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Characterization and optimization of a novel vaccine for protection against Lyme borreliosis

Pär Comstedt, Markus Hanner, Wolfgang Schüler, Andreas Meinke, Robert Schlegl, Urban Lundberg*

Valneva Austria GmbH, Campus Vienna Biocenter 3, 1030 Vienna, Austria

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ABSTRACT

Lyme borreliosis (LB) is the most common vector-borne disease in the northern hemisphere and there is no vaccine available for disease prevention. The majority of LB cases in Europe are caused by four different *Borrelia* species expressing six different OspA serotypes, whereas in the US only one of these serotypes is present. Immunization with the outer surface protein A (OspA) can prevent infection and the C-terminal part of OspA is sufficient for protection against infection transmitted by *Ixodes* ticks. Here we show that the order of the stabilized monomeric OspA fragments making up the heterodimers in our LB vaccine does not influence the induced immunogenicity and protection. Using bioinformatics analysis (surface electrostatics), we have designed an improved version of an LB vaccine which has an increased immunogenicity for OspA serotype 3 and an optimized expression and purification profile. The OspA heterodimers were highly purified with low amounts of endotoxin, host cell proteins and host cell DNA. All three proteins were at least 85% triacylated which ensured high immunogenicity. The LB vaccine presented here was designed, produced and characterized to a level which warrants further development as a second generation human LB vaccine.

1. Introduction

Lyme borreliosis (LB) is a zoonotic disease and the most common vector-borne infection in the northern hemisphere. Results from the Center for Disease Control and prevention (CDC) suggest that in the United States (US) the number of LB cases is around 300,000 per year [1]. Since LB is not a notifiable disease in most European countries, the number of annual cases has been reported to be between 65,000 and 85,000 which is most likely an under estimate [2–4]. In the US, LB is caused by *Borrelia burgdorferi* in contrast to Europe where four *Borrelia* species cause the majority of LB cases. These four *Borrelia* species are associated with six OspA serotypes, *B. burgdorferi* (serotype 1), *B. afzelii* (serotype 2), *B. garinii* (serotypes 3, 5 and 6) and *B. bavariensis* (serotype 4) [5–7]. In addition, in Europe *B. valaisiana* and *B. spielmanii* have rarely been isolated from patients diagnosed with LB, showing that the disease can be caused also by less common *Borrelia* species [8,9].

LB can be prevented by vaccination with the outer surface protein A (OspA) and a vaccine targeting LB (LYMErix) based on OspA from *B. burgdorferi* (serotype 1) was available in the US from 1998 to 2000 [10–12]. One of the functions assigned to OspA is the binding to TROSPA in the tick mid-gut [13]. OspA is expressed when the spirochetes are located in the mid-gut of unfed ticks and is down-regulated during tick feeding, allowing the migration of the bacteria to the salivary glands and further into the vertebrate host [14]. Thus, antibodies directed against OspA can recognize and neutralize spirochetes in the tick mid-gut in a complement independent manner [15,16].

We have designed an LB vaccine based on the notion that the C-terminal half of OspA plays an essential role for protection [17]. This vaccine consists of three OspA heterodimers, each heterodimer is a fusion of the C-terminal part of OspA from two different serotypes, with a 21 amino acid long linker. Here we provide data for the selection of the order of OspA serotypes in the heterodimers and we have modified the heterodimer containing OspA serotype 3 which resulted in improved production yields. In addition, the purification and characterization of the OspA-derived vaccine components are presented.

2. Materials and methods

2.1. Protein modeling

The structure models of the stabilized monomeric OspA (*B. burgdorferi* (OspA serotype 1 (B31)), *B. afzelii* (OspA serotype 2

* Corresponding author. Tel.: +43 1 20620 1289; fax: +43 1 20620 81289.
E-mail address: urban.lundberg@valneva.com (U. Lundberg).

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(K78)), *B. bavariensis* (OspA serotype 4 (PBi)), *B. garinii* (OspA serotypes 3 (PBr), 5 (PHae) and 6 (DK29)) and *B. valaisiana* (VS116) were generated with the open-source version of PyMOL (http://sourceforge.net/projects/pymol/) [18] using the crystal structure of OspA serotype 1 (PDB: 1OSP) [19] as the starting point and available homology models [20]. Fold compatibility was verified with molecular mechanics simulation [21].

The electrostatic potential isocontours of the stabilized monomeric OspA, *B. burgdorferi* (serotype 1), *B. afzelii* (serotype 2), *B. bavariensis* (serotype 4), *B. garinii* (serotypes 3, 5 and 6) and *B. valaisiana*, were calculated with the adaptive Poisson–Boltzmann solver (APBS [22], pdb2pqr [23]).

2.2. Cloning

The pET expression plasmids for the heterodimers (abbreviated "D" followed by the corresponding OspA serotype and a letter (B) indicating the stabilization of the first and second monomer) were previously described by Comstedt et al. [17]. Two new heterodimers have been generated by gene synthesis (GeneArt, Germany), Lip-D4B3B^{233} wherein the proline at position 233 in serotype 3 was changed to a threonine (P233T) and Lip-D4Bva3B wherein the first one-third of the serotype 3 portion was substituted with the corresponding sequence from *B. valaisiana* OspA (amino acid 126 to 176) in addition to the P233T substitution in the serotype 3 part.

2.3. Mouse experiments

All animal experiments were performed in accordance with Austrian law (BGB1 No. 114/2012) and approved by "Magistratsabteilung 58". Eight-week-old female C3H/HeN mice were used for all studies (Janvier, France) [17].

Mice were challenged either s.c. with *B. burgdorferi* strain N40 (OspA serotype 1) at doses corresponding to between 5- and 10-fold the ID_{50} [17] or with infected ticks. The infection prevalence (OspA serotype 2, strain IS1) of the ticks was >90% for all experiments, as assessed by qPCR. The feeding status of the ticks was monitored and only mice from which at least one fully fed tick was collected were included in the final readout [17].

The infection status was determined by analyzing sera with VlsE ELISA and DNA samples with qPCR targeting the *recA* gene [17]. A mouse was regarded as infected when at least one of the two methods was positive, the consistency between the two methods was >95%.

2.4. OspA ELISA

The OspA ELISA was performed as previously described [17]. Briefly, ELISA plates were coated with 50 ng stabilized monomeric OspA (M1B-His, M2B-His, M3B-His or M4B-His). Five-fold serial dilutions of sera were analyzed in duplicate. The half-max titer (the reciprocal of the dilution that corresponds to the mean absorbance between highest and lowest dilution) was determined.

2.5. Flow cytometry

Flow cytometry was performed as previously described [17]. Briefly, spirochetes (1×10^6) were fixed with paraformaldehyde prior to staining. Serum pools were sterile filtered before incubation with the spirochetes. PE-conjugated secondary antibody was used for surface staining and spirochete DNA was stained with LDS 751 (Life technologies, Germany). Labeled spirochetes were measured with a Beckman Coulter Cytomics FC500 flow cytometer and gated for LDS 751 positive events.

2.6. Upstream process

One hundred mL preculture medium was inoculated with 1 mL culture of BL21(DE3) expressing the different OspA heterodimers (Lip-D1B2B, Lip-D4Bva3B or Lip-D5B6B). The cultures were incubated for 3–4 h at 37 °C. The preculture was used to inoculate 8 L batch medium in a 19 L fermenter (Bioengineering Type NLF22). A fed-batch process, consisting of a batch phase (11–13 h; growth until depletion of glucose) and an exponential feed phase (7 h; controlled glucose feed at $\mu = 0.20$/h, and 3–4 h induction with 1 mM IPTG) was used. The yield of wet biomass was about 900 g from 11 L culture which was stored in aliquots at −80 °C until further processing.

2.7. Downstream process

Cell paste was resuspended in a 10-fold volume of lysis buffer (50 mM Tris, 500 mM NaCl, 5 mM EDTA pH 8.0). A high pressure homogenizer (Panda 2K, Niro Soavi S.p.A, Italy) was used for cell disruption. Triton X-114 was added to the crude lysate to a final concentration of 6% and incubated at 4 °C overnight. After removal of cell debris by centrifugation at 7000 × *g* for 3 h at 4 °C, phase separation was performed at 28 °C followed by recovery of the lipid phase by centrifugation at 7000 × *g* for 45 min at 28 °C. The lipidated proteins were enriched in the lipid phase. The lipid phase was diluted 20-fold (5 mM $NaPO_4$, 20 mM Tris, 20 mM NaCl, 10% EtOH, 0.05% Tween 20, pH 8.0) prior to loading onto Q-Sepharose. The Q-Sepharose flow-through was loaded onto a ceramic hydroxyapatite column. Three washing steps were performed, step 1 (5 mM $NaPO_4$, 20 mM Tris, 20 mM NaCl, 0.05% Tween 20, pH 8.0) removed remaining feed solution, step 2 contained Triton X-114 for removal of remaining endotoxins and wash step 3 removed Triton X-114 from the column. Elution was performed with a linear gradient from 15% to 85% elution buffer with 180 mM $NaPO_4$. The hydroxyapatite pool was stored at 4 °C overnight and then diluted approximately 4- to 6-fold with Milli-Q water and loaded onto a DEAE column and the flow through was collected and concentrated with a Pellicion 50 kDa membrane (PES membrane) followed by an AmiconUltra-15 device. The concentrated protein was loaded onto a Superdex 200 column, and the product was eluted between 0.45 and 0.6 CV with a total elution volume of approximately 0.15 CV.

The LB vaccine, comprising the three OspA heterodimers (Lip-D1B2B, Lip-D4Bva3B and Lip-D5B6B) in a 1:1:1 ratio, is formulated in 10 mM $NaPO_4$, 150 mM NaCl, 10 mM L-Methionine, 5% Sucrose, 0.05% Tween 20 and 0.15% (w/v) aluminum hydroxide at pH 6.7 for optimal stability at 2–8 °C. All buffer components are listed by U.S. Food and Drug Administration (FDA) as generally recognized as safe (GRAS) for human use. Tween 20 is included to eliminate aggregate formation of the OspA heterodimers and L-Methionine is added to quench modifications of the lipid moiety of the OspA heterodimers caused by Tween 20.

2.8. Analyses of the lipidation status and fatty acid composition

Reverse-phase HPLC: Protein samples were analyzed by HPLC using a cyanopropyl-bonded column (Zorbax 300SB-CN 4.6 × 150 mm, Agilent) equilibrated at 1 mL/min with 35% acetonitrile/0.1% TFA/water at 80 °C. Elution was performed by linear gradient to 60% acetonitrile/0.1% TFA/water over 10 min. Column effluent was monitored at 214 and 280 nm.

GC–MS: Fatty acid analysis of purified OspA heterodimer Lip-D5B6B was done by an external service provider (M-Scan, UK). In short, fatty acids were cleaved off from the purified protein with sodium hydroxide, spiked with an internal standard (heptadecanoic acid (C17) in heptane), freeze-dried and methylated to the corresponding methyl ester followed by GC–MS analysis