

Multicomponent Lyme vaccine: Three is not a crowd

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Abstract

Lyme disease is caused by the spirochete *Borrelia burgdorferi* and it is the most common vector-borne disease in the United States. Disseminated spirochetes can persist in various tissues and can result in a variety of different disease manifestations. Vaccination trials testing various lipoprotein candidates have yielded mixed results despite the generation of robust antibody titers. Data presented in this report demonstrate that a combination vaccine composed of DbpA, BBK32 and OspC is more effective than single or double component formulations and that the ratio of each component dramatically impacts vaccine efficacy when tested in protection experiments against *Borrelia* following needle inoculation.

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1. Introduction

Lyme disease is caused by the spirochete *Borrelia burgdorferi* sensu lato; a family of organisms that includes *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*. The spirochetes are transmitted to humans during the blood meal of infected ticks [1,2]. During a blood meal, an infected tick will deposit the bacteria in the dermis of the mammalian host, and in a process that can take several weeks, the bacteria subsequently disseminate throughout the body of an infected individual. The severity of the disease can vary substantially following dissemination. Symptoms can be multisystemic or localized to specific tissues, and Lyme disease is often difficult to diagnose [1,3,4]. Late stages of Lyme disease can include neurological, ocular, cutaneous, and cardiac disease in addition to arthritis [1]. Early antibiotic therapy is often

an effective treatment of Lyme disease, however, up to 10% of affected individuals do not respond to antibiotic therapy and are classified as treatment resistant patients [3,5]. In the absence of antibiotic intervention, disseminated *B. burgdorferi* can persist in an individual for months if not years even in the face of an apparently strong immune response against the spirochete.

Despite a plethora of highly antigenic and apparently immunologically accessible *Borrelia* surface proteins [6–18], the only vaccine formulation tested and approved for human use was derived from the outer surface protein A (OspA) [19]. This formulation (LYMERix), however, has been removed from the market (Spring, 2002) primarily because of low sales resulting from claims alleging that LYMERix alone induced arthritis in vaccinated patients uninfected with the Lyme spirochete [20–23].

Although OspA-based vaccines have been the focus of many immunization trials [11,15,24–27], various other lipoproteins have also been tested as vaccine candidates against *B. burgdorferi* infection with varying results [10,28–40]. Table 1 describes various active or passive vaccination trials using either the decorin binding protein (DbpA) [9,41], the fibronectin-binding protein (BBK32) [42,43], or

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Table 1
Summary of DbpA, BBK32, or OspC vaccination trials

Vaccinogen	Strain source ^a	Adjuvant ^b	Immunization	Mouse strain	Infection type ^c		Protection	B/T ^d	Protection ^e (%)	Reference
rDbpA ^f (50 µg)	297	CFA/IFA	2×	C3H/HeN	Tick (10) ⁱ	297	No	11/12	8 (2 weeks)	[33]
rDbpA ^f (50 µg)	297	CFA/IFA	2×	C3H/HeN	Needle (10 ³)	297	Yes	2/9	78 (2 weeks)	[34]
rDbpA ^f (20 µg)	297	CFA/IFA	1×	C3H/Hen	Needle (10 ⁴)	297	Yes	0/5	100 (2 weeks)	[32]
lDbpA ^f (20 µg)	297	CFA/IFA	1×	C3H/Hen	Needle (10 ⁴)	297	Yes	0/5	100 (2 weeks)	[32]
lDbpA ^f (20 µg)	N40	CFA/IFA	1×	C3H/Hen	Needle (10 ⁴)	N40	Yes	0/5	100 (2 weeks)	[32]
PreDbpA ^f (20 µg)	297	CFA/IFA	1×	BALB/c	Needle (10 ⁴)	B31	Yes	0/5	100 (2 weeks)	[32]
PreDbpA ^f (20 µg)	297	CFA/IFA	1×	C3H/Hen	Needle (10 ⁴)	B31	No	13/15	13 (2 weeks)	[32]
lDbpA ^f (20 µg)	297	CFA/IFA	1×	C3H/Hen	Needle (10 ⁴)	B31	No	5/5	0 (2 weeks)	[32]
rDbpA ^f (20 µg)	297	CFA/IFA	1×	C3H/Hen	Needle (10 ⁴)	B31	Yes	1/5	80 (2 weeks)	[32]
rDbpA ^f (20 µg)	B31	CFA/IFA	1×	C3H/Hen	Needle (10 ⁴)	B31	Yes	1/4	75 (2 weeks)	[32]
DbpA antiserum	297	None	1×	C3H/Hen	Needle (10 ⁴)	B31	Yes	0/6	100 (2 weeks)	[32]
rBBK32 ^f (10 µg)	N40	CFA/IFA	2×	C3H/Hen	Needle (10 ⁴)	N40	No	5/9	44 (2 weeks)	[39]
BBK32 antiserum	N40	None	1×	C3H/Hen	Needle (10 ⁴)	N40	No	4/5	20 (2 weeks)	[39]
BBK32 antiserum	N40	None	1×	C3H/Hen-C.B.-17- <i>scid</i>	Tick (20) ⁱ	N40	No	9/10	10 (2 weeks)	[28]
DNA-OspC (100 µg)	ZS7	±CpG ^h	Multiple	C3H/HeJ	Needle (10 ⁴)	ZS7	Yes	0/20	100 (12 weeks)	[37]
DNA-OspC (2 µg)	ZS7	±CpG ^h -Gene-gun	Multiple	C3H/HeJ	Needle (10 ⁴)	ZS7	Yes	2/19	89 (12 weeks)	[37]
rOspC ^f (5 µg)	ZS7	±CpG ^h	Multiple	C3H/HeJ	Needle (10 ⁴)	ZS7	Yes	0/20	100 (12 weeks)	[37]
OspC antiserum	ZS7	None	1×	AKR/N-C.B.-17- <i>scid</i>	Needle (10 ³)	ZS7	Yes	0/5	100 (40 days)	[38]
rOspC ^f (100 µg)	B31	Alum	1×	Outbred mice	Tick (10) ⁱ	B31	No	11/12	8 (4 weeks)	[30]
rOspC ^f (undefined ^g)	B31	TiterMax [®]	2×	Outbred mice	Tick (10) ⁱ	B31	Yes	0/12	100 (4 weeks)	[10]
OspC antiserum	B31	None	1×	Outbred/C3HeN	Tick (10) ⁱ	B31	Yes	0/12	100 (4 weeks)	[35]

^a Strain from which vaccinogen was derived.

^b CFA/IFA, complete Freund's adjuvant/incomplete Freund's adjuvant.

^c Mice were infected either by needle inoculation or by allowing *B. burgdorferi*-infected ticks to feed to repletion. Strain used for infection indicated.

^d Borrelia-positive cultures/total cultures examined.

^e Percent Borrelia-positive cultures identified at the indicated time post infection (parenthesis).

^f r, recombinant His-tag protein; Pre, recombinant His-tag protein containing leader sequence; l, lipidated His-tag recombinant protein.

^g *E. coli* containing the appropriate vector were induced to express rOspC, lysed and then emulsified with TiterMax[®] prior to immunization.

^h CpG, oligodeoxynucleotides containing CpG-motifs (potent stimulators of cellular immunity) [37].

ⁱ Number of *B. burgdorferi*-infected ticks allowed to feed/mouse.

the outer surface protein (OspC) [44] (DBO) that yielded mixed results with respect to vaccine efficacy. The DBO components were chosen for several reasons: DbpA and BBK32, are *Borrelia* adhesins or MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules) [9,41–43,45] and OspC appears to be involved in spirochete migration from the tick midgut into the salivary glands during a blood meal [30,46,47]. Because MSCRAMMs like DbpA and BBK32 mediate bacterial adherence to the host's extracellular matrix (ECM) components (decorin and fibronectin, respectively), MSCRAMM-based vaccines have become attractive targets for vaccine design because anti-MSCRAMM antibodies can serve as conventional opsonins as well as inhibitors of bacterial adherence to host ECM components [48]. In addition, regions of MSCRAMMs critical for ligand and binding must remain conserved if protein function is to be maintained; therefore, protective antibodies generated against these regions should be more likely to protect against infections by heterologous *Borrelia* strains. All three of the selected proteins are highly antigenic and are significantly up-regulated during the transition between the tick vector and the mammalian host [49] and combination vaccines composed of DbpA/OspA or OspC/OspA have been tested, demonstrating increased vaccine efficacy compared to single component immunizations [31,50].

Humoral immunity has been long believed to be protective against *B. burgdorferi* infections [18,26,40,51,52], and numerous antigens can elicit high antibody titers without conferring protection, however, the reasons for this are unknown [10,29,30,32,40]. One explanation is the prozone effect [53], a phenomenon that describes high antibody titers but no protection or enhanced infection. Prozone effects have been observed primarily in passive vaccine studies for other pathogens such as *Cryptococcus neoformans*, *Brucella abortus*, *Pseudomonas aeruginosa*, *Escherichia coli* and *Varicella-Zoster* [54–58] and may be a reason for the lack of protection observed in the presence of anti-*Borrelia* antibodies directed to specific antigens (Table 1). Data presented in this report suggests that multicomponent formulations composed of recombinant forms of DbpA, BBK32, and OspC administered at defined concentrations are more effective than single or double component formulations of any combination.

2. Materials and methods

2.1. Mice

Specific pathogen-free (MTV–) BALB/c mice were obtained from an in-house BALB/c breeding colony. The animals were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care in accordance with current regulations and standards of the United States Department of Agriculture, Department of Health and Human Services, and National Institutes of

Table 2
Multicomponent vaccine formulations used

Designation	Composition	Component Concentrations (μg)
DbpA	–	20
BBK32	–	20
OspC	–	20
DbpA/BBK32	–	10/10
DbpA/OspC	–	10/10
BBK32/OspC	–	10/10
DBO.0	DbpA/BBK32/OspC	7/7/7
DBO1	DbpA/BBK32/OspC	14/8/8
DBO2	DbpA/BBK32/OspC	8/14/8
DBO1.1	DbpA/BBK32/OspC	14/14/14
DBO4	DbpA/BBK32/OspC	15/10/5
DBO6	DbpA/BBK32/OspC	10/15/5
DBO8	DbpA/BBK32/OspC	5/10/15

Health. All animal procedures were approved by the Institutional Animal Care and Use Committee. Female mice ages 8–10-week-old were used at the start of each experiment.

2.2. Bacterial strains, culture and materials

Low-passage *B. burgdorferi* strain B31 (passage 5) were used in this study and cultured in BSK II (Barbour-Stoenner-Kelly) medium at 34 °C [59]. Bacterial cultures were incubated in a CO₂-enriched atmosphere in a GasPak chamber (BBL, Baltimore, MD) containing BBL GasPak Plus envelopes and a GasPak anaerobic indicator (Beckton Dickinson, Cockeysville, MD) until the cells reached log phase. The density of bacteria was determined using dark field microscopy and a Petroff-Hausser chamber. *E. coli* strain JM101 (Qiagen, Chatsworth, CA) were grown at 37 °C in Lennox broth (LB) (Difco, Detroit, MI) [60], containing the appropriate antibiotics as described [61,62].

2.3. Recombinant protein vaccinations

Mice were immunized subcutaneously (100 μl) with DbpA, BBK32 or OspC or combinations thereof (Table 2) in complete Freund's adjuvant, with adjuvant alone, or left untreated (negative controls) as described previously [32]. Mice were boosted subcutaneously (100 μl) 4 weeks post primary immunization in incomplete Freund's adjuvant as described above and infected 2 weeks later. This vaccination schedule was adopted from Hanson et al. [32].

2.4. Expression and purification of recombinant proteins

Recombinant DbpA (strain 297) [9] and BBK32 (strain B31) [43] were expressed in *E. coli* (JM101) harboring the corresponding plasmid as recombinant His-tag proteins as described previously. *OspC* (strain B31) was amplified by polymerase chain reaction (PCR) using *B. burgdorferi* B31 DNA as template. The following oligonucleotide primers were used: 5'-GCG GGA TCC AAT AAT TCA GGG AAA GAT GGG AAT-3' forward primer and 5'-GCG CTG CAG

TCA TTT TTT TGG ACT TTC TGC CAC AAC-3' reverse primer containing a *Bam*HI and *Pst*I restriction site (underlined), respectively. The resulting PCR amplifications were ligated with plasmid pQE-30 (Qiagen) and transformed by heat shock into competent *E. coli* JM101 (Qiagen) cells. Recombinant OspC was expressed and purified as described previously [9,61]. Protein concentrations were determined by the Bicinchoninic Acid (BCA) Protein Assay (Pierce, Rockford, IL) and stored at -20°C until use.

2.5. *Borrelia* infections

Needle inoculation was performed by injecting 10^4 *B. burgdorferi* strain B31 passage 5 (100 μl volume) i.d. into shaved dorsal skin at the base of the tail 2 weeks after the last recombinant protein immunization. Mice were lightly anesthetized with IsoFlo[®] (isoflurane) (Abbott Laboratories, North Chicago, IL) during the infection procedure.

2.6. Culturing of tissues

Two weeks post infection; ear, heart, bladder and one joint devoid of skin were harvested under a laminar flow biosafety containment hood as described [63,64]. Tissues were cultured in BSK II media and incubated at 34°C . The cultures were checked for the presence of spirochetes 2 weeks post-culture inoculation as described previously [64].

2.7. Enzyme-linked immunosorbent assay (ELISA)-type binding assays and serum isotype analysis

Isotype-specific (see below) anti-*Borrelia* responses for mice immunized with D-B-O components or with DBO formulations were characterized as described previously [63,65]. Briefly, ELISA-type assays were performed using Immulon-1B microtiter plate wells (Dynatech Laboratories, Chantilly, VA) coated overnight at 4°C with 0.25 μg whole, inactivated *B. burgdorferi* B31 [63–65]. All incubations were carried out at 37°C except for the final step (addition of substrate). All reagents were diluted in Super Block (Pierce, Rockford, IL) with the exception of the substrate. Wells were washed five times between each incubation using 0.05% Tween 20 (Sigma Chemical Co., St. Louis, MO) in phosphate-buffered saline. The titer of the serum samples was previously determined to be 1:1000. After the overnight incubation, the wells were blocked with 200 μl of Super Block, washed, and then incubated with 100 μl of the serum samples. Triplicate serum dilutions were performed for each sample and then tested for the presence of each antibody class/subclass. Serum samples (1:1000 dilution) were incubated for 2 h, washed, and 100 μl of the respective biotin-conjugated rat anti-mouse IgG1, IgG2a, IgG2b and IgG3, IgE, IgA, or IgM (Pharmingen, San Diego, CA) (1:2000 dilution) were added to the corresponding wells for 30 min. After washing, 100 μl of avidin-AP (alkaline phos-

phatase) (1:5000) was added for 30 min, washed and then developed by adding 100 μl of a 1 mg/ml Sigma 104 phosphatase substrate (Sigma) dissolved in 1 M diethanolamine, 0.5 mM MgCl_2 , pH 9.8 for 1 h. Plates were read at 405 nm using a microplate reader (Molecular Devices, Menlo Park, CA) and the values are expressed as the mean absorbance \pm S.E.

3. Results

3.1. Single, double, and triple component vaccinations

The data presented in Fig. 1a–c represents pooled data (428 BALB/c mice total) collected from each vaccination trial involving single, double or triple vaccine component experiments, respectively. Since the infection only and complete Freund's adjuvant/incomplete Freund's adjuvant (CFA/IFA) groups were the only groups that remained constant throughout the course of the study, these groups have a significantly higher pooled number of mice (72 and 96 mice total for CFA/IFA and infection only groups, respectively) when compared to the individual vaccination groups. Single and double vaccine component experiments did not elicit significant

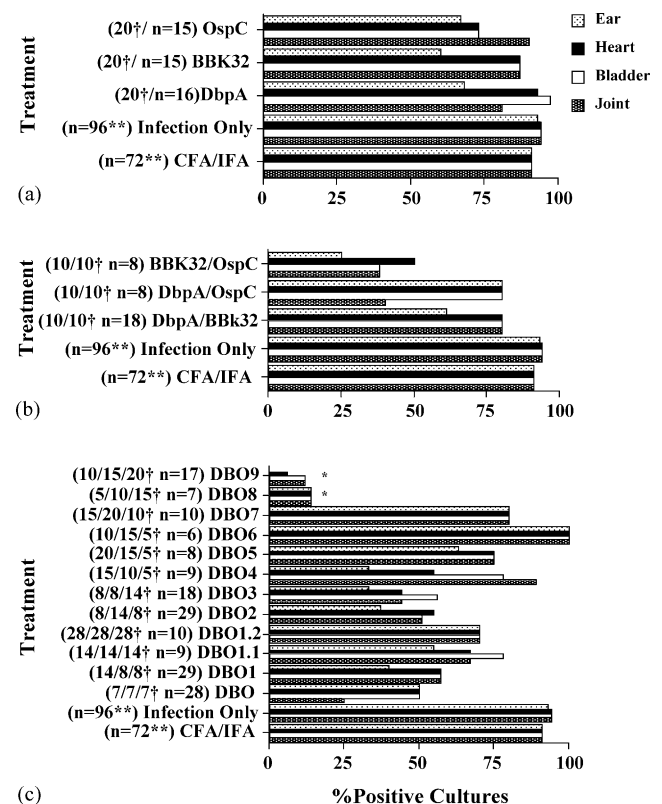


Fig. 1. Percent *Borrelia*-positive cultures following (a) single, (b) double or (c) triple component vaccinations. Tissues were harvested and cultured for the presence of *Borrelia* 2 weeks post infection. (**) Infection only and CFA/IFA groups were pooled from all experiments. (†) Concentration of DBO or individual components (Table 2). * $p < 0.0001$ Fisher's exact test vs. infection only or CFA/IFA control groups. The number of mice (n) used for each experiment in indicated in parenthesis.

protection against *Borrelia* infection (Fig. 1a and b) although the numbers of *Borrelia*-positive cultures were slightly decreased in double component-vaccinated mice (Fig. 1b) compared to single component-vaccinated mice (Fig. 1a). A similar lack of protection was also observed in various DBO experiments (DBO-DBO6) (Fig. 1c). For each of these vaccine experiments (DBO-DBO6), the concentration of DbpA was equal to or greater than the BBK32 or OspC concentrations, respectively. However, when DbpA was administered at concentrations below those of both BBK32 and OspC (DBO8 and DBO9), significant differences in *Borrelia*-positive tissues were observed (Fig. 1c). The data presented in Fig. 1c for DBO9-vaccinated mice is pooled from 2 experiments (DBO8 vaccination was performed once). The infection only and CFA/IFA groups infected at the time of the DBO8/DBO9 experiments (15 and 13 mice/group, respectively) were 100% *Borrelia*-positive for all tissues examined. An OspC only vaccine group (10 mice) was also included in the DBO8/DBO9 vaccination trial and tissues from this group were between 60 and 70% *Borrelia*-positive (data not shown). DBO9-vaccinated mice ($n = 17$) were (0, 6, 12 and 6% *Borrelia*-positive for Ear, Heart, Bladder and Joint tissues, respectively) and DBO8-vaccinated mice were 14% *Borrelia*-positive for all the tissues examined (Fig. 1c). The percent protection for both DBO8- and DBO9-vaccinated groups was statistically significant ($p < 0.0001$, Fisher's exact test) when compared to the infection only and CFA/IFA controls used in these experiments or when compared to the pooled infection only or CFA/IFA groups depicted in Fig. 1. These data suggested that at certain ratios, DBO formulations could protect mice against *Borrelia* infection.

3.2. Antibody responses

It has been demonstrated by many studies that the simultaneous injection of two or more antigens can decrease or increase the immune response to one (or more) of the components when compared to the immune response generated when only one component is administered [66–69]. As a result, these multicomponent immunizations directly affect the nature of the resulting immune response, i.e. cellular (Th1) or humoral (Th2) which can be defined in part by the resulting antibody response to the respective vaccine components [70–73]. For example, IgG2a is associated with Th1-type of responses and IgG1 is associated with Th2 responses [70,71]. Therefore, antibody profiles were analyzed during the course of the vaccine trials described here. In this study, mice immunized with DbpA/BBK32 had a dominant IgG1 response to either component (Fig. 2a and b), however, the IgG2b response against BBK32 was slightly higher in mice vaccinated with DbpA/BBK32 than in mice treated with BBK32 alone (0.143 ± 0.035 compared to 0.90 ± 0.089 ; Fig. 2b). In contrast, the IgG1 response against BBK32 was higher in BBK32-immunized mice than in the DbpA/BBK32-immunized group (0.620 ± 0.074 compared to $0.338 \pm .119$; Fig. 2b).

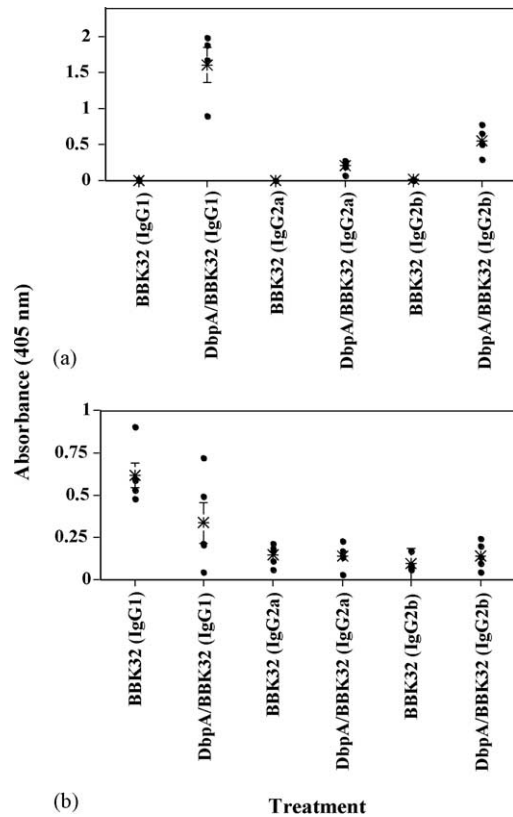


Fig. 2. Antibody profile following vaccination with either BBK32 or DbpA/BBK32. One week after secondary immunization, serum was collected and examined for antibodies reactive to DbpA (a) or BBK32 (b). Only IgG1-, IgG2a-, and IgG2b-reactive antibodies were detected. The data are expressed as the mean absorbance values of triplicate dilutions of sera from individual mice. The data are expressed as the mean \pm S.E. of 10 mice/group. The mean for each treatment group is indicated by *.

Double component formulations containing OspC in combination with either DbpA or BBK32 were also dominated by IgG1 responses and the presence of OspC did not affect the anti-DbpA (Fig. 3a) or anti-BBK32 (Fig. 3b) responses, respectively, although the IgG1 response in general in the presence of OspC was low (Fig. 3a–c). The anti-OspC response, however, was unchanged regardless of whether OspC was administered alone or in the presence of either DbpA or BBK32 (Fig. 3c).

The addition of a third component to the vaccine formulation, in combination with varying the concentrations of individual components (Table 2), further altered the antibody profiles. As shown in Fig. 4, the IgG1, IgG2a and IgG2b responses against DbpA (the component in the least amount) in DBO9-immunized mice, was significantly greater than the respective responses to either BBK32 or OspC ($p < 0.0002$, Student's *t*-test). This was surprising considering that the antibody responses (IgG1, IgG2a, or IgG2b) observed in response to each component in DBO.0-vaccinated mice (equal amounts of each protein, Table 2) displayed a profile where the IgG2a and IgG2b responses against DbpA were only moderately higher than the responses observed against BBK32

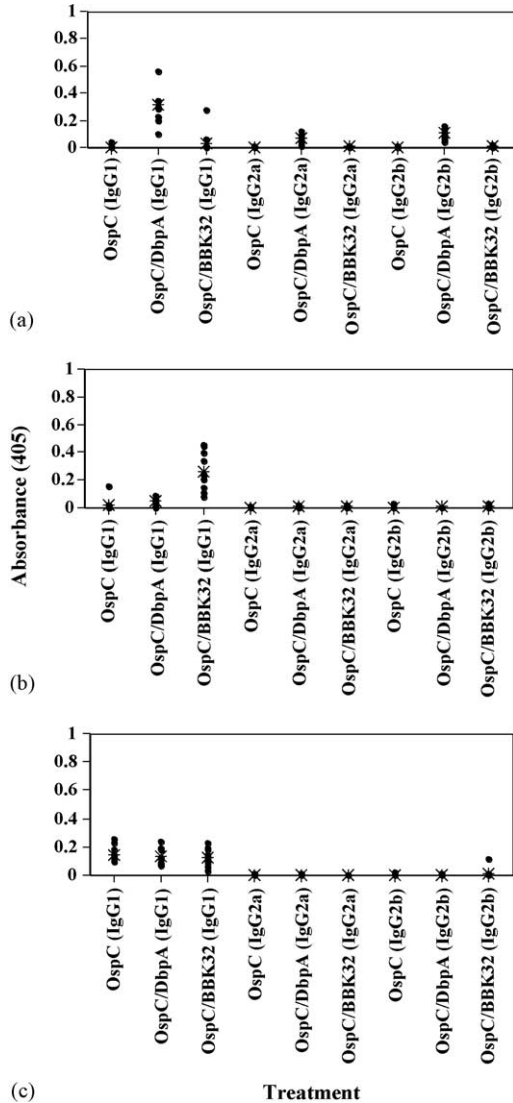


Fig. 3. Antibody profile following vaccination with either DbpA/OspC, BBK32/OspC or OspC. One week after secondary immunization, serum was collected and examined for antibodies reactive to DbpA (a), BBK32 (b), or OspC (c). Only IgG1-, IgG2a-, and IgG2b-reactive antibodies were detected. The data are expressed as the mean absorbance values of triplicate dilutions of sera from individual mice. The data are expressed as the mean \pm S.E. of 10 mice/group. The mean for each treatment group is indicated by *.

or OspC (Fig. 5). Furthermore, the IgG1 anti-OspC response was significantly greater than the IgG1 anti-DbpA or -BBK32 responses ($p < 0.0001$ and $p < 0.0002$, respectively, Student's *t*-test) (Fig. 5). How the concentration of one component can affect the nature of the antibody response to the other components, i.e. isotype profile and the fine specificity of the antibodies generated against each vaccine component as it relates to the different immunization dosages used is unknown. However, the effects on efficacy and in defining the antibody profile that develops can be severely impacted by the use of different component concentrations. No IgG3, IgE, IgA or IgM with specificity to *Borrelia* were detected for any group (data not shown).

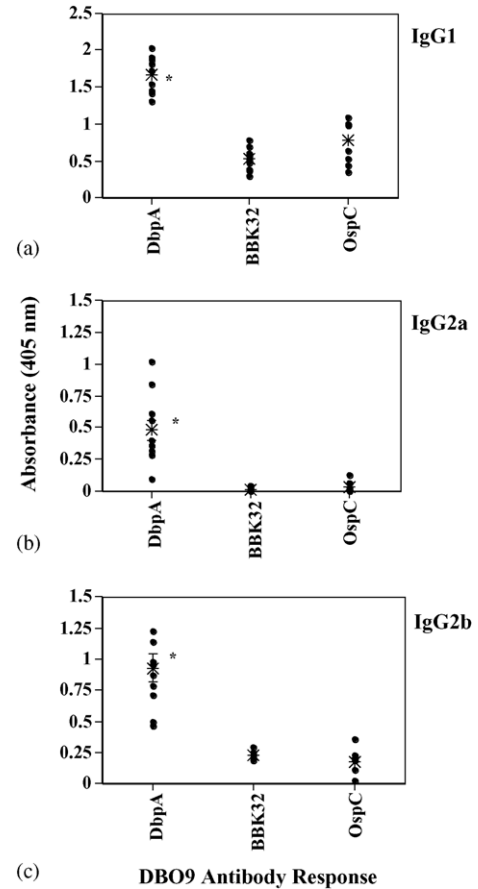


Fig. 4. Antibody profile following vaccination with DBO9. One week after secondary immunization, serum was collected and examined for antibodies reactive to DbpA (a), BBK32 (b), or OspC (c). Only IgG1-, IgG2a-, and IgG2b-reactive antibodies were detected. The data are expressed as the mean absorbance values of triplicate dilutions of sera from individual mice. The data are expressed as the mean \pm S.E. of 10 mice/group. The mean for each treatment group is indicated by *. Significant differences between the antibody response raised against DbpA compared to the response generated against either BBK32 or OspC is indicated by the asterisk ($*p < 0.05$, Student's *t*-test).

4. Discussion

One hypothesis that can be put forth to explain the differences in vaccine efficacy observed for the different DBO immunization groups relates to the concentrations of antigen-specific antibodies and their ability to confer protection following each different DBO vaccination. Although a minimum amount of specific serum IgG is necessary for protection against certain pathogens [74], there is also evidence that antibody-protective efficacy can decline in conditions of antibody excess. This observation, referred to as the prozone effect, was described in a *Streptococcus pneumoniae* infection model whereby administration of antibodies at concentrations larger than the optimally protective dose resulted in a loss of protection [75,76]. The work of Tabora et al. [53,54] has reminded us of observations made as early as 1913, that “antibody protection studies did not obey the law of multiple

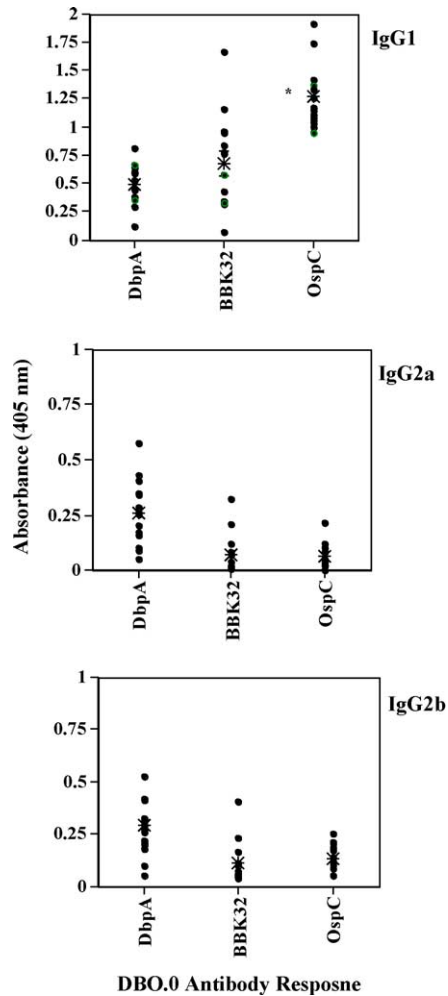


Fig. 5. Antibody profile following vaccination with DBO.0. One week after secondary immunization, serum was collected and examined for antibodies reactive to DBO components. Only IgG1-, IgG2a-, and IgG2b-reactive antibodies were detected. The data are expressed as the mean absorbance values of triplicate dilutions of sera from individual mice. The data are expressed as the mean \pm S.E. of 15 mice/group. The mean for each treatment group is indicated by \bar{x} . Significant differences between the antibody response raised against DbpA compared to the response generated against either BBK32 or OspC is indicated by the asterisk (* $p < 0.05$, Student's t -test).

proportions with respect to infecting inocula" [53,54]. Even though the prozone effect has been described for numerous pathogens [55–58], this phenomenon has largely been overlooked in numerous infection and vaccination scenarios as a possible explanation for immune failure. It is difficult to ascertain based on the current literature whether or not the prozone effect is primarily a function of passive immunization or if the principles of this phenomenon can be applied to active immunization. Regardless, understanding the 'type' of antibody response that develops with respect to a certain vaccine formulation can be useful for tailoring novel vaccine formulations, e.g. when testing different adjuvants or routes of immunization. Specifically, if it is known that an antibody response (e.g. similar to what is shown in Fig. 4) is protective, then 'aiming' for this profile when testing a new adjuvant

or protein combination(s) can facilitate effective vaccine design. Conversely, understanding what type of a response is detrimental would be of equal value.

The different concentrations of each DBO protein not only affected the antibody titers to each individual component, but also the nature of the IgG subclasses detected. Most of the single and double vaccine groups (data not shown and Figs. 2 and 3) resulted in an IgG1-dominated response with little or no IgG2a or IgG2b detected. In contrast, most DBO regimens elicited a mixed antibody response and the DBO8- and DBO9-vaccinated mice (data not shown and Fig. 5) had antibody profiles containing high titers of IgG1, IgG2a, and IgG2b. Remarkably, these antibodies were primarily against DbpA (the component in the lowest concentration [10 μ g]) compared to low-levels of IgG1 anti-BBK32 and anti-OspC and almost undetectable IgG2a and IgG2b responses against BBK32 and OspC (15 and 20 μ g, respectively) (Fig. 5). This suggested that varying the relative concentrations of each component likely affected the resulting cytokine response that would directly shape the developing immune response towards a protective or non-protective phenotype and is one explanation for the lack of protection observed during the single component D-B-O vaccinations (Fig. 1a). In addition, these observations may also help explain the varied results observed in vaccination trials against *B. burgdorferi* (Table 1) since simply changing the concentration of a component, the use and type of adjuvant, the genetic background, age and species of animal used, the immunization regimen (i.e. number of boosts) and the strain of *Borrelia* utilized likely impacted the results of each study (Table 1).

In the *C. neoformans* model, high antibody titers correlated with five- to eight-fold increases in brain colony forming units compared to mice receiving lower antibody concentrations, suggesting that pathogen dissemination could be affected by the level (and type) of antibodies present at the time of infection [54]. It has already been demonstrated that antibody-mediated immune pressure can change the antigenic profile of *Borrelia* in vivo [77,78]. Can changing an antibody fine specificity or its isotype, by manipulating the doses of different vaccine components, e.g. DBO, prevent antigen profile changes or the dissemination pattern of the infecting organism?

At a time when multicomponent vaccines are becoming more widely used, understanding the impact of each component on the subsequent immune response following vaccination will be critical in defining the composition of an effective vaccine formulation [79–81].

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