Oral vaccine that breaks the transmission cycle of the Lyme disease spirochete can be delivered via bait

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Abstract

Borrelia burgdorferi causes Lyme disease, a potentially debilitating human disease for which no vaccine is currently available. We developed an oral bait delivery system for an anti-*B. burgdorferi* vaccine based in OspA. Mice were immunized orally via gavage and bait feeding. Challenge was performed via *Ixodes scapularis* field nymphs carrying multiple *B. burgdorferi* strains. Vaccination protected 89% of the mice and the systemic immune response was skewed toward IgG2a/2b production. Moreover, this oral vaccine reduced the pathogen in the tick vector by eight-fold. We conclude that this oral vaccine induces a protective systemic immune response against a variety of infectious *B. burgdorferi* strains found in nature and therefore it can eliminate this zoonotic pathogen from its major host reservoirs. Because we observed elimination of the spirochete from the tick vector, a broad delivery of this oral vaccine to wildlife reservoirs is likely to disrupt the transmission cycle of this pathogen.
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1. Introduction

Zoonotic pathogens circulate in several vertebrate reservoir hosts and occasionally cause human disease due to incidental infection. Examples of such diseases are Rocky Mountain spotted fever, Lyme disease and West Nile virus. The pathogens that cause these diseases are transmitted to man via an arthropod vector, such as a tick or a mosquito. The best approach to restrain most vector-borne infectious diseases is through vector control. However, the major method used so far, organochloride pesticides [1], produce unacceptable environmental risks.

One way to prevent human infection is to block transmission of the pathogen in the vertebrate reservoir and/or within the arthropod vector via the development of vaccines to disrupt its zoonotic transmission cycle. Successful vaccination of vertebrate reservoirs should lead to the decrease of the infection prevalence in the arthropod vector, and thus, to a lower probability of pathogen transmission to man. Widespread immunization of vertebrate reservoirs in their natural habitat can only be achieved via the oral route because it is not invasive and is suitable for cost-effective mass vaccination campaigns [2]. Immunizing wildlife against zoonotic pathogens via oral bait has been successfully attempted before to control rabies and plague [3–6]. *Borrelia burgdorferi* cycles in nature between vector ticks of the genus *Ixodes* and a variety of vertebrate reservoir species, such as white-footed mouse, eastern chipmunk and white-tailed deer [7–12]. Lyme disease occurs when *Ixodes scapularis* ticks periodically feed on and transmit the bacteria to man [13]. Here, we set out to develop an oral bait vaccine to control *B. burgdorferi* in wildlife reservoirs and therefore used the natural transmission cycle of this zoonotic pathogen and Lyme disease as a model to test this hypothesis.

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Outer surface protein A (OspA) inoculated via oral or parenteral administration has been studied as a vaccine against *Lyme borreliosis* [14–21,2,22]. Recently, it has been demonstrated that vaccinating wild mice reservoirs via subcutaneous inoculation reduced the prevalence of *B. burgdorferi* in *I. scapularis* nymphs [23]. However, subcutaneous inoculation of wildlife is not practical. Although OspA vaccination represents an exciting strategy to prevent zoonotic transmission of *B. burgdorferi*, until now, there was no practical system to deliver such vaccines in the wild.

In this study, we demonstrate that our oral vaccine induced a protective systemic (IgG) immune response against multiple wildtype *B. burgdorferi* strains and drastically reduced the pathogen in its tick vector. Thus, this system is an exciting candidate to distribute vaccines in the wild to disrupt the transmission cycle of *B. burgdorferi*, a zoonotic vector-borne pathogen. Here, we describe for the first time, an oral bait vehicle for delivery of vaccines that could be adapted to disrupt the transmission cycle of several other human vector-borne diseases.

2. Methods

2.1. Lyophilization and viability of E. coli expressing B. burgdorferi antigens

B. burgdorferi full length OspA, strain B31, was cloned into the Nde I-Bam HI restriction sites of pET9c and was transformed into *Escherichia coli* BL21 (DE3) pLysS. The parental *E. coli* strain was used as control. After protein induction, cells were harvested, resuspended in 10 ml of TBY containing 24% sucrose [24] and quickly frozen. The antigen was placed in a lyophilizer (Labconco) overnight and stored at $-70\,^{\circ}$C for future use. The viability of the lyophilized bacteria was determined by adding 1 ml of PBS to lyophilized bacteria to get an $OD_{600} = 1$ and plating 1/10 of the suspension onto TBY plates supplemented with proper antibiotic. Protein extraction from lyophilized antigen was done using 200 μl of CELLytic solution (Sigma).

2.2. Oral immunization

B. burgdorferi susceptible 6–8 week female C3H/HeJ inbred mice (Charles River, Boston) were used. Lyophilized, previously induced bacteria, were weighed and resuspended in TBY to an $OD_{600} = 1$ (approximately 10^9 cells/ml). For oral gavage, 250 μl (2.5×10^8 *E. coli* cells in TBY) were inoculated with a ball tipped disposable feeding needle. Mice received the first immunization daily on days 1, 2, 3, 4 and 8, 9, 10, 11. Two weeks later, mice were bled (day 27) and on days 30, 31, 32, 33 received the 1st boost. On day 45, mice were bled for the second time and on days 52, 53, 54, 55 received the second boost. On day 64, mice were bled for the 3rd time. Challenge was performed on day 67. One month later (day 97), mice were euthanized and blood, ear, heart and

bladder tissues were obtained to assess for spirochete dissemination. For oral bait, mice received the first round of bait on days 1, 2, 3, 4, the second round on days 23, 24, 25, 26, the third round on days 43, 44, 45, 46 and were bled on days 17, 39 and 60 to compare the anti-OspA immune response with mice immunized by oral gavage. Lyophilized bacteria (10 mg, 50 mg or 100 mg) were mixed with 500 mg of either peanut butter or oatmeal. Mice were placed in individual cages and to train them, the food was removed the night before bait feeding in the first week of the immunization. Next morning, a dose of the bait was provided for ingestion ad libidum. The same dose was provided every day and the mice were allowed to eat for 3 h, after which empty containers were removed from the cage and mouse chow was offered. Water was provided at all times. For the subsequent rounds of bait feeding the food was not removed the night before immunization, the bait was presented the same way and at the same time as the previous round. If given bait and mouse chow at the same time, mice always finished the bait first. All experiments involving animals were performed after obtaining proper IRB approval.

2.3. B. burgdorferi challenge

Needle challenge was performed by intradermal inoculation of 2×10^3 of a *B. burgdorferi* sensu stricto strain cultured from a patient biopsy. This is a highly infectious strain, characterized with an OspC group K, that has not been subjected to further laboratory passages. This strain is used in our laboratory to perform both infections of mice and needle challenges directly from the 1st passage. Tick challenge was performed by placing 9–10 *B. burgdorferi* infected nymphal field ticks on the back of the head of restrained mice that were allowed to feed for 2 h. Three days later (d3 to d5), engorged ticks were collected after naturally falling off, counted and a daily record was kept for each mouse.

2.4. Collection and maintenance of Ixodes scapularis ticks

The field ticks were obtained from *I. scapularis* larva collected from ears of infected white-footed mice trapped at Millbrook, NY in the summer of 2003. Larva were allowed to molt to the nymphal stage in glass flasks, where they were kept at room temperature in a humid environment. The tick rate of infection was checked by darkfield microscopy and by *ospC*-PCR using the following primers: OC6 (+) AAAGAATACATTAAGTGCGATATT; OC623 (−) TTAAGGTTTT TTTTGGACTT TCTGC.

2.5. Determination of vaccine efficacy

Antibody assays: Total IgG and IgG subclass isotyping was performed in immunized mouse sera using either alkaline phosphatase (1:1600) (KPL, Gaithersburg, MD) or horseradish peroxidase secondary antibody (1:50,000) (Bethyl, Montgomery, TX) by ELISA. Because we planed

to do tick challenges and therefore could not use a specific *B. burgdorferi* strain lysate for immunoblots, we normalized the procedure by using a standard test commonly used for diagnostic of Lyme disease in humans. The MarDx test (*B. burgdorferi*, strain B31 (MarDx Diagnostics Inc., Carlsbad, CA) was used to test for anti-*B. burgdorferi* IgG antibodies in serum from vaccinated mice after challenge, by immunoblot. We considered a pattern of five out of ten bands positive (93 kDa, 66 kDa, 58 kDa, 45 kDa, 41 kDa, 39 kDa, 30 kDa, 28 kDa, 23 kDa, 18 kDa) as evidence of infection. Because we immunized mice with the OspA vaccine, we considered the presence of a single OspA band (~30 kDa) to be indicative of protection. The LA2 equivalency assay was performed as described [25] using 300 ng/ml biotinylated mAb LA2.2 and neutravidin, alkaline phosphatase conjugated, at 1:1000 (Pierce, Rockford, IL).

B. burgdorferi Culture: The heart and bladder were individually cultured in BSKH medium with and without antibiotic mixture for Borrelia (Sigma) for up to 6 weeks at 34 °C. Cultures were checked every week and positive cultures were frozen in 10% glycerol.

PCR from culture, mouse tissue and from ticks: Single round PCR was used to test cultures. Bladder, heart and ear tissue was weighed (<25 mg) and DNA extraction was performed using the DNAeasy tissue kit (Qiagen) according to the manufacturer's instructions. PCR from tissue and ticks was performed in blinded samples at least in two independent experiments by semi-nested PCR according to published protocol [7].

2.6. Statistics

McNemar's exact test for correlated proportions [26] was used. *p* values <0.05 are considered statistically significant. This test evaluates whether discordant results are evenly distributed or biased one way or the other.

3. Results

3.1. The lyophilized vaccine preserves its structural integrity and cell viability

To evaluate the structural integrity of the lyophilized antigen we compared the protein extracts of the lyophilized culture with the freshly induced culture by Coomassie blue staining of SDS-Page (Fig. 1A) and by immunoblot using a panel of anti-OspA monoclonal antibodies: 184.1, LA2.2 and 336.1 (Fig. 1B–D, respectively). MAb 184.1 binds to the N-terminus of OspA in a region centered around amino acid 61 (aa61) [27]; MAb LA2.2 defines an important protective B cell epitope of OspA [28,29] and binds to aa143–273 [30], and finally MAb 336.1, binds with low avidity to the most C-terminus alpha-helix of OspA [31]. Because all monoclonal antibodies bound to lyophilized extract with the same avidity as to purified OspA and to freshly induced extract,
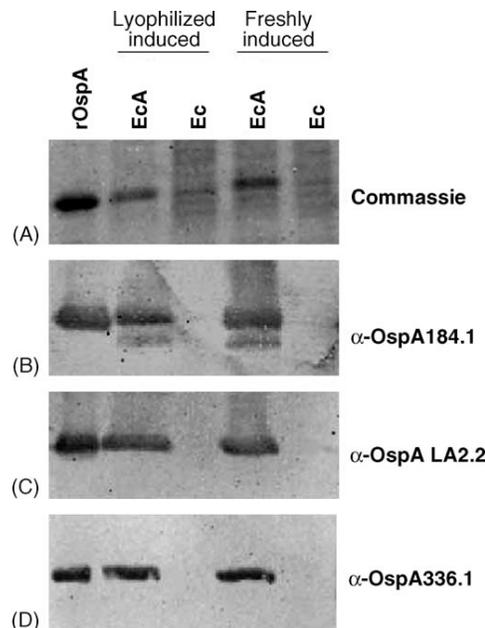

Fig. 1. The lyophilized oral vaccine displays the same antigenic determinants as the purified vaccine control. The protein extract obtained from lyophilized induced *E. coli* expressing OspA (EcA) was compared to freshly induced culture and to ~300 ng of purified recombinant OspA (rOspA). Controls included lyophilized and freshly induced *E. coli* lacking OspA (Ec). (A) Coomassie Blue staining of SDS-Page. (B)–(D) Western Blot analysis against anti-OspA monoclonal antibodies 184.1, LA2.2 and 336.1, respectively.

we conclude that the process of lyophilization was undisruptive to the OspA protein. In addition, we determined the cell viability of the lyophilized EcA vaccine antigen at $OD_{600} = 1$ as 5×10^4 colony forming units per ml (CFU/ml) compared to 10^5 CFU/ml of freshly induced culture. The viability of the lyophilized control at $OD_{600} = 1$ was determined as 4×10^7 CFU/ml as compared to 10^8 CFU/ml of freshly induced culture.

3.2. Oral immunization with lyophilized vaccine results in anti-OspA IgG seroconversion and OspA-specifc IgG subclass distribution is skewed toward IgG2a/2b

To determine the protective effect of the vaccine delivered via the oral route we characterized the anti-OspA systemic immune response in vitro by testing serum titer levels of total IgG and its subclasses by ELISA.

We first determined the intensity of the anti-OspA systemic immune response by testing levels of total IgG in serum diluted at 1:500 (Fig. 2A). Mice vaccinated by oral gavage with lyophilized *E. coli* expressing OspA (EcA) had detectable anti-OspA IgG antibody as early as 2 weeks post immunization. This response increased dramatically from d45 to d64, when it started to decline due to lack of boosting, but at termination (d97), the IgG response to OspA was still considerably high. Compared to OspA immunization via the parenteral route where anti-OspA titers are readily detected
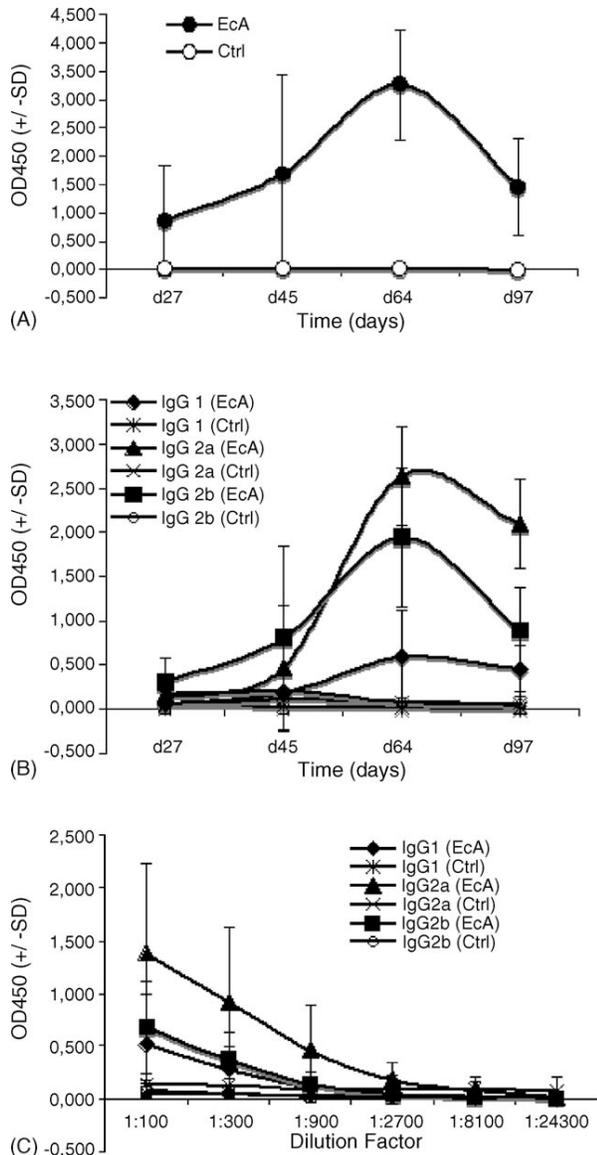
Fig. 2. Oral gavage immunization induces a systemic anti-OspA IgG response with an IgG subclass distribution skewed toward IgG2a/2b. To determine the total level of anti-OspA IgG we tested serum harvested from mice immunized by oral gavage, diluted at 1:500, against purified recombinant OspA coated on ELISA microwells (A). Alkaline phosphatase labeled secondary antibody was used. The average of three OD_{405} values of eight mice per group is represented and standard deviations have been determined. Next, we determined the OspA-specific antibody isotype distribution by testing sera collected on days d27, d45, d64, and d97 diluted at 1:100 (B) and the IgG isotype titer was determined by testing three-fold serial dilutions of sera collected at termination, d97 (C). Horseradish peroxidase labeled secondary antibody was used. The average of three OD_{450} values of six mice per group is represented and standard deviations have been determined.

at ∼1:25,000 [32], the intensity of the systemic immune response to OspA induced by the oral route was about one order of magnitude lower (∼1:2500). No anti-OspA IgG titer was detected in mice that were inoculated with control *E. coli* that did not express the OspA antigen.

Then, we determined the OspA-specific antibody isotype distribution by testing sera (1:100) collected on days 27, 45, 64, and 97 (Fig. 2B) and the isotype titer was determined by testing three-fold serial dilutions of sera collected at termination, d97 (Fig. 2C). In this assay, we included sera from mice that had total IgG response to OspA throughout the entire immunization schedule. We observed that in contrast to immunizations via the parenteral route, in which IgG1 is the most detected IgG subclass [32], oral immunization with *E. coli* expressing OspA, resulted in an OspA-specifc IgG subclass distribution skewed toward IgG2a immediately followed by IgG2b. The lowest subclass detected was IgG1. These results indicate that the oral administration of our lyophilized vaccine elicited a sustainable anti-OspA systemic immune response (IgG) in mouse serum for over 2 months (70 days) and that immune response was skewed towards IgG2a/2b production.

3.3. OspA-specific LA2 concentration is directly proportional to vaccine dose

To determine the optimal bait vaccine dose we administered several concentrations of lyophilized vaccine antigen (EcA) and tested the sera harvested at days 28, 45, and 60, from two mice per group (Fig. 3A). Here, we established that a concentration ranging from 50 mg to 100 mg of lyophilized EcA is the optimal vaccine dose to be administered via oral bait, which is about 10–20-fold higher than the concentration effective for oral gavage (∼5 mg/dose).

Next, we determined the LA2 equivalency in serum from mice subjected to oral gavage versus oral bait immunization with lyophilized EcA (Fig. 3B and C). This assay is an in vitro clinical correlate of a protective anti-OspA response and it defines the ability of serum from immunized mice to competitively block binding of OspA-specific LA2 to OspA [25]. We verified that an increase of vaccine dose led to an increase of OspA specific LA-2 antibody, in mice immunized with EcA via oral bait (Fig. 3B) as well as in mice immunized via oral gavage (data not shown). We conclude that the protective antibody response is directly proportional to the vaccine dose administered. When the optimized oral immunization protocols are compared, we show that oral bait immunized mice generated a higher concentration of LA2 equivalent antibodies than our comparison group, mice immunized via oral gavage (Fig. 3C). This data indicates that a much higher concentration of antigen was needed to induce a protective level of anti-OspA antibodies via oral bait immunization.

3.4. OspA antibody response results in protection from B. burgdorferi infection

Next, we wanted to determine if the systemic anti-OspA immune response elicited by our oral vaccine could protect mice from *B. burgdorferi* infection in vivo. We used two protocols to challenge mice with *B. burgdorferi:* by needle inoculation with an infectious strain of *B. burgdorferi* or by
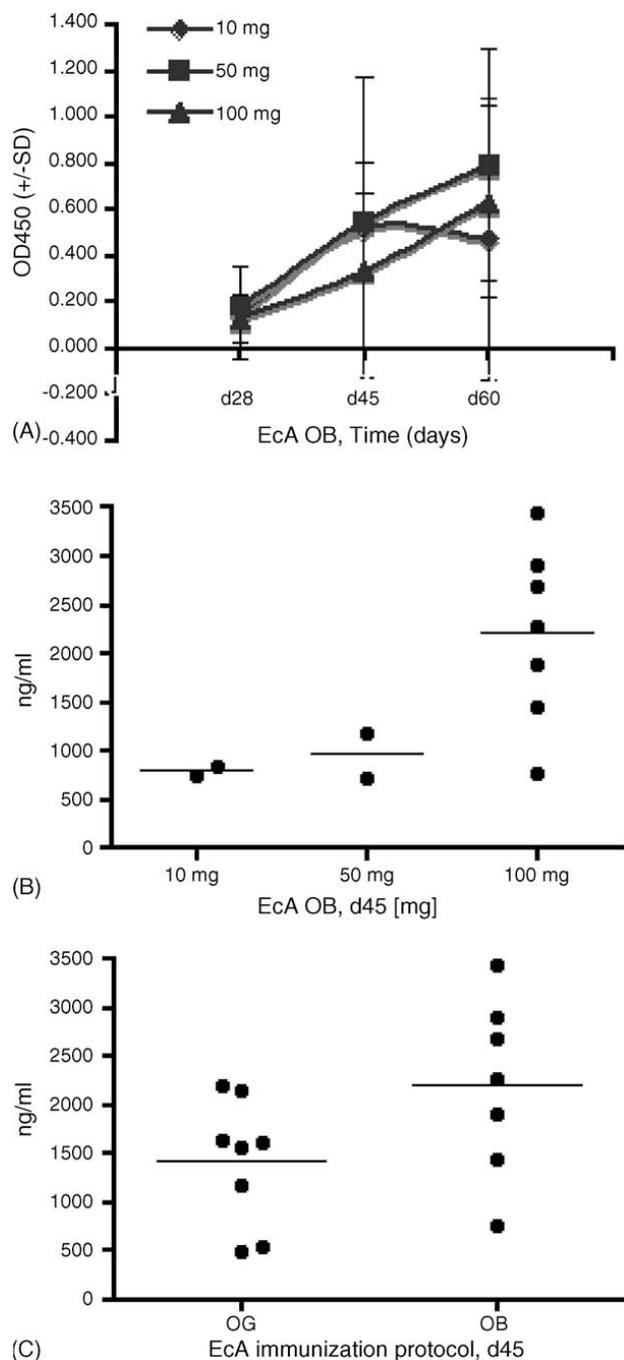
Fig. 3. LA2 equivalent antibody production is directly proportional to the vaccine dose administered. The optimal bait vaccine dose was determined by feeding mice with 10 mg, 50 mg or 100 mg of lyophilized vaccine antigen (EcA) in peanut butter. Total anti-OspA IgG was determined in sera harvested at days 28, 45, and 60 by ELISA (A). Horseradish peroxidase labeled secondary antibody was used. The average of three OD$_{450}$ values of two mice per group is represented and standard deviations have been determined. To determine protective antibody levels the "LA2 equivalency test" was done with serum from mice immunized with several concentrations of vaccine per dose by oral bait in oatmeal, 10 mg ($n=2$), 50 mg ($n=2$), 100 mg ($n=7$) (B). OspA-specific LA2 antibody concentration was compared in serum from mice immunized by our oral gavage protocol (OG, 5 mg EcA, $n=8$) and by our newly devised oral bait protocol containing oatmeal (OB, 100 mg EcA, $n=7$) (C). Serum harvested at day 45 from mice presenting an immune response to OspA was used (1:20) and LA2 equivalency was detected with neutravidin, alkaline phosphatase conjugated (Pierce). Legend: OB, oral bait; OG, oral gavage; EcA, lyophilized E. coli expressing OspA.

infestation with *I. scapularis* nymphs carrying multiple *B. burgdorferi* strains. Infection or protection was determined by the presence or absence of *B. burgdorferi* dissemination, respectively. We monitored the anti-OspA systemic immune response by determination of total IgG by ELISA. Immediately after challenge the probability of exposure to *B. burgdorferi* via *I. scapularis* nymphal infestation was determined. One month after challenge, spirochete dissemination in vaccinated and control mice was detected by immunoblotting serum against whole cell sonicate of *B. burgdorferi*, by culture of *B. burgdorferi* from infected heart and bladder tissues and by PCR amplification of *B. burgdorferi* ospC DNA from cultures and from heart, bladder and ear tissues. The primers used for OspC-PCR bind to the flanking conserved regions of *ospC*. These have been used to determine and characterize the genetic diversity of *ospC* within populations of *B. burgdorferi* isolated from the tick vector and from several hosts. Based on the broad sequence divergence determined with these primers [33–35,7] we believe that we can amplify any *B. burgdorferi* strain.

For the first spirochete challenge protocol, eight mice (EcA, $n=4$ and control, $n=4$) were immunized via oral gavage with lyophilized vaccine, or the parental *E. coli* strain, and challenged via needle inoculation with an infectious *B. burgdorferi* strain isolated from skin biopsy obtained from a patient with Lyme disease. Data is summarized in Table 1. We achieved 75% protection in the group of mice vaccinated orally with EcA in contrast with the control group that was inoculated with lyophilized *E. coli* lacking OspA in which there was no protection.

For the second spirochete challenge protocol, 18 mice were immunized via oral gavage with lyophilized vaccine, or the parental *E. coli* strain (EcA, $n=9$ and control, $n=9$) and challenged with *B. burgdorferi* via field-tick feeding. Field ticks represent a more authentic challenge to the immunized mice than needle inoculation because they harbor an array of infectious and non-infectious strains of *B. burgdorferi* [36–38,12] that the vertebrate host of the spirochete would be exposed to in the wild.

Because we cannot quantify the amount of *B. burgdorferi* transmitted by tick challenge, the probability of exposure to *B. burgdorferi* via *I. scapularis* nymphal infestation was determined immediately after challenge. The probability that a mouse was not exposed to *B. burgdorferi* by any of the ticks that were placed on it was calculated as follows: $(1-NIP)^{NR}$, where NIP is the observed infection prevalence of the nymphs [39], estimated at 85% in this study, and NR is the number of engorged ticks that were rescued after naturally falling off. We calculated that the probability of a mouse not being exposed to *B. burgdorferi* via nymphal infestation is extremely low (10^{-2} to 10^{-8}). This data strongly indicates that both groups of mice, orally inoculated with either the vaccine or with control, were exposed to *B. burgdorferi* via tick challenge.

One month after challenge, we euthanized mice and collected blood to test for the presence of anti-OspA or anti-*B.*

Table 1
Anti-OspA IgG response protects mice from *B. burgdorferi* infection

Challg	Immunoblot		Culture			Tissue PCR		
	Pos/Ttl	%(I/P)	Pos/Ttl		% (I/P)	Pos/Ttl		% (I/P)
			Heart DFM:PCR	Blad DFM:PCR		Heart:Blad	Ear	
EcA								
Needle	1/4		1:1/4				0/4	
Tick	1/9		0:0/9	0:1/9		0:1/9	0/4	
Total	2/13	15/85	1:1/13	0:1/9	8–11/92–89	0:1/9	0/8	11–0/89–100
Ctrl								
Needle	4/4		4:4/4				4/4	
Tick	8/9		8:8/9	3:8/9		9:8/9	2/4	
Total	12/13	92/8	12:12/13	3:8/9	92–89/8–11	9:8/9	6/8	100–75/0–25
p	<0.05		<0.05:<0.05	>0.05:<0.05		<0.05:<0.05	<0.05	

Lyophilized *E. coli* expressing OspA (EcA); lyophilized *E. coli* lacking OspA (Ctrl); Pos/Ttl, number of positive samples/total; % (I/P), % infected/protected; blad, bladder; challg, challenge; DFM, dark field microscopy; *p*-value determined between vaccine and control groups by McNemar exact test for correlated proportions.

burgdorferi antibodies, and tissues to assess for *B. burgdorferi* dissemination (Figs. 4 and 5, respectively). The anti-OspA total IgG titer was determined in serum diluted at 1:2500 on ELISA and it was correlated with the immunoblot result obtained for the same sample (Fig. 4). In the group orally immunized with lyophilized EcA we observed that 8 of 9 sera and cultures were free of spirochetes, as determined by immunoblot and culture of *B. burgdorferi* from tissues confirmed by PCR, indicating 89% protection (Fig. 5). The mouse that did not seroconvert to OspA, displayed an immunoblot profile indicative of *B. burgdorferi* dissemination and spirochete DNA was amplified from bladder culture and tissue. However, *B. burgdorferi* was not successfully cultured from either heart or bladder in this case. In the control group, eight of nine mice had an immunoblot profile indicative of *B. burgdorferi* dissemination confirmed by *B. burgdorferi* culture and PCR. Furthermore, we amplified *B. burgdorferi* DNA from heart tissue harvested from the mouse that developed negative serologic assay or cultures and therefore we presume 0% protection in the control group. Even though an immune response against a wildtype *B. burgdorferi* strain was not detected in one of the control mice, that strain disseminated to the heart. It is well documented that *B. burgdorferi* strains disseminate differently in humans [34,40–43] and in other vertebrate hosts [7]. This data indicates that a series of techniques is necessary to assess *B. burgdorferi* dissemination and determine vaccine efficacy or *B. burgdorferi* tissue dissemination accurately. These results show that the *B. burgdorferi* strains harbored by the ticks were able to disseminate through mouse tissues upon transmission, indicating that the field-ticks used in the challenge were colonized by infectious *B. burgdorferi* strains. The differences observed between the oral vaccine groups and the controls were statistically significant ($p < 0.05$) by McNemar's exact test for correlated proportions.
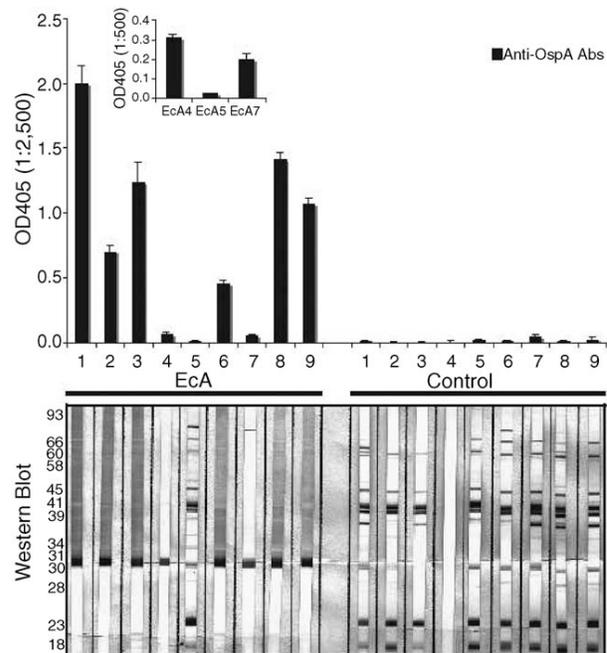

Fig. 4. ELISA and immunoblot serology 1 month after tick challenge (d97) correlates with protection. Mice were inoculated by gavage with lyophilized vaccine and sera tested at d97, 1 month after tick challenge. ELISA: mouse serum was tested at different dilutions (1:500 and 1:2500) against purified recombinant OspA. The average of three OD$_{405}$ values for each sample is represented and standard deviations have been determined. Immunoblot: the MarDx test was used. The strips have been aligned with the ELISA titer for the same serum sample. We considered a pattern of 5/10 positive bands (93 kDa, 66 kDa, 58 kDa, 45 kDa, 41 kDa, 39 kDa, 30 kDa, 28 kDa, 23 kDa, 18 kDa) as evidence of infection. Inset graphic: to fine tune anti-OspA antibody titer, we re-tested the sera presenting low anti-OspA antibodies (EcA 4, 5, 7) at a lower dilution (1:500). Legend: EcA, lyophilized *E. coli* expressing OspA; control, lyophilized *E. coli* lacking OspA; Abs, antibodies.
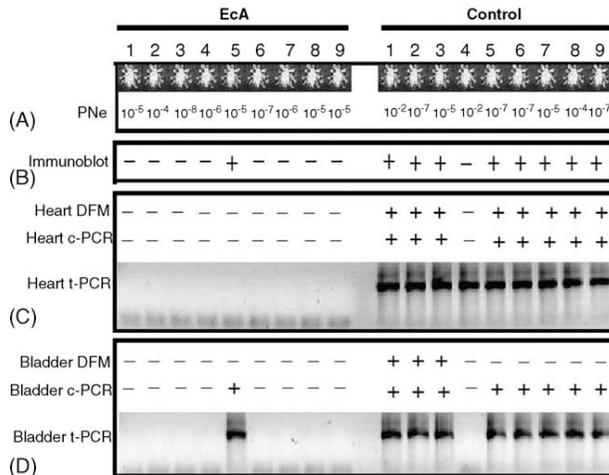
Fig. 5. *B. burgdorferi* does not disseminate in orally immunized mice. We compared the probability of non-exposure to *B. burgdorferi* through nymphal infestation obtained immediately after tick challenge (A), with the immunoblot, heart and bladder culture results of tissues harvested 1 month after challenge ((B)–(D), respectively). Culture results were determined by dark field microscopy confirmed by PCR (OspC, 594 bp). PCR was performed twice in blinded samples of the culture and tissue independently. Legend: PNe, probability of non-exposure to *B. burgdorferi* through nymphal infestation, $PNe = (1 - NIP)^{NR}$, where NIP is the observed infection prevalence of the nymphs used for tick challenge (85%) and NR is the number of engorged nymphs that were rescued; EcA, lyophilized *E. coli* expressing OspA; control, lyophilized *E. coli* lacking OspA; DFM, dark field microscopy; c-PCR, culture PCR; t-PCR, tissue PCR.

Taken together, these results suggest that oral immunization with our lyophilized vaccine resulted in an anti-OspA systemic seroconversion for a period superior to 2 months with an IgG subclass skewed towards IgG2a/2b production. In addition, using the LA2 equivalency test, we show that higher levels of protective antibody correlate with higher vaccine doses. This systemic immune response protects vaccinated mice from *B. burgdorferi* infection by needle challenge, and more importantly, by feeding of *I. scapularis* field ticks with 89% efficacy (data summarized in Table 1 and Fig. 6)
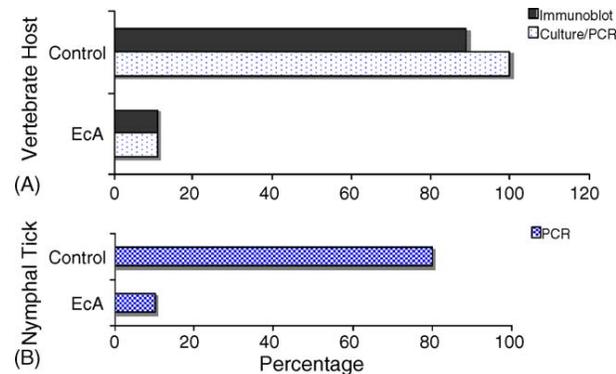

Fig. 6. Oral vaccine protects the vertebrate host from *B. burgdorferi* infection (A) and clears *B. burgdorferi* from the infected tick vector (B). Legend: EcA, lyophilized *E. coli* expressing OspA; control, lyophilized *E. coli* lacking OspA.

and high anti-OspA antibody titer does not seam an important determinant of protection. Moreover, the lyophilized OspA vaccine cleared a spectrum of *B. burgdorferi* strains that are present in field ticks, a necessary component of a bait vaccine that would be used to prevent transmission of the spirochete in wild mouse populations.

3.5. *B. burgdorferi* load is drastically reduced in ticks that fed on orally vaccinated mice

Ticks retrieved from the wild, such as the ticks used in this study, are infected with multiple *B. burgdorferi* strains [36–38,12]. The prevalence of infection of *I. scapularis* ticks used in this study was determined by PCR from 20 nymphs that took their larval bloodmeal from feral white-footed mice in the summer of 2003, all of which were infected [7]. These larvae were allowed to molt to the nymphal stage and these nymphs were then tested for prevalence of infection and used in our challenge studies. Seventeen of the 20 nymphs (85%) tested positive for *B. burgdorferi* by ospC-PCR. We tested engorged ticks recovered after feeding on orally immunized and control mice. Eight of ten ticks (80%) recovered from control mice tested positive for *B. burgdorferi* by PCR. Of the ticks that fed on EcA immunized mice, only one of ten (1/10), tested positive for *B. burgdorferi* DNA. This reduction from 85% prevalence of *B. burgdorferi* infection to 10% indicates that OspA antibodies drastically reduced *B. burgdorferi* infection from ticks. These results suggest that our oral vaccine not only protected the immunized vertebrate host from *B. burgdorferi* infection, with a decrease from 100% to 10% infection in control versus orally immunized mice, but also cleared a variety *B. burgdorferi* strains from the nymphal tick vector, where we observed a decrease from 85% to 10% prevalence of infection (Fig. 6), another important feature of a field vaccine to abrogate pathogen transmission in the wild.

4. Discussion

We developed a system to deliver a vaccine to a vertebrate host carrier of a zoonotic pathogen, i.e. *B. burgdorferi*, via the oral route. We used lyophilized *E. coli* expressing OspA in liquid form (gavage) or in dry form (bait) as a vehicle for immunogen delivery to test our hypothesis against *B. burgdorferi* on a Lyme disease model. This oral vaccine raised the production of IgG2a and IgG2b antibody isotypes that not only prevented infection of the vertebrate host upon tick challenge, as it cleared a variety of *B. burgdorferi* strains from the infected vector thus abrogating transmission of this vector-borne pathogen. Here, we show for the first time, that a vaccine proven to prevent transmission of the vector-borne pathogen *B. burgdorferi* can be delivered via oral bait. Thus, our baiting system is an exciting candidate to distribute the vaccine in the wild to prevent zoonotic transmission of *B. burgdorferi*. If effective after field studies, this vaccination strategy may prove useful as a new way to block trans-

mission of vector-borne pathogens of other zoonotic human diseases.

Many of the most important reservoir hosts for *B. burgdorferi*, such as the white-footed mice live less than 1 year, and no host can pass *B. burgdorferi* to its offspring transplacentally [44]. The incidence of Lyme disease in humans correlates with the prevalence of *B. burgdorferi* infection of the tick vector, *I. scapularis* [45–48]. The white-footed mouse and the short-tailed shrew are two of the vertebrate host species that carry the highest *I. scapularis* larval burden, with the white-footed mouse carrying the highest burden of infected larvae [39,7]. In addition, the white-footed mouse is the only host infected by *B. burgdorferi* carrying all four of the *ospC* Major Groups (oMGs) previously isolated from secondary disseminated sites of Lyme disease infection [34,7]. Recently, it has been demonstrated that subcutaneous vaccination of wildlife reservoirs against *B. burgdorferi* reduced vector infection prevalence [23]. Thus, vaccines aimed at preventing vertebrate host infection and at reducing the vector infection prevalence with *B. burgdorferi,* should be targeted to the vertebrate host, such as mice, in a format that can be distributed to wildlife via oral bait. Distribution of an anti-*B. burgdorferi* vaccine via the oral route allows us to target the treatment to multiple vertebrate host reservoirs if others are proven important carriers of *B. burgdorferi*.

An effective anti-*B. burgdorferi* wildlife vaccine needs only to raise an immune response in the reservoir host during the larval host-seeking period, which is about 2 months in the northeastern Unites States [23]. In this study, we demonstrate that mice started developing an anti-OspA IgG immune response 2 weeks after immunization and that the antibody titer was sustained for about two and a half months. Our results indicate that this oral vaccine raised a systemic immune response for a period of time superior to the larval host-seeking season and therefore it could potentially break the mouse-tick transmission cycle in the wild within several years.

In mice, IgG2a and IgG2b which are induced by Th1 cytokines, such as IFNg, activate complement and bind Fc receptors that in turn promotes phagocytosis and antibody dependent cytotoxicity [49,50], while IgG1 which is induced by Th2 cytokines, such as IL4, does not bind complement. Subcutaneous immunization with OspA induces primarily the IgG1 subclass [51]. In contrast, IgG2a and IgG2b are the IgG subclasses primarily induced by our OspA oral vaccine. It has been proven that in OspA vaccinated mice, an intact complement system in the host was not required to kill spirochetes within feeding nymphs. However, the acquisition of *B. burgdorferi* by larval ticks was considerably impaired in mice vaccinated with OspA [52]. These results suggest that an anti-OspA antibody response skewed toward IgG2a/2b production will not increase *B. burgdorferi* killing within the feeding nymphal tick via complement-dependent antibody-mediated lysis. On the other hand, because complement plays a role in the OspA antibody-mediated block in larval acquisition of spirochetes, an OspA antibody response skewed toward

IgG2a/2b production will be beneficial in the overall strategy to reduce Lyme disease incidence via decrease of *B. burgdorferi* prevalence from larval ticks. However, we should stress that different inbred mice, such as C3H and B6, can produce different IgG subclass antibody responses to *B. burgdorferi* [53] and therefore wild-type mice may not produce the same subset of IgG antibody responses after administration of the oral vaccine.

Using the "LA2 equivalency test" [25], which defines protective levels of antibodies produced in response to a vaccine antigen, we verified that mice vaccinated by oral bait needed to receive a higher dose of vaccine to generate a slightly higher concentration of LA-2 equivalent antibodies compared to our oral gavage protocol. In addition, we determined that protective serologic antibody concentration is directly proportional to vaccine dose.

It is reasonable to expect that some wild vertebrate *B. burgdorferi* reservoirs will take fewer than the two immunizations needed to generate high anti-OspA antibody titers given that only about 60% of mice return to feeding or capture stations [11]. However, given the efficient low antibody titer raised by our oral vaccine, we conclude that even mice that take only one immunization are likely to be protected which further raises the potential effectiveness of the vaccine in the field.

Lastly, in *I. scapularis* nymphs, we estimated a reduction from 85% prevalence of infection to 10% after the feeding on immunized mice. Because we used field ticks that carry multiple *B. burgdorferi* strains [36–38,7] to challenge immunized mice our results demonstrate that the majority of the wild-type strains were neutralized and cleared by OspA antibodies within the tick. Hence, this vaccine, if delivered properly, could prove effective in the wild.

In the studies reported here, the oral administration of a lyophilized vaccine based in OspA prevented the zoonotic transmission of *B. burgdorferi*, from the tick vector to the vertebrate host, and cleared a variety of *B. burgdorferi* strains from the infected tick. Therefore, these data support the hypothesis that an oral bait vaccine could drastically reduce *B. burgdorferi* prevalence from its major wildlife reservoirs and from *I. scapularis* ticks. Because it is lyophilized, this vaccine can be produced in large quantities and could be distributed to a broad range of *B. burgdorferi* reservoir hosts in the wild.

We are now in the process of testing this oral bait vaccine in the field so that it can be distributed to a range of wildlife reservoirs including *Peromyscus leucopus* and other ground dwelling mammal species and birds. Such broadcast delivery of an oral vaccine could enhance its potential to disrupt the transmission cycle of this pathogen. We are aware that introducing drug resistant *E. coli* into the environment might be cause for some concern. However, we used a non-pathogenic *E. coli* strain extensively studied in the laboratory that is sensitive to other commonly used antibiotics. If the field studies turn out to be successful we will explore alternative methods of selecting for *ospA* in *E. coli*. Given the current problems

in developing a safe Lyme disease vaccine for human use, this study provides the proof-of-concept for the development of a non-pesticide way of arresting *B. burgdorferi* transmission in wildlife reservoirs in endemic areas for this disease. This could be an inexpensive and efficient way to control the disease in reservoir hosts without the use of acaricides or wildlife culling.

Acknowledgements

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