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Oral Vaccination With Vaccinia Virus Expressing the Tick Antigen Subolesin Inhibits Tick Feeding and Transmission of *Borrelia burgdorferi* Vaccination

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Abstract

Immunization with the *Ixodes scapularis* protein, subolesin, has previously been shown to protect hosts against tick infestation and to decrease acquisition of *Anaplasma marginale* and *Babesia bigemina*. Here we report the efficacy of subolesin expressed from Vaccinia virus for use as an orally delivered reservoir-targeted vaccine for prevention of tick infestation and acquisition/transmission of *Borrelia burgdorferi* to its tick and mouse hosts. We cloned subolesin into Vaccinia virus and showed that it is expressed from mammalian cells infected with the recombinant virus in vitro. We then vaccinated mice by oral gavage. A single dose of the vaccine was sufficient for mice to generate antibody response to subolesin. Vaccination with the subolesin expressing Vaccinia virus inhibited tick infestation by 52% compared to control vaccination with Vaccinia virus and reduced uptake of *B. burgdorferi* among the surviving ticks that fed to repletion by 34%. There was a reduction in transmission of *B. burgdorferi* to uninfected vaccinated mice of 40% compared to controls. These results suggest that subolesin has potential as a component of a reservoir targeted vaccine to decrease *B. burgdorferi*, *Babesia* and *Anaplasma* species infections in their natural hosts.

Keywords

Vaccinia virus; *Borrelia burgdorferi*; Lyme disease; Reservoir-targeted vaccine; *Ixodes scapularis*; Tick; OspA; Subolesin

Introduction

Lyme disease is the most common vector borne disease in the U.S. It is caused by infection with the spirochete, *Borrelia burgdorferi*. It is transmitted by ticks *Ixodes scapularis* ticks [1]. There are three human infections in the United States that are commonly transmitted by *I. scapularis*: Lyme borreliosis, babesiosis, and human granulocytic ehrlichiosis/anaplasmosis (HGE). Despite efforts to control and reduce these tick borne diseases, the incidence of all three infections has risen steadily over last several decades [2–5]. There is currently no available human vaccine for any of these three diseases [6]. As a result, control of these diseases has focused on control and avoidance of the tick vector. Spraying of

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acaricides on vegetation as well as application of acaricides directly on tick hosts such as mice and deer have been shown to be successful in certain settings [6–9]. However, none of these strategies has gained wide acceptance. Acaricidal resistance in ticks, the potential for negative impacts on non-target insects and other animals in the environment and the need for repeated applications present obstacles for these strategies [6, 10]. Studies have shown efficacy of distribution of antibiotics such as doxycycline to reservoir animals [11]. However, widespread distribution of antibiotics that are used for treatment of human diseases raises concerns for the development of resistance in both target and non-target pathogens.

Reservoir targeted vaccine approaches have been used very successfully to eradicate rabies from foxes and raccoons in endemic areas using a vaccinia virus (VV) vector in oral baits [12–15]. Our laboratory has been interested in applying similar approaches to reduction of *B. burgdorferi* infection in the wild. Tsao et al have shown that injection of the *B. burgdorferi* protein outer surface protein A (OspA) is effective in reducing tick carriage of the organism in the season following distribution of the vaccine [16]. We have previously cloned OspA into VV and shown that oral vaccination with this vaccine can decrease transmission of *B. burgdorferi* by infected ticks to uninfected mice as well as reduce acquisition of *B. burgdorferi* by uninfected ticks feeding on infected mice [17, 18]. However, protection against *B. burgdorferi* transmission/acquisition using this oral OspA vaccine was not complete and in addition this vaccine offers no protection against transmission or acquisition of *B. microti* or *A. phagocytophilum* suggesting that the inclusion of additional antigens that protect through different mechanisms may be complementary [17, 18]. Vaccines against tick feeding and transmission offer promise as strategies for protecting against multiple tick-borne infections. This approach has been used for the development of commercially available bovine vaccines currently marketed as Gavac and TickGARD. These vaccines target the Bm86 midgut antigen from tick *Boophilus microplus*. The vaccine successfully helped control tick infestation in cattle and reduced the transmission of some tick borne pathogens including *Babesia* sp [19].

Several tick antigens have been shown to prevent tick feeding and disease transmission by *Ixodes* ticks [6]. One very promising *Ixodes* antigen is subolesin, which has been extensively studied by de La Fuente's group [20–24]. Immunization using recombinant subolesin protected hosts against tick infestation by reducing survival, weight and oviposition and decrease the vector competency of *I. scapularis* ticks for *Anaplasma phagocytophilum*, *Anaplasma marginale*, and *Babesia bigmina* [25–27]. The biological function of subolesin is not fully understood. However, previous studies have shown that subolesin can be involved in the regulation of NF- κ B-dependent and independent gene expression [23, 28]. Recent studies have shown that subolesin knockdown by RNA interference (RNAi) caused degeneration of tick tissues including the midguts, salivary glands, and reproductive tissues [25, 29, 30]. No studies have yet reported on the effect of subolesin vaccine against transmission or acquisition of *B. burgdorferi*. In this study we report the use of VV as a vector for oral delivery of subolesin as an anti-tick vaccine to reduce tick infestation as well as the transmission and acquisition of *B. burgdorferi*.

2. Materials and methods

2.1 Vaccinia virus, bacteria, tick and mouse strains

VV strain vRB12 [31], which is a vp37 deleted strain derived from the mouse adapted WR strain of VV, was the kind gift of Dr. Bernard Moss (NIH). VV was grown and maintained in HeLa cells as described previously [17, 18, 32]. *B. burgdorferi* (strain N40 D10E9) was cultivated in Barbour-Stoener-Kelly H (Sigma Chemical Co.) at 37°C as described previously [33]. Female C3H/HeN mice (6–8 weeks old), were purchased from Charles River Laboratories (Boston, MA). *I. scapularis* larvae were obtained from National Tick

Research and Education Center, Oklahoma State University (Stillwater, OK). *B. burgdorferi*-infected nymphs were generated as previously described [18].

2.2 Construction of Vaccinia virus vaccine expressing subolesin

To construct VV expressing subolesin (VV-Sub), the *sub* gene was amplified as follows: RNA was extracted from nymphal *I. scapularis* ticks using TRIZOL® Reagent (Invitrogen). cDNA was generated using the RNA as a template using ImProm-II™ (Promega). Subolesin was amplified from cDNA using primers subF, and subR (Sub-HA tag) (Table 1), and cloned into pCR2.1 (Invitrogen). An HA epitope tag was introduced by encoding it into the reverse primer. The amplicons were inserted into the cloning plasmid pCR2.1 (TopoTA Cloning kit, Invitrogen). Clones were selected and sequenced. Plasmid DNA was purified, restricted with the appropriate enzymes and the fragment ligated to a similarly restricted pRB21 plasmid (kind gift of Bernard Moss) [34]. The clones containing the correct insertion of the *sub* gene were confirmed by restriction mapping and gene sequencing.

To generate recombinant vaccinia, the pRB21/*sub* construct was transformed into infected cells as described by Moss et al. [32] with the exception that lipofectamine 2000 (Invitrogen) was used for transfection as described by the manufacturer's instruction. After four rounds of plaque selection, the presence of subolesin insert in VV was analyzed by PCR using the primers Sub-F and Sub-R to detect the presence of the insert and vRB12Int-F and vRB12Int-R specific to *E. coli gpt* segment found only in the parental vRB12 to determine contamination (Table 1) [17].

2.3 Expression of recombinant subolesin

The subolesin gene was amplified using cDNA as a template. The forward and reverse primers used for PCR were designed to include *NheI* and *XhoI* restriction sites respectively and to permit in frame cloning to the pET28C expression vector (Table 1). The *E. coli* BL21 (DE3) pLysS was transformed with pET28C to over-express subolesin protein. The bacteria cells were grown at 37°C in culture media containing appropriate antibiotic to an OD of 0.6. Thereafter, the cells were induced with 1mM IPTG for 2 hours. The cells were harvested by centrifugation at 5000g for 10 min and the pellet was resuspended in lysis buffer (50 mM NaH₂PO₄; 300mM NaCl).

2.4 Western blot analysis

For western blot analysis, lysates of cells infected with VV-Sub or VV-vp37 were collected and analyzed by SDS-PAGE. Western blots were performed as previously described [18] with the following modifications: 1) the primary antibody was a rat monoclonal anti-HA antibody (Roche) used at 1:4000 dilution; 2) the secondary antibody was HRP-linked anti-Rat antibody (Roche) used at 1:8000 dilution. The antibody response by VV-Sub vaccinated and VV-vp37 control mice was evaluated by western blot against recombinant subolesin expressed from *E. coli*. Quantitative analysis of antibody development was performed by scanning densitometry with Bio-Rad Quantity One analysis software.

2.5 Determination of VV-Sub efficacy

Female C3H/HeN mice were infected with VV-Sub or control VV-vp37 by gavage with 10⁸ plaque-forming units (pfu) of virus. Efficacy of the VV-Sub vaccine was determined for inhibition of larval infestation, larval tick acquisition, and for inhibition of transmission to uninfected mice by infected nymphal ticks. 3 weeks after vaccination mice with either VV-Sub or control VV-vp37, mice were infected with *B. burgdorferi* subcutaneously using a 1 ml syringe and 27½ ga needle. Infection was confirmed by culture of an ear punch in BSK at week 2. The infected control and vaccinated mice were infested with 100–150 *I. scapularis*

larvae per mouse [24, 35]. The larvae were collected daily for 1 week [35]. The larval ticks were collected and kept in 95% humidity chamber at 21°C. After 5 days, a subset of larval ticks from each mouse were homogenized and cultured in BSK media [18]. The presence of *B. burgdorferi* was determined by observing the cultures under microscope. The remaining ticks were allowed to molt. The inhibition of larvae infestation (*I*) was calculated with respect to controls as described previously by Almazan et al. [35, 36].

To test the efficacy against transmission to uninfected mice, mice were vaccinated with VV-Sub or control (VV-vp37). Antibody response was determined 4 weeks post vaccination. 5 weeks post-vaccination, mice were challenged by allowing 5 *B. burgdorferi* infected nymphal ticks to feed to repletion as previously described [17, 18]. The nymphs were collected and cultured for *B. burgdorferi* infection. The mice were tested for *B. burgdorferi* infections by culture of an ear punch in BSK media 2 weeks after infestation and at time of sacrifice at 4 weeks when heart, joint and skin tissue was cultured. Cultures were monitored by darkfield microscopy. Mice were considered infected if there was a single positive culture from any tissue. The inhibition of nymphal infestation was determined with respect to the number of engorged nymphs recovered from the control as described previously by Almazan *et al.* [35].

2.6 Statistics

The number of attached ticks per mouse and the ratio replete ticks over attached ticks were compared by Fisher's exact test between vaccinated and control mice. Fisher's exact test was used to determine if significant differences in experiments that resulted in discrete data points (positive and negative *B. burgdorferi* infection of mice and ticks).

3. Results

3.1 Construction of recombinant virus

VV-Sub was constructed using the VV strain vRB12. This VV strain that has a deletion in the *vp37* gene that encodes a 37-KDa outer envelope protein which is essential for virus packaging and plaque formation [34]. The strategy for creating a recombinant VV that expresses Subolesin tagged with an HA epitope is shown in Fig. 1A. The successful recombination and presence of the subolesin gene was confirmed by PCR (Fig. 1 B). To confirm the expression of subolesin, recombinant virus was used to infect HeLa cells. The infected cell lysates were subjected to western blot to show expression of subolesin containing the HA tag (Fig. 2).

3.2 Immunogenicity of VV-Sub administered to mice by gavage

Prior studies with the VV-OspA vaccine have shown that optimal antibody responses to oral gavage was achieved with 10^8 pfu of VV [37]. We administered 10^8 pfu of VV-Sub or the VV-vp37 control to C3H/HeN by oral gavage. Blood samples were collected at various time points and analyzed by scanning densitometry of Western blots (Fig. 3 A). The majority of mice vaccinated with a single dose of VV-Sub developed antibody responses to recombinant subolesin by week 4. Antibody responses appeared stable to 8 weeks.

3.3 Efficacy of VV-Sub in prevention of tick infestation and *B. burgdorferi* acquisition by larvae ticks from infected mice

To evaluate the efficacy of VV-Sub on tick larvae infestation and acquisition of *B. burgdorferi*, we vaccinated mice with 10^8 pfu control (VV-vp37) or VV-Sub. All of the vaccinated mice developed antibody responses to subolesin (Fig. 3 B). The mice were infested with uninfected larval ticks 5 weeks after administration of the vaccine. There was a

significant reduction in larval infestation in VV-Sub vaccinated mice compared with control vaccinated mice (Table 2). Only 38% of ticks placed on VV-Sub vaccinated mice fed to repletion and were recovered versus 72% from control vaccinated mice (52% decrease in VV-Sub vaccinated mice, $p<0.0001$). Among the recovered ticks, a small but significant effect on larval molting to the nymphal stage between VV-Sub and control vaccinated mice was seen (77 vs. 95% respectively, $p<0.0001$). In addition to the effects on tick repletion, there appeared to be a separate effect on acquisition of *B. burgdorferi* by feeding ticks. Of the ticks that fed to repletion and were recovered, only 66% of those fed on VV-Sub vaccinated animals were infected with *B. burgdorferi* versus 100% infection in tested ticks recovered from control vaccinated mice. Combining the effect on larval feeding with the effect on *B. burgdorferi* acquisition, vaccination decreased the chance of an uninfected larval tick becoming a *B. burgdorferi* infected nymphal tick by more than 60%.

3.4 Efficacy of VV-Sub in prevention of *B. burgdorferi* transmission to uninfected mice

To determine the efficacy of VV-Sub for protection of uninfected mice from transmission of *B. burgdorferi* from infected ticks, we vaccinated mice with VV-Sub or VV-vp37 control vaccine. Nine out of 15 vaccinated mice developed strong antibody responses to subolesin (Fig. 3 C). At weeks 5 post vaccination, *B. burgdorferi* infected nymphs were allowed to feed on each mouse. Vaccination with VV-Sub inhibited nymphal feeding by a small but statistically significant amount (31%, $p<0.01$) (Table 3). Transmission of *B. burgdorferi* to vaccinated mice was also inhibited. 9 out of 15 (60%) VV-Sub vaccinated mice were infected compared to 15 out of 15 (100%) of the control mice (40% decrease in infection, $p<0.01$). Of note, the nine VV-Sub vaccinated mice that developed strong antibody responses, 6 were protected from infection and only 3 were infected. Of the 6 mice with low antibody responses, all 6 became infected. Removing the mice with low antibody responses from the calculations, antibody to subolesin provided 67% protection against *B. burgdorferi* infection.

4. Discussion

The control of infections through the vaccination of wild reservoirs is an emerging strategy for reducing human disease. There are several groups attempting to develop reservoir targeted vaccines for Lyme disease using the *B. burgdorferi* antigen, OspA [7, 38, 39]. While vaccination of animals with OspA can both prevent acquisition of *B. burgdorferi* by uninfected ticks feeding on vaccinated, infected animals as well as prevent transmission of the organism from an infected tick to an uninfected animal, the protection is not complete [17, 18]. Nor does vaccination with OspA prevent transmission of *B. microti* or *A. phagocytophilum*, two other *Ixodes* borne diseases that are commonly co-transmitted with *B. burgdorferi*. Vaccination with tick antigens has the potential to affect transmission of multiple pathogens. Vaccination with the BM86 antigen of the *Boophilus microplus* tick has been shown to prevent transmission of *Babesia sp* and *Anaplasma Sp* to cattle [19] and vaccination with TRP64, a tick cement protein, has been shown to prevent transmission of tick borne encephalitis virus (TBE) [1, 40]. While BM86 prevents feeding of ticks on vaccinated animals, prevention of TBE in TRP64 vaccinated animals does not seem to work through decreasing tick feeding [41, 42]. It has been postulated that vaccination with TRP64 results in increased inflammation at the tick bite site which then inhibits transmission of the virus [1, 40].

Subolesin is a tick antigen that has previously been identified as an anti-tick vaccine candidate [24, 36, 43]. Vaccination with subolesin has been shown to prevent tick feeding and molting. Vaccination with subolesin has already been shown to decrease acquisition of *A. phagocytophilum*, *A. marginale*, and *B. bigmina* [25–27, 36]. The protective mechanism

of subolesin vaccine is not fully understood [44], but may be due to effects of antibodies on protein function and [29, 28, 44, 45] the various effects caused by antibodies to subolesin may have a cumulative effect on the infection and multiplication of the pathogens in tick tissue [46, 47].

Here, we have shown that subolesin can be successfully expressed from a VV vector and results in antibody generation when administered orally to mice. Oral administration of VV-Sub protects mice against tick feeding with a similar level of protection to that reported for recombinant subolesin administered percutaneously [36]. In addition to its effects on successful tick feeding to repletion, there appeared to be separate vaccine effects on both acquisition of *B. burgdorferi* by uninfected larval ticks feeding on infected, vaccinated mice as well as transmission of *B. burgdorferi* from infected ticks to vaccinated mice. While individually each of these effects were modest, the combined effects on successful tick feeding, acquisition and transmission of *B. burgdorferi* have the potential to interrupt the transmission cycle of *B. burgdorferi* in the wild in additive ways at multiple points.

The mechanism by which vaccination with subolesin affects *B. burgdorferi* acquisition and transmission separate from its effects on tick feeding are unknown. It has been demonstrated previously that inhibition of inflammatory mediators such as IL-1 and TNF- α can affect the establishment of *B. burgdorferi* infection from an inoculation site [48]. In addition, *B. burgdorferi* has been shown to take advantage of multiple factors in tick saliva that suppress host immune responses to establish early infection [49–52]. If vaccination with subolesin results in increased inflammation at the tick bite site, or if antibodies to subolesin alter expression of tick salivary factors that are utilized by *B. burgdorferi* to establish infection, it may become more difficult for *B. burgdorferi* to establish infection in the host.

Given the incomplete protection against either tick feeding or acquisition/transmission of *B. burgdorferi* by ticks, it is unlikely that subolesin can be used as a stand-alone antigen for reservoir vaccination against Lyme disease. We anticipate that it would be used in conjunction with other antigens to offer broader protection against Lyme and other organisms. One of the advantages of VV as a vector for vaccine delivery is that it is can stably accommodate multiple gene insertions. Including Subolesin in a VV vaccine that expresses *B. burgdorferi* OspA has the potential to improve its efficacy in blocking *B. burgdorferi* acquisition and transmission by *Ixodes* ticks. In addition, although not studied in our system, subolesin has been shown to inhibit acquisition/transmission of *Anaplasma* and *Babesia*. In this study, we administered VV-sub at the same doses and challenged at the same time points that we have found effective for the VV-OspA vaccine. Given the multiple potential effects of a combined OspA/subolesin reservoir targeted vaccine on tick feeding, pathogen acquisition and pathogen transmission, even partial efficacy against acquisition and transmission of these three organisms, carried out over multiple cycles can have significant effects on the population dynamics of these pathogens in ticks and potentially reduce the incidence of these expanding emerging infectious diseases.

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Fig. 1. Strategy for construction of VV-Sub

(A) The subolesin gene was amplified from cDNA produced from RNA from nymphal *I. scapularis* ticks using primers that encoded a HA epitope tag, (B) PCR showing presence of subolesin gene with HA tag insert in different clones of VV-Sub obtained after four rounds of plaque selection. Lane 1 and 2: different clones of VV-Sub, Lane 3: The purity of VV-Sub recombinant was tested by amplifying *gpt* sequence, which is present only in VV- ν RB12 and lost with proper insertion of the subolesin gene. M: 1Kb DNA ladder (Invitrogen Inc).

Fig. 2. Expression of subolesin protein from recombinant vaccinia virus *in vitro*

Confluent Hela cells were infected with 10^8 pfu/ml VV-Sub or VV-vp37 control. Cell lysate were subjected to SDS-PAGE and Western blot. (A) Coomassie stain. (B) Western blot probed with an anti-HA antibody to test for subolesin expression. M: molecular weight marker, Lane1: negative control, cell lysate infected by VV-vp37 control, Lane 2: VV-Sub infected cell lysate. The subolesin protein (~22 kDa) is indicated by an arrowhead.

Fig. 3. Antibody responses to vaccination with VV-Sub

(A) Time course of anti-sub response in VV-Sub vaccinated mice (—) and control mice (-----). Western blots were analyzed by scanning densitometry. The relative anti-subolesin antibody concentrations are expressed in arbitrary density units. (B) Anti-subolesin antibody response on week 5 by VV-Sub and VV-vp37 vaccinated mice used in experiments of larval tick infestation and acquisition of *B. burgdorferi* by ticks. (C) Anti-subolesin antibody response on week 5 by VV-Sub and VV-vp37 vaccinated mice used in studies of *B. burgdorferi* transmission by infected nymphal ticks. Western blots were analyzed by scanning densitometry. The relative anti-subolesin antibody concentrations are expressed in

arbitrary density units. Note that the arbitrary units in figures are specific to each blot and absolute units cannot be compared across graphs.

Table 1

Primers used in the generation and characterization of VV-Sub.

Primer name	Sequence (5'----3')	Function
Sub-F	ATGGCTTGCGCA ACATTAAA	Forward primer for cloning subolesin into VV
Sub-R-HA	TTAAGCGTAGTCTGGGACGTCGTATGGGTAT GACAA ATA GCT TGG AGTGGC	Reverse primer for subolesin with an HA epitope tag
vRB12Int-F	TTTAGCGATATAGCCGATGA	Forward primer to detect presence of vRB12
vRB12Int-R	GATGAAGCCTTCGCCATC	Forward primer to detect presence of vRB12

Table 2Efficacy of VV-sub vaccine against larval tick infestation and acquisition of *B. burgdorferi* by ticks

	Fed larvae recovered /total placed (%)	Successful molted larvae /recovered larvae* (%)	<i>B. burgdorferi</i> infected larvae / total cultured larvae (%)	**% Inhibition of tick larval infestation
VV-vp37	492/682 (72)	391/412 (95)	80/80 (100)	-
VV-Sub	333/948 (35)	199/253** (77)	28/80** (66)	52

Eight control and 10 VV-Sub vaccinated mice were infested with 100–150 larvae.

Results showed a significant degree of protection from spirochete infection in VV-Sub vaccinated mice vs. control.

Inhibition of tick infestation was calculated as described by Alamzan et al. 2005.

* Eighty recovered larvae were removed for testing (see column 4)

**
 $p < 0.0001$ compared to control

Table 3

Efficacy of VV-sub vaccine against in prevention of *B. burgdorferi* transmission to mice.

	Total mice	#Mice infected	**Inhibition of acquisition (%)	**Inhibition of nymphal infestation (%)
VV-vp37	15	15	-	-
VV-Sub	15	9	40	22

-Results are from 15 mice from each group. The mice were vaccinated with 10^8 pfu VV-sub or VV-vp37 control. Statistical analyses (Fisher's exact test**) showed a significant degree of protection from spirochete infection in VV-sub vaccinated mice vs.control

**
 $p < 0.01$ compared to control