens.

Acknowledgements

We thank Phil Stewart and Patti Rosa for providing the B31-A3 strain, and the Division of Vector Borne Diseases Animal Resources group.

Funding

Funding was provided by CDC.

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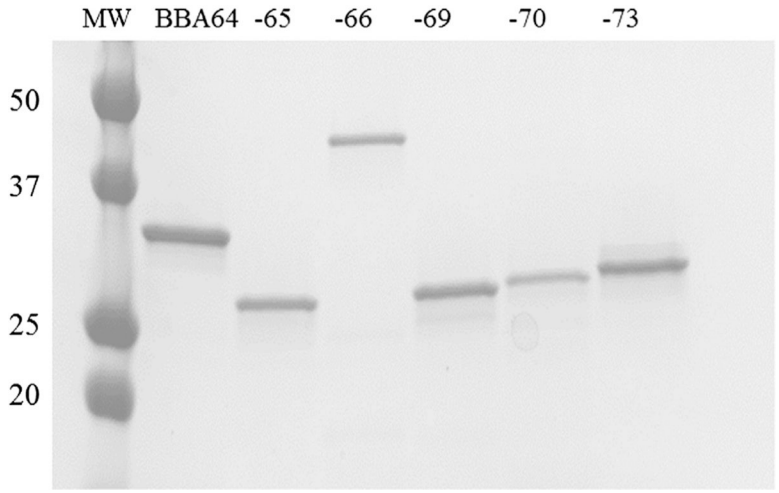

Fig. 1.

GelCode Blue (ThermoFisher) stained SDS-PAGE of the purified recombinant proteins used for experimental mouse immunizations (labelled above each lane). MW = molecular weight markers. Numbers on the left denote molecular mass in kilodaltons.

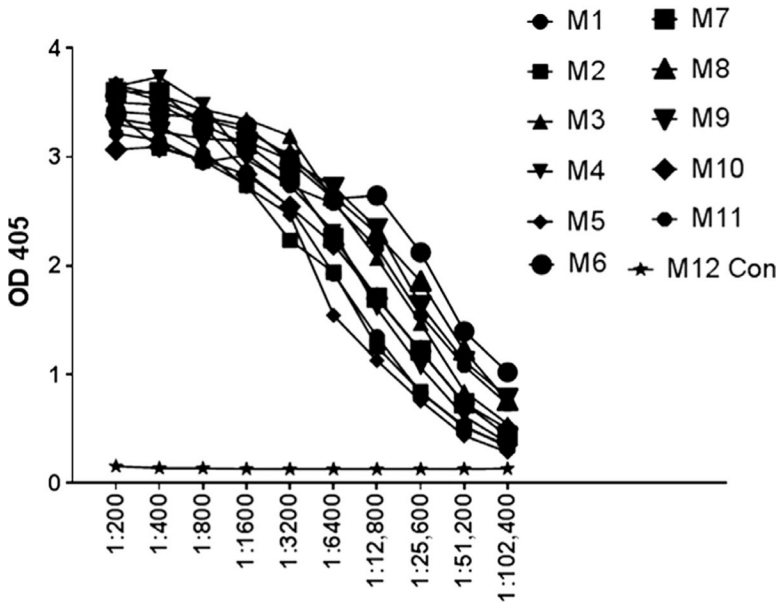

Fig. 2.

Representative graph of ELISA results demonstrating antibody titers from individual mice prior to *B. burgdorferi* challenge by tick transmission. The data presented is from mice immunized with recombinant BBA70. Antibody titers from mice immunized with each antigen demonstrated similar high titers prior to challenge. M = mouse (number); M12 Con = control mouse mock immunized with PBS plus adjuvant only.

Table 1

Active immunizations with recombinant antigens.

Antigen	Challenge	Mouse strain	Number mice infected[a]/number mice challenged		p value[b]
			Immunized	Non-immunized control	
rBBA66	Tick	CD-1	10/12	2/2	0.73
rBBA64 + 65+66 + 73	Tick	CD-1	8/11	4/5	0.63
rBBA69 + 70	Tick	CD-1	11/11	5/5	1.0
rBBA65	Tick	CD-1	11/11	5/5	1.0
r OspC	Tick	CD-1	2/9[c]	4/4[c]	0.02

[a]Positive by both serology and ear culture.

[b]One-tailed Fisher Exact Probability Test.

[c]Combined two trials.

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