




Enhanced Protective Immunogenicity of Homodimeric *Borrelia burgdorferi* Outer Surface Protein C

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ABSTRACT Lyme borreliosis is caused by tick-transmitted spirochetes of the *Borrelia burgdorferi sensu lato* group and is the most common vector-borne disease in the United States and Europe. Outer surface protein C (OspC) is a 23-kDa outer surface lipoprotein expressed during spirochete transmission from the tick to the vertebrate host. In a previous study, we found that immunization with a recombinant disulfide-bridged dimeric form of OspC (D-OspC) stimulates increased antibody responses relative to immunization with commonly employed monomeric OspC. Here, we report that mice immunized with dimeric OspC proteins also exhibited enhanced protection against infection with the cognate *B. burgdorferi* strain. Mice were protected by four immunizations containing as little as 100 ng of dimeric OspC, suggesting that this form of the protein can induce protective immunity within a dose range reasonable for a human or veterinary vaccine. In contrast, monomeric OspC was only partially protective at much higher doses. IgG subclass analysis revealed that D-OspC-immunized animals mainly possessed anti-OspC-IgG1. In contrast, infected animals develop anti-OspC restricted to the IgG3 isotype. A subset of antibodies generated by dimeric OspC immunization did not recognize the monomeric variant, indicating that unique epitopes exist on the dimeric form. Moreover, monoclonal antibodies that recognized only dimeric OspC protected mice from *B. burgdorferi* challenge, whereas another monoclonal that recognized both immunogens was not protective. These studies suggest that this dimeric OspC presents distinctive epitopes that generate antibodies protective against *B. burgdorferi* infection and could be a useful vaccine component.

KEYWORDS OspC, vaccine, ELISA, immunoblot, Lyme disease, *Borrelia burgdorferi*, *Borrelia*

Lyme borreliosis (LB) is the most commonly reported arthropod-borne illness in North America and Europe (1), with an estimated annual incidence of 300,000 cases in the United States (2). Caused by spirochetes of the *Borrelia burgdorferi sensu lato* group (*B. burgdorferi sensu stricto*, *B. afzelii*, *B. garinii*, and related species), these bacteria are transmitted by hard-bodied ticks of the genus *Ixodes* in wooded areas of moderate climate. They can infect many mammals but generally find reservoir hosts in rodent and bird populations. In the United States, *B. burgdorferi sensu stricto* is the most common LB-associated strain, while the most common species in Europe are *B. afzelii* and *B. garinii*.

LB is a multisystemic bacterial infection and is characterized by localized, disseminated, and late manifestations. Localized disease often presents as erythema migrans, an expanding red rash that develops at the site of the tick bite. Patients may also

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experience fatigue, headaches, fever, chills, and muscle and joint pain. After a period of several days or weeks, the bacteria disseminate by hematogenous or lymphatic routes to other organs and cause systemic manifestations (3–5). These patients may present with multiple erythema, Lyme neuroborreliosis (LNB), arthritis, and cardiac symptoms. Late manifestations (6, 7) vary but may consist of arthritis, neuroborreliosis, or carditis (7, 8). In North America, a significant proportion of late-manifestation patients develop chronic arthritis, especially in the large joints. The disease phenotype varies somewhat between the different genospecies; for example, *B. burgdorferi* infection tends to favor arthritic symptoms, whereas neurologic manifestations are more commonly associated with *B. garinii* infections. Antibiotic therapy is frequently effective, but 10 to 20% of patients, especially those diagnosed late in the disease process, develop posttreatment Lyme disease syndrome and continue to have symptoms of fatigue, joint and muscle pain, and cognitive deficits (9–11).

At present, the prevention of LB is restricted to personal protection measures, including the use of pesticides and personal tick checks. Plans to eradicate *Borrelia* or tick populations on a large scale are only in conceptual stages and appear to be both impractical and cost-ineffective. LYMERix, a Lyme disease vaccine, was approved for human use in 1998 (12). It was composed of a recombinant form of the *B. burgdorferi* outer surface protein A (OspA). Although OspA is primarily expressed when the spirochete is in the *Ixodes* tick, LYMERix was found to be effective at preventing Lyme borreliosis by protecting against the transmission of *B. burgdorferi* from the tick to the human. LYMERix had some limitations, including a vaccine efficacy of <80%, uncertainty about the length of vaccine-induced immunity (potential need for booster doses), and efficacy only against the predominant North American *Borrelia* strain (13). In addition, concerns were raised by antivaccine groups regarding vaccine safety, and in response to these concerns and low public demand, the manufacturer voluntarily withdrew LYMERix from the market in 2002. Currently, new strategies employing mixtures of chimeric OspAs from several strains are being tested as vaccine candidates (14).

Another vaccine candidate is the outer surface protein C (OspC). The expression of OspC is induced during tick feeding and transmission of *Borrelia* and is essential for the initial colonization of mammalian hosts (15–18). In the tick host, OspC binds to the Salp15, a tick salivary protein (19). This interaction appears to facilitate the localization of the spirochetes to the salivary gland from which the spirochetes enter the mammalian host. The OspC-Salp15 interaction further enhances invasion of the host and may also enhance evasion of the host immune system (20). Furthermore, OspC appears to interact with plasminogen, perhaps utilizing this interaction to facilitate hematologic dissemination to tissues distant from the infection site (21, 22). Finally, mutagenesis studies support the existence of a ligand binding domain in OspC that interacts with an unidentified mammalian factor that is critical for survival and dissemination in early infection (23).

OspC is highly immunogenic and is one of the primary proteins against which host immune memory is developed (24, 25). It generates an IgM antibody response early during the course of infection, and the detection of anti-OspC IgM antibodies is useful in the diagnosis of early LB (26, 27). However, the amino acid sequence of OspC is highly diverse, even within strains of the same *Borrelia* species (28), and at least 25 families of OspC variants have been defined, with each geographic area harboring as many as 10 to 15 different types (29). The membrane-proximal N and C termini of OspC tend to be more conserved, especially a 10-amino-acid epitope near the C terminus. Both humans and mice can produce borreliacidal IgM to this C-terminal epitope (30–32).

OspC has previously been evaluated as a vaccine candidate and was found to be mostly protective against the *Borrelia* strain from which it originated; however, it failed to provide satisfactory cross-protection against strains with divergent OspC sequences (33–37). To overcome this problem, tetra- or octavalent recombinant OspC proteins

have been designed but thus far have not been tested in depth in animal infection models (38, 39).

In previous studies, Probst et al. (40) found that a recombinant OspC dimer has higher binding efficiency for anti-OspC antibodies in patient specimens and elicits an improved antibody response compared to monomeric OspC. OspC contains an intrinsic signal sequence followed by a cysteine residue, the attachment site of the lipid membrane anchor. Removal of the signal sequence allows isolation of a recombinant OspC homodimer that is covalently linked via an N-terminal disulfide bridge. The goal of the current study was to examine whether recombinant dimers of OspC (e.g., dimeric recombinant OspC from *B. burgdorferi* strain B31 [D-OspC_{B31}]) result in enhanced protection in comparison with the corresponding recombinant monomers (such as monomeric recombinant OspC from *B. burgdorferi* strain B31 [M-OspC_{B31}]) against challenge with LB *Borrelia* by either needle inoculation or tick transmission. Our data indicate that this dimeric form of OspC is a more protective immunogen than monomeric OspC and produces protective antibodies to epitopes unique to the dimeric form.

RESULTS

Characterization of recombinant OspCs. We constructed six different expression vectors to produce OspC variants from several *Borrelia burgdorferi* strains as monomers or as dimers. In all cases, sequences encoding the lipoprotein leader (amino acids 1 to 18) were omitted from the recombinant sequence, resulting in a soluble cytoplasmically expressed protein product. An N-terminal octahistidine tag was added to aid in protein purification. Three recombinant constructs lacked any cysteine residues and therefore were predicted to form monomers (M-OspC_{B31}, monomeric recombinant OspC from *B. burgdorferi* strain 297 [M-OspC₂₉₇], and monomeric recombinant OspC from *B. burgdorferi* strain N40 [M-OspC_{N40}]). An additional three recombinant forms (D-OspC_{B31}, D-OspC₂₉₇, and D-OspC_{N40}) contained cysteines at position 19 and were predicted to form dimers, based on prior results (40). Details of the vector construction and protein expression and purification are described in the supplemental material. Mass spectrometry verified the identity and purity of the prepared recombinant OspC variants (data not shown).

In nonreducing SDS-PAGE, M-OspC_{B31}, M-OspC₂₉₇, and M-OspC_{N40} showed the same migration behavior as in reducing SDS-PAGE and migrated with apparent molecular masses in the range of 25 to 30 kDa. In contrast, the OspC variants with intact Cys19 migrated more slowly in nonreducing gels and produced 50- to 60-kDa bands (Fig. 1C, left panel). Less than 5% of the total protein of the dimer constructs migrated, with an M_r of 25,000 to 30,000, as calculated from densitometric analysis of nonreducing gels. Individually, their apparent molecular masses were twice those observed under reducing/alkylating conditions, in agreement with the formation of disulfide-bridged dimers. The 50- to 60-kDa bands could be reduced to 25 to 30 kDa by heating the sample with reducing agents prior to electrophoresis (Fig. 1C, right panel).

To determine the size distribution of OspC variants in solution, M-OspC_{B31} and D-OspC_{B31} were also analyzed by dynamic light scattering in reducing and nonreducing buffer in the presence of various amounts of sodium chloride (Fig. S1). In all cases, a model for globular proteins was applied. The hydrodynamic radius (H_R) of M-OspC_{B31} was calculated as ~5 nm under all conditions. D-OspC_{B31} showed a hydrodynamic radius of >10 nm under nonreducing conditions in buffers of low ionic strength (≤ 150 mmol/liter NaCl). However, the H_R of D-OspC_{B31} was reduced to ~5 nm under reducing conditions or when the NaCl concentration was ≥ 500 mmol/liter, indicating that D-OspC_{B31} is capable of forming multimeric structures not observed for M-OspC_{B31}.

Immunization with dimeric OspC protects mice from *B. burgdorferi* challenge better than immunization with monomeric OspC. Since previous experiments indicated that dimerized OspC was more immunogenic than monomeric OspC (40), we undertook a series of immunization and challenge experiments to determine whether dimeric OspC could protect against *B. burgdorferi* challenge better than monomeric OspC. A total of nine experiments were performed that tested various parameters of

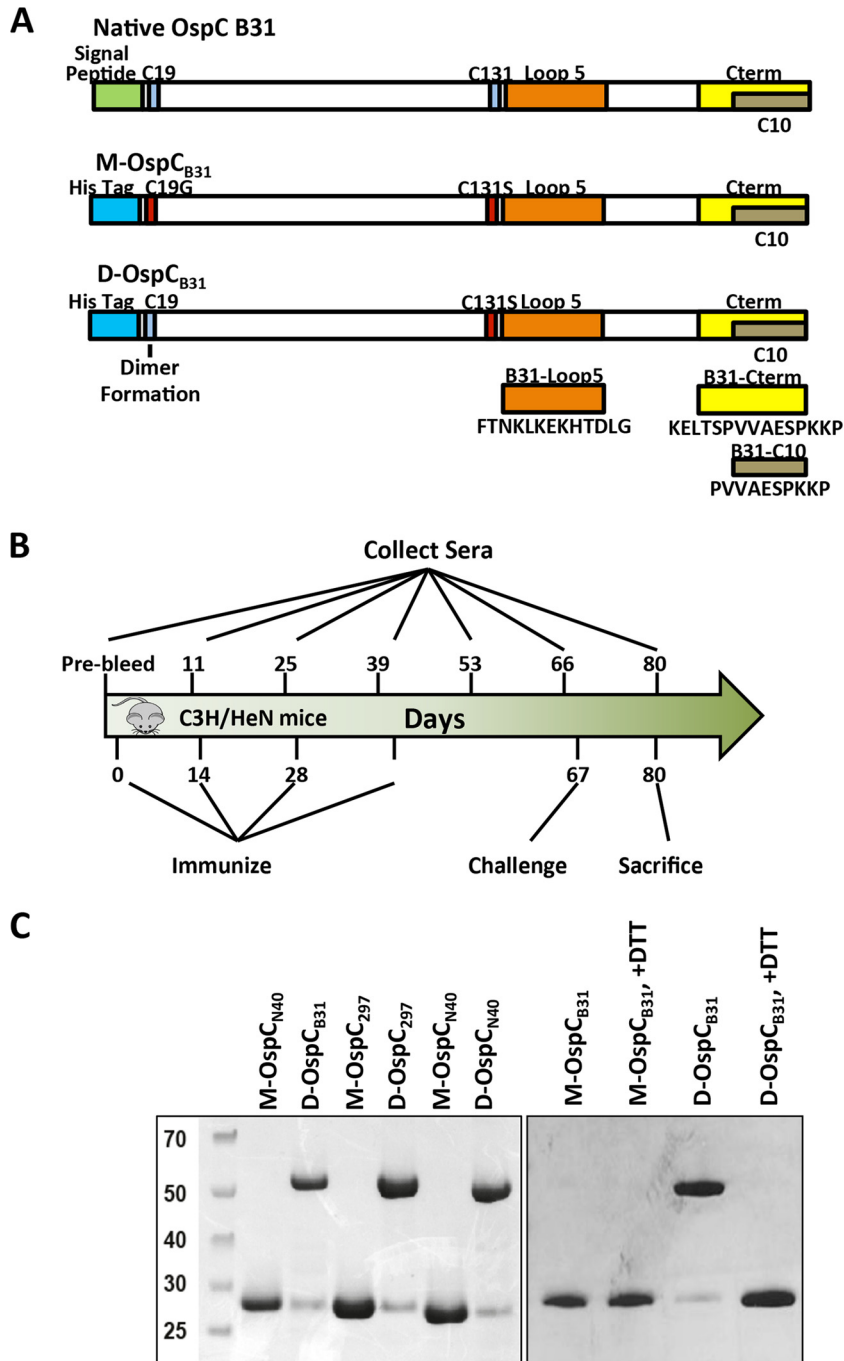


FIG 1 Antigens and a representative immunization protocol used in this study. (A) Schematic of the recombinant monomeric and dimeric OspC proteins. OspC from strain B31 is given as an example. The signal peptide (amino acids [aa] 1 to 18) is replaced with an oligohistidine tag. Monomeric OspC also carries a C19G mutation. Both monomeric and dimeric variants carry a C→S mutation of the central cysteine, thereby preventing aggregation. Peptides B31_{loop5} and B31_{Cterm} are indicated in orange and yellow, respectively. (B) Schematic overview of a representative immunization schedule. Mice were immunized up to 4 times using up to 100 μ g of protein per injection with adjuvant (typically alum). Adjuvant only was used as a control. Sera were sampled before the initial immunization, ~12 days after each immunization, on the day of challenge, and on the day of sacrifice. Challenge was done either by subcutaneous injection of *in vitro*-cultured *B. burgdorferi* (needle inoculation) or by applying *B. burgdorferi* B31-infected *I. scapularis* ticks onto each individually caged mouse for a period of 14 days. (C) SDS-PAGE of monomeric and dimeric recombinant OspC proteins, showing dimerization mediated by Cys19. Left panel, SDS-PAGE of M-OspC_{B31}, D-OspC_{B31}, M-OspC₂₉₇, D-OspC₂₉₇, M-OspC_{N40}, and D-OspC_{N40} in the absence of dithiothreitol (DTT) reducing agent. Right panel, SDS-PAGE migration of recombinant B31 OspC proteins in the presence and absence of DTT. Each lane contains 1 μ g of purified recombinant protein. M, molecular mass (in kilodaltons) ladder;

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protection: required dosage, effect of adjuvants, number of required immunizations, lot-to-lot variability, tick challenge, and cross-strain protection. In most experiments, alum was used as the adjuvant, and 4 immunizations per animal were used. A summary of all the experiments is shown in Table 1.

An initial needle inoculation experiment indicated that high doses of M-OspC_{B31} (10 to 100 μg per immunization) could protect some immunized mice (33%) from *B. burgdorferi* infection, but protection was not observed when doses lower than 100 μg per immunization were used (Tables 1 and 2, experiments 1 and 2). In contrast, D-OspC_{B31} protected challenged animals with immunization doses as low as 0.1 μg (Tables 1 and 2, experiments 1 and 2). When large doses of antigen were used (10 μg or more of either recombinant OspC), we observed a nodular reaction at the site of immunization. This reaction was diminished or absent when lower doses of antigen (0.1 μg to 1.0 μg) were used. Since 1 μg per immunization of D-OspC_{B31} routinely protected all animals, we chose this dose for subsequent immunizations. No variation in protection was seen between different lots of OspC proteins (Table 1, experiment 6). Fisher's exact test was used to examine protection bias between animals immunized with 1- μg doses of D-OspC_{B31} or M-OspC_{B31}. The result was significant at a *P* value of <0.01.

We also tested the ability of the recombinant monomeric and dimeric OspCs to protect against tick challenge (Table 3). Immunization with M-OspC_{B31} failed to protect mice from *B. burgdorferi* infection via tick bite. However, as with needle inoculation, D-OspC_{B31} protected all tick-inoculated mice (10/10) from infection.

OspC amino acid sequences vary considerably, with 63 to 90% sequence identity among different LB species and strains (29, 41). To determine whether the dimerization of OspC from other strains also enhanced protective immunogenicity, we immunized animals with monomeric or dimeric OspC variants derived from *B. burgdorferi* strains 297 and N40 (recombinant proteins M-OspC₂₉₇, M-OspC_{N40}, D-OspC₂₉₇, and D-OspC_{N40}). Similar to D-OspC_{B31}, D-OspC₂₉₇ provided superior protection against the cognate challenge strain, 297, compared to M-OspC₂₉₇ (Table 1, experiments 7 and 8). All animals immunized with D-OspC₂₉₇ were protected from 297 challenge, while most animals (5/6) immunized with M-OspC₂₉₇ became infected in response to 297 challenge. In the case of OspC_{N40}, immunization with either monomeric or dimeric OspC_{N40} protected against needle challenge with the *B. burgdorferi* N40. Thus, in cases where monomeric OspC was unable to protect from *B. burgdorferi* challenge, dimerization improved protection.

We also tested different immunization protocols to determine the effect of reducing the number of immunizations or using different adjuvants. A reduction in the number of immunizations from four injections to three injections with 1 μg of D-OspC_{B31} led to protection in 10 of 12 mice (83%). Two injections did not induce reliable protection (6 of 12 animals protected) (Table 1, experiments 3 and 4).

We also tested monophosphoryl lipid A (MPL) as an adjuvant, because it has been reported to be more effective than alum in promoting T-cell differentiation (42). Using the standard dose of antigen (1 μg), we tested protection from B31 challenge with two or three immunizations of D-OspC_{B31} (Table 1, experiment 8). Three immunizations gave 100% protection from *B. burgdorferi* challenge. Two immunizations protected less well (67% protection). Analysis of sera from mice receiving two doses of antigen showed relatively low antibody titers compared to those obtained after three immunizations (data not shown).

The ability of dimeric OspCs to provide protection to challenge by nonhomologous strains varied. Using our standard protocol, immunization with D_{N40} and D₂₉₇ led to no protection (0/3) against *B. burgdorferi* B31 challenge and 33% (2/6) protection against *B. burgdorferi* 297 challenge. Immunization with D-OspC_{B31} protected poorly against

FIG 1 Legend (Continued)

M-OspC_{B31}, monomeric OspC of strain B31; D-OspC_{B31}, dimeric OspC of strain B31; M-OspC₂₉₇, monomeric OspC of strain 297; D-OspC₂₉₇, dimeric OspC of strain 297; M-OspC_{N40}, monomeric OspC of strain N40; and D-OspC_{N40}, dimeric OspC of strain N40.

TABLE 1 Relative protection of mice against *Borrelia* infection through immunization with dimeric and monomeric recombinant OspC constructs

Antigen	Challenge	No. of animals	Dosage (μ g)	No. of immunizations	No. infected/ total no.	% protection
Summary, experiments with B31 immunization and challenge						
Alum only	B31	27	0	4	27/27	0
Alum + OspC-D _{B31}	B31	63	0.1–100	4	2/60	97
Alum + OspC-M _{B31}	B31	45	0.1–100	4	32/39	18
MPL only	B31	3	0	3	3/3	0
MPL + OspC-D _{B31}	B31	6	1	3	0/6	100
MPL + OspC-M _{B31}	B31	6	1	3	3/6	50
Experiment 1, antigen dosage effects						
Alum only	B31	6	0	4	6/6	0
Alum + OspC-D _{B31}	B31	6	100	4	0/6	100
Alum + OspC-D _{B31}	B31	6	30	4	0/6	100
Alum + OspC-D _{B31}	B31	6	10	4	0/6	100
Alum + OspC-M ₃₁	B31	6	100	4	5/6	17
Alum + OspC-M _{B31}	B31	6	30	4	5/6	17
Alum + OspC-M _{B31}	B31	6	10	4	4/6	33
Experiment 2, antigen dosage effects						
Alum only	B31	6	0	4	6/6	0
Alum + OspC-D _{B31}	B31	6	10	4	1/6	83
Alum + OspC-D _{B31}	B31	6	1	4	0/6	100
Alum + OspC-D _{B31}	B31	6	0.1	4	1/6	83
Alum + OspC-M _{B31}	B31	6	100	4	4/6	33
Alum + OspC-M _{B31}	B31	3	10	4	2/3	33
Alum + OspC-M _{B31}	B31	3	1	4	3/3	0
Alum + OspC-M _{B31}	B31	3	0.1	4	3/3	0
Experiment 3, no. of immunizations						
Alum only	B31	3	0	4	3/3	0
Alum + OspC-D _{B31}	B31	6	1	4	0/6	100
Alum + OspC-D _{B31}	B31	6	1	3	1/6	83
Alum + OspC-D _{B31}	B31	6	1	2	2/6	67
Alum + OspC-M _{B31}	B31	6	1	4	6/6	0
Experiment 4, no. of immunizations						
Alum only	B31	3	0	4	3/3	0
Alum + OspC-D _{B31}	B31	6	1	4	0/6	100
Alum + OspC-D _{B31}	B31	6	1	3	1/6	83
Alum + OspC-D _{B31}	B31	6	1	2	4/6	33
Experiment 5, cross-strain challenge						
Alum only	B31	3	0	4	3/3	0
Alum + OspC-D _{B31}	B31	6	1	4	0/6	100
Alum only	N40	6	0	4	6/6	0
Alum + OspC-D _{B31}	N40	6	1	4	6/6	0
Alum only	297	6	0	4	6/6	0
Alum + OspC-D _{B31}	297	6	1	4	6/6	0
Experiment 6, different lots of recombinant proteins						
Alum only	B31	3	1	4	3/3	0
Alum + OspC-D _{B31} (lot 1)	B31	3	1	4	0/3	100
Alum + OspC-D _{B31} (lot 2)	B31	3	1	4	0/3	100
Alum + OspC-D _{B31} (lot 3)	B31	3	1	4	0/3	100
Alum + OspC-M _{B31} (lot 2)	B31	3	1	4	3/3	0
Alum + OspC-M _{B31} (lot 3)	B31	3	1	4	1/3	67
Experiment 7, OspC from different strains						
Alum only	297	3	0	4	3/3	0
Alum + OspC-D ₂₉₇	297	6	1	4	0/6	100
Alum + OspC-M ₂₉₇	297	6	1	4	5/6	17
Alum only	N40	3	0	4	3/3	0
Alum + OspC-D _{N40}	N40	6	1	4	0/6	100
Alum + OspC-M _{N40}	N40	6	1	4	0/6	100

(Continued on next page)

TABLE 1 (Continued)

Antigen	Challenge	No. of animals	Dosage (μg)	No. of immunizations	No. infected/total no.	% protection
Experiment 8, Cross-strain challenge, MPL adjuvant, and reduced dimer immunization						
MPL only	B31	3	1	3	3/3	0
MPL + OspC-M _{B31}	B31	6	1	3	3/6	50
MPL + OspC-D _{B31}	B31	6	1	3	0/6	100
MPL + OspC-D _{B31}	B31	6	1	2	2/6	67
MPL + OspC-D _{N40}	B31	3	1	3	3/3	0
None	297	3	1	3	3/3	0
MPL + OspC-D _{N40}	297	3	1	3	2/3	33
MPL only	N40	3	1	3	3/3	0
MPL + OspC-D _{N40}	N40	3	1	3	0/3	100

challenge with either *B. burgdorferi* 297 (1/6) or against *B. burgdorferi* N40 (0/6) (Table 1, experiments 5 and 8).

Characterization of antibody response to OspC immunization. In an effort to understand the difference in protective immunogenicity between monomeric and dimeric forms of recombinant OspC, we characterized the antibody responses of immunized animals. In examining total IgG, all OspC variants produced a strong anti-OspC response (see Fig. 2A for a representative example). While there was significant variability between experiments and among individual animals, immunization with dimeric OspCs generally led to a faster development of endpoint titers than with the monomeric homologues. However, the monomers often produced higher endpoint titers. The specificity of the enzyme-linked immunosorbent assay (ELISA) reactivity against OspC was confirmed by immunoblotting (Fig. 2B, lanes 3 and 5). As expected, infected animals (i.e., positive controls and unprotected vaccinated mice) developed an IgG response against VlsE, p39/BmpA, and p18/DbpA, as determined by immunoblotting (Fig. 2B, lanes 2 and 6 for examples) and ELISA (Table S3). In addition, varied reactions against unidentified antigens occurred in individual animals.

IgG subclass analysis revealed that immunization of mice with either the dimeric or monomeric form of OspC_{B31} predominantly generated anti-OspC antibodies of subclass IgG1, with minor contributions of IgG2a and IgG2b (Fig. 3 and data not shown). In contrast, infected animals developed a strong IgG3 response to OspC that was absent in uninfected immunized animals (Fig. 3, top panels, and Table S3). In OspC-immunized mice that became infected, antibody titers against OspC increased significantly, while the IgG reactivity remained nearly constant in protected animals (Fig. 2A and 3 top panels, compare prechallenge and postchallenge). A significant difference in antibody reactivity was observed between control animals (alum only) and OspC-immunized mice that were infected upon challenge. OspC-naïve mice developed a more pronounced IgG3 response against recombinant OspCs than did infected M-OspC_{B31}-immunized mice (Fig. 3, top panels). Overall, mice immunized with either monomeric or dimeric forms of OspC had predominant IgG1 responses, whereas infected animals exhibited IgG3 responses.

TABLE 2 Protection obtained by immunization with dimeric OspC at clinically relevant dosages

Group	No. of cultures positive/total no.						No. of mice positive/total no.	% mice protected
	Skin	Ear	Joint	Bladder	Heart	All sites		
Alum only	6/6	6/6	6/6	6/6	6/6	30/30	6/6	0
0.1 μg D-OspC _{B31} + alum	1/6	1/6	0/6	1/6	1/6	4/30	1/6	83
1.0 μg D-OspC _{B31} + alum	0/6	0/6	0/6	0/6	0/6	0/30	0/6	100
10 μg D-OspC _{B31} + alum	1/6	0/6	1/6	1/6	1/6	4/30	1/6	83
0.1 μg M-OspC _{B31} + alum	3/3	3/3	3/3	3/3	3/3	15/15	3/3	0
1.0 μg M-OspC _{B31} + alum	3/3	3/3	3/3	3/3	3/3	15/15	3/3	0
10 μg M-OspC _{B31} + alum	2/3	2/3	2/3	2/3	2/3	10/15	2/3	33
100 μg M-OspC _{B31} + alum	4/6	4/6	4/6	4/6	4/6	20/30	4/6	33

TABLE 3 Immunization with dimeric OspC protects mice against tick challenge

Challenge	Group	No. of mice infected/total no.	No. of tissues infected/total no. ^a	% mice protected
Needle inoculation with B31 5A4	Alum	12/12	57/60	0
	M-OspC _{B31} + alum	12/12	56/60	0
	D-OspC _{B31} + alum	0/12	0/60	100
Bite of B31-infected ticks	Alum	9/11 ^b	36/44	20
	M-OspC _{B31} + alum	5/6 ^b	20/24	17
	D-OspC _{B31} + alum	0/10 ^b	0/40	100

^aEar, joint, bladder, and heart tissues were analyzed for the presence of viable *Borrelia* bacteria in all animals as well as skin from the injection site of needle-inoculated animals.

^bMice for which no *Borrelia*-infected fed ticks could be detected after challenge were disregarded.

Liquid-phase inhibition studies were performed to determine the degree of cross-reactivity of anti-OspC antibodies generated through immunization with D-OspC_{B31} and M-OspC_{B31}. As shown in Fig. S2, preincubation of sera from immunized mice with the antigen used in immunization resulted in nearly complete inhibition of antibody binding to antigen-coated ELISA plates (94% inhibition for D-OspC_{B31} and 93% for M-OspC_{B31}). In general, cross-absorption (e.g., preincubation of sera from D-OspC_{B31}-immunized animals with M-OspC_{B31} antigen) resulted in less inhibition. These results indicate that, as expected, antibodies from the immunized animals are absorbed more effectively by the antigen used for immunization. However, sera from dimer-immunized mice preincubated with either the dimeric or monomeric forms of recombinant OspC exhibited nearly equivalent inhibition of binding (83% and 87%) in the M-OspC_{B31} ELISA (Fig. S2, lower panel, left side), suggesting that D-OspC_{B31} possesses the epitopes found in M-OspC_{B31}. None of the differences are statistically significant ($P > 0.05$), so they can be interpreted only as trends.

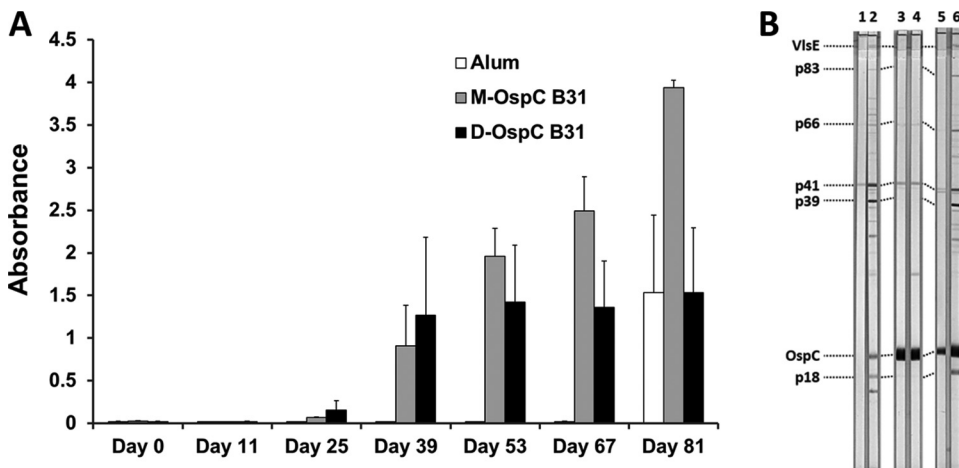


FIG 2 Mice exhibit a strong differentiated immune response to vaccination with recombinant monomeric and dimeric OspC forms and subsequent *Borrelia burgdorferi* B31 challenge. (A) ELISA assessment of pan-IgG anti-OspC response to immunization with alum only (white bars), M-OspC_{B31} (gray bars), and D-OspC_{B31} (black bars). Animals were injected with 1 μ g of antigen on days 0, 14, 28, and 42 and were needle inoculated with 10^4 *B. burgdorferi* B31 bacteria on day 67; day 81 thus represents a 14-day postchallenge time point. Each group consisted of 6 animals. Sera were tested at a 1:4,000 dilution against dimeric OspC. Mean \pm standard error (SE) absorbance is indicated. (B) Immunoblot analysis of murine sera. Sera from immunized and challenged mice were tested for reactivity to various *Borrelia* antigens using anti-*Borrelia* Euroline-WB. Immunization was with alum only (lanes 1 and 2), with D-OspC_{B31} and alum (lanes 3 and 4), and with M-OspC_{B31} and alum (lanes 5 and 6). Odd lanes (pre) were incubated with sera collected after immunization but prior to *Borrelia* challenge. Sera incubated in even lanes (post) were collected on day 14 postchallenge. Sera were tested in 1:500 dilutions. Immunization with both forms of OspC resulted in strong prechallenge reactivity with OspC (lanes 3 and 5); nonspecific reactivity to P41 (flagellin) was also observed. After *B. burgdorferi* challenge, animals immunized with the alum only (strip 2) and M-OspC_{B31} (strip 6) groups exhibited antibody reactivity to additional *B. burgdorferi* proteins (VlsE, p39, OspC, and p18) indicative of active infection.

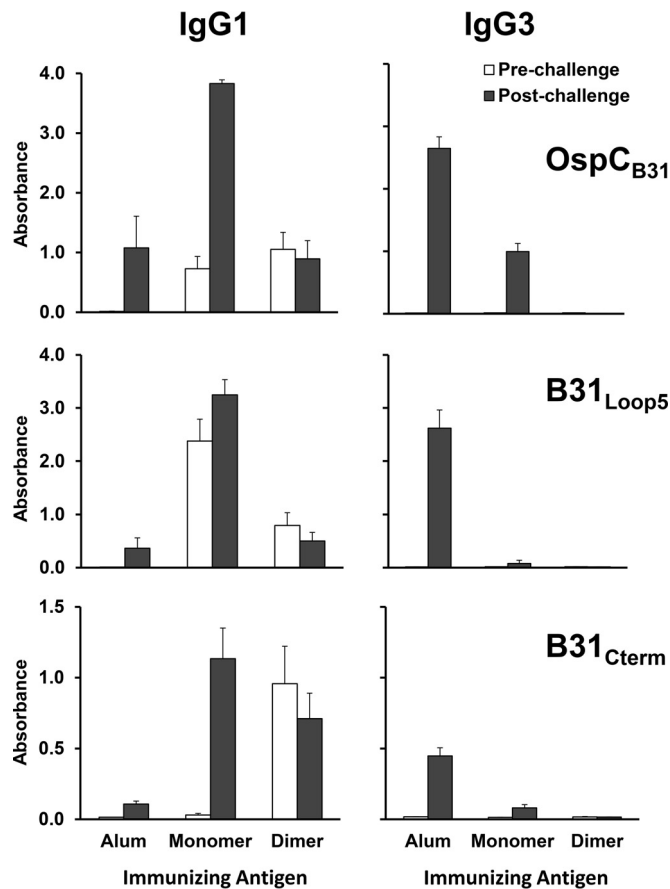


FIG 3 Animals immunized with OspC can be distinguished from animals with active infection by antibody isotype. IgG1 and IgG3 reactivity after immunization with alum only, M-OspC_{B31}, and D-OspC_{B31} is shown. All animals were injected with 1 μ g at days 0, 14, 28, and 42, needle inoculated with 10^4 *B. burgdorferi* B31 bacteria at day 67, and sacrificed at day 81. Each group consisted of 6 animals. Top, IgG1 and IgG3 reactivity against D-OspC_{B31}. Middle, IgG1 and IgG3 reactivity against peptide B31_{loop5}. Bottom, IgG1 and IgG3 reactivity against peptide B31_{Cterm}. Sera were tested at dilutions of 1:4,000 for IgG1 and 1:200 for IgG3. Values represent mean \pm SE absorbance.

To determine whether antibodies generated by immunization with an OspC monomer or dimer could recognize OspC from other *B. burgdorferi* strains, sera from animals immunized with M-OspC_{B31} and D-OspC_{B31} were tested by immunoblotting against whole-cell extracts from *B. burgdorferi* N40 and *B. burgdorferi* 297. Sera from D-OspC_{B31}-immunized animals recognized a single band of the predicted OspC size in both N40 and 297 whole-cell extracts by immunoblotting (Fig. 4A, left panel). In contrast, sera from M-OspC_{B31}-immunized animals did not yield a detectable reaction with extracts from either N40 or 297 (Fig. 4A, right panel). By ELISA, each serum recognized its cognate OspC ortholog most strongly but also reacted with the other orthologs. Cross-reactivity with heterologous OspCs was varied and reached 10 to 30% of the total IgG reactivity against the immunizing proteins, as determined by ELISA (Fig. 4B).

Reactivity with OspC epitopes. In an effort to identify protective epitopes, we performed PepScan analysis of sera from five representative animals after immunization with D-OspC_{B31} (data not shown). All animals possessed high-titer antibodies against the loop 5 peptides FTNKLKEKHTDL and TNKLKEKHTDLG. The loop 5 domain of OspC has been identified as surface exposed and highly antigenic in both humans and mice (43). Three animals also had varied reactivity against the loop 5 overlapping peptides NKLKEKHTDLGK, KLKEKHTDLGKE, LKEKHTDLGKEG, and KEKHTDLGKEGV. Reactivity against other peptides was low and varied in comparison to the reactivity observed for the loop 5 region.

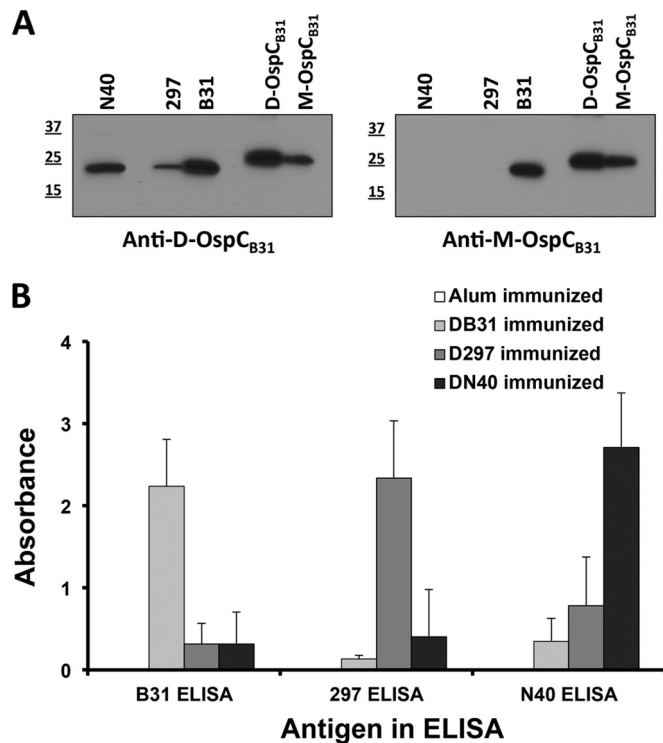


FIG 4 IgG cross-reactivity against recombinant dimeric OspC proteins from different *B. burgdorferi* strains. (A) Immunoblot analysis of murine sera. Sera from D-OspC_{B31}-immunized (left panel) (pool of sera from two animals) or M-OspC_{B31}-immunized (right panel) (pool of sera from two animals) mice were tested for reactivity to different *Borrelia* strains. N40, 10⁷ *Borrelia burgdorferi* N40; 297, 10⁷ *Borrelia burgdorferi* 297; B31, 10⁷ *Borrelia burgdorferi* B31, M, molecular mass (in kilodaltons) standards; D-OspC_{B31}, 50 ng recombinant D-OspC_{B31}; M-OspC₂₉₇, 50 ng recombinant M-OspC_{B31}. (B) Limited IgG cross-reactivity against recombinant dimeric OspC proteins from different *B. burgdorferi* strains by ELISA. Sera from immunized animals were tested against the indicated antigen: alum only (white, pool of sera from 9 animals), D-OspC_{B31} (black, pool of sera from 3 animals), D-OspC₂₉₇ (shaded, pool of sera from 12 animals), and D-OspC_{N40} (dotted, pool of sera from 12 animals). Bars represent mean \pm standard deviation (SD) absorbance.

We also tested the sera of all animals by ELISA for reactivity to a synthetic peptide corresponding to the B31_{loop5} region (FTKNKLKEKHTDLG) and with a second peptide, B31_{Cterm} (KELTSPVVAESPCKP). B31_{Cterm} overlaps the C-terminal 10 amino acids of OspC_{B31} (C10, PVVAESPCKP), which are well conserved between OspC orthologs and are surface exposed. Antibodies to this region are frequently seen in the sera of early Lyme borreliosis patients and in patients with neuroborreliosis (44, 45). Due to the chemical nature of the peptide linkage in the PepScan, this C-terminal peptide was not well analyzed in the PepScan assay.

Both M-OspC_{B31} and D-OspC_{B31} induced high-titer IgG1 against B31_{loop5}, but M-OspC_{B31}-immunized animals exhibited \sim 3-fold higher absorbance values (see Fig. 3, middle panels, prechallenge values). In contrast, the absorbance values against B31_{Cterm} were higher in D-OspC_{B31}-immunized mice, although significant variation was found between individual animals (Fig. 3, lower panels, prechallenge values). There was no discernible relationship between prechallenge reactivity with either B31_{loop5} or B31_{Cterm} and protection against infection. Many D-OspC_{B31}-immunized animals that were protected against infection had low reactivity to either peptide, and conversely, M-OspC_{B31}-immunized animals with high antibody levels to either peptide were frequently not protected from infection (Fig. 5). These data indicate that the protective effect conferred by immunization with D-OspC_{B31} is not due to the induction of antibodies against these linear epitopes.

To further examine the relationship between the epitopes represented by B31_{loop5} and B31_{Cterm} and protective immunity, the correlation between ELISA reactivity of sera

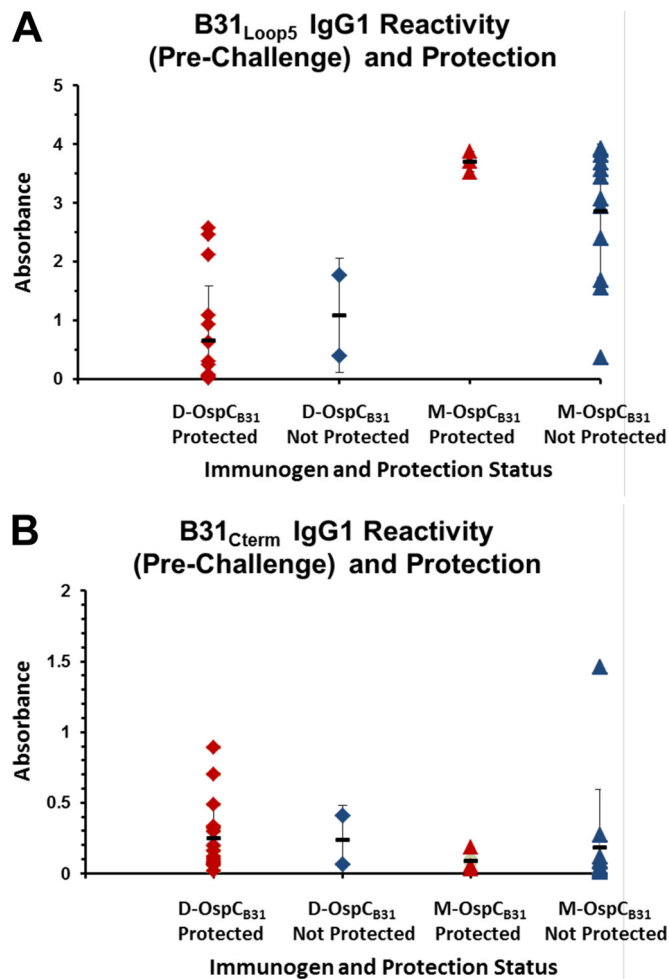


FIG 5 Lack of relationship between serologic reactivity to the OspC peptide B31_{loop5} or B31_{Cterm} and protection against infection with *B. burgdorferi* B31. Data points indicate the ELISA absorbance obtained for B31_{loop5} (A) and B31_{Cterm} (B) in prechallenge sera from individual mice protected against infection (red symbols; no cultures positive) and not protected (blue symbols; one or more cultures positive). Diamonds, animals immunized with D-OspC_{B31}; triangles, animals immunized with M-OspC_{B31}.

from immunized mice with these peptides and D-OspC_{B31} or M-OspC_{B31} was determined. B31_{Cterm} binding did not correlate well with binding of antibodies to either antigen ($R^2 = 0.02$ to 0.17 ; data not shown). The ELISA reactivity to B31_{loop5} of sera from mice immunized with either D-OspC_{B31} or M-OspC_{B31} correlated well with reactivity to M-OspC_{B31} ($R^2 = 0.7044$ to 0.7615) (Fig. S3C and D). When sera from D-OspC_{B31} mice were examined (Fig. S3B), there was a poor correlation between reactivity to the dimer form of the antigen and the B31_{loop5} ($R^2 = 0.117$). In contrast, the correlation of reactivities was high when reactivity of sera from M-OspC_{B31} mice with the dimer form and the B31_{loop5} were compared (Fig. S3A; $R^2 = 0.7257$). These results again support the concept that D-OspC_{B31} contains additional epitopes that contribute substantially to the protective antibody response.

Characterization of monoclonal antibodies. We speculated that we might be able to generate monoclonal antibodies to the unique epitopes in D-OspC_{B31} and that these antibodies might passively protect mice from *B. burgdorferi* challenge. Thus, we immunized four mice with D-OspC_{B31} using the standard protocol. As expected, all mice developed a strong antibody response to D-OspC_{B31}, M-OspC_{B31}, B31_{loop5}, and B31_{Cterm} at the end of the 56-day immunization protocol (data not shown). Two mice were randomly chosen for spleen fusion. Of the resulting 1,572 hybridoma cultures, 36 cultures produced supernatants that reacted strongly with D-OspC_{B31} and/or

TABLE 4 Protection against *B. burgdorferi* challenge by passive immunization with monoclonal antibodies

Antibody source	No. positive/total no.						No. of mice positive/total no.	% mice protected
	Skin	Joint	Heart	Bladder	Ear	All sites		
Normal mouse serum	3/3	3/3	3/3	3/3	3/3	15/15	3/3	0
1-5A10	0/5	0/5	0/5	0/5	0/5	0/25	0/5	100
1-8B7	0/5	1/5 ^a	0/5	0/5	0/5	1/25	1/5	80
2-8C10	0/5	0/5	0/5	0/5	0/5	0/25	0/5	100
2-2H8	5/5	3/3	5/5	5/5	5/5	23/23	5/5	0
Serum pool M-OspC _{B31}	3/3	3/3	3/3	3/3	3/3	15/15	3/3	0
Serum pool D-OspC _{B31}	1/5	1/5	1/5	1/5	1/5	5/25	1/5	80

^aCulture did not grow out, but spirochetes were observed in one field on day 7 attached to a fragment of tissue debris.

M-OspC_{B31} and expressed antibodies of the IgG1 subclass. Four clones were selected for further study: 1-5A10, 1-8B7, 2-2H8, and 2-8C10. Clones 1-5A10, 1-8B7, and 2-8C10 reacted strongly with D-OspC_{B31} and only weakly with M-OspC_{B31} (Table S4). Clone 2-2H8 reacted equally well with D-OspC_{B31} and M-OspC_{B31}. Similar results were obtained in the immunoblot analyses. After nonreducing SDS-PAGE, clones 1-5A10, 1-8B7, and 2-8C10 reacted with the putative dimeric OspC (50- to 60-kDa form) more strongly than with the smaller 25- to 30-kDa monomeric form of OspC. Conversely, immunoblots after reducing/alkylating SDS-PAGE showed equal reactivity for all 4 clones (data not shown). Only clone 2-2H8 showed reactivity against the B31_{loop5} peptide (Table S4).

To determine whether clones 1-5A10, 1-8B7, and 2-8C10 recognized the same epitope, we performed a cross-reaction analysis. After incubation with one of the monoclonal antibodies, D-OspC_{B31}-coated ELISA plates were incubated with a second biotinylated monoclonal antibody and binding was detected with streptavidin-horseradish peroxidase (HRP). We found that clones 1-5A10, 1-8B7, and 2-8C10 inhibited binding of each other, indicating that they recognize the same or nearby epitopes (data not shown). Clone 2-2H8 was neither inhibited by, nor inhibited, the binding of the other three clones. Likewise, sera collected after immunization with D-OspC_{B31} inhibited the binding of 1-5-A10, 1-8-B7, and 2-8-C10 to a greater extent than binding of 2-2H8 was inhibited (data not shown). Similarly, monoclonal 2-2H8 was inhibited to a greater extent by sera from M-OspC_{B31}-immunized mice. These data indicate that the three monoclonal antibodies 1-5A10, 1-8B7, and 2-8C10 may recognize a unique epitope present or revealed in D-OspC_{B31}.

Passive protection with monoclonal antibodies. To investigate whether the monoclonal antibodies 1-5-A10, 1-8-B7, and 2-8-C10 that recognize unique D-OspC_{B31} epitopes could protect against *B. burgdorferi* challenge, we performed a passive immunization experiment. Mice were injected with each one of the monoclonal antibodies individually, normal mouse serum, serum from D-OspC_{B31}-immunized animals, or serum from M-OspC_{B31}-immunized animals and then challenged with *B. burgdorferi* B31. We found that monoclonal antibodies 1-5-A10, 1-8-B7, 2-8-C10, and serum from D-OspC_{B31}-immunized mice protected mice from challenge (Table 4; Fig. 6). In contrast, monoclonal antibody 2-2-H8 that recognizes the loop 5 epitope and serum from M-OspC_{B31}-immunized mice were not able to protect mice from infection. ELISA using sera from passively immunized mice demonstrated that all mice still had significant levels of anti-OspC reactivity (data not shown). Taken together, these results demonstrate that unique antibodies that protect against *B. burgdorferi* challenge can be generated by immunization with dimeric OspC and suggest that D-OspC_{B31} contains distinctive protective epitopes.

DISCUSSION

OspC is expressed at high levels during the first few days to weeks of *B. burgdorferi* infection and is a highly immunogenic lipoprotein (24, 37, 46, 47). These properties indicate that OspC is potentially useful as a vaccine antigen. Indeed, prior reports had indicated that OspC has immunoprotective activity, but the protective activity is allele specific (34, 37, 48). This limitation is important, in that OspC sequences in different *B.*

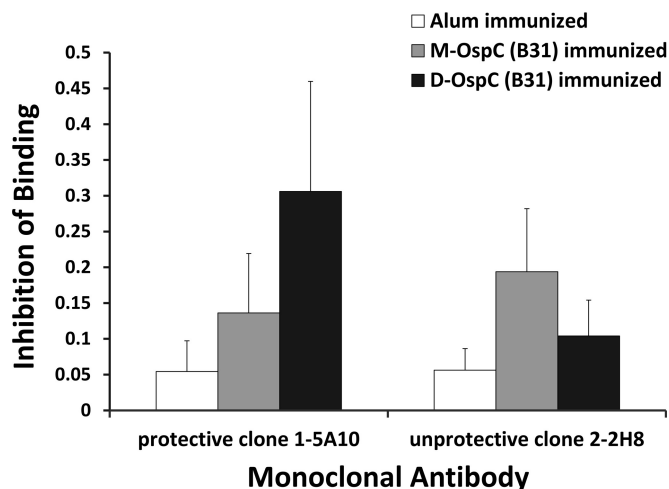


FIG 6 Murine sera from protected D-OspC_{B31}-immunized animals inhibited binding of monoclonal antibodies protective against *B. burgdorferi* B31 challenge. Sera from mice immunized with alum only, D-OspC_{B31}, or M-OspC_{B31} were used in ELISA to compete for binding with biotinylated monoclonal antibodies 1-5A10 and 2-2H8, and the proportion of inhibition was plotted. The antigen on the ELISA plates was D-OspC_{B31}. Sera were from mice immunized with alum only (white), M-OspC_{B31} (gray), or D-OspC_{B31} (black). Clone 1-5A10 was protective against *B. burgdorferi* challenge. Clone 2-2H8 reacted with the loop 5 epitope and was not protective. Each group consisted of 12 animals. Bars represent the mean \pm SD proportion of inhibition.

burgdorferi sensu lato strains are heterogeneous, with a degree of amino acid sequence variability that is second only to that of the antigenic variation protein VlsE (49). Probst et al. (40) previously showed that dimerization of OspC through an N-terminal disulfide bridge greatly enhanced reactivity with serum antibodies from humans with Lyme borreliosis and increased its ability to induce anti-OspC antibody responses in experimentally vaccinated animals.

The current study demonstrates that vaccination with the dimer form of recombinant OspC was much more effective than the monomeric form in inducing protective immunity in a mouse model. We used alum as an adjuvant because of its compatibility with use in humans. High dosages of 10 to 100 μ g were utilized initially. Even at these high doses, monomeric OspC was only partially protective against *B. burgdorferi* B31 infection, whereas the dimeric form was uniformly protective (Table 1). We found that D-OspC_{B31} at doses as low as 0.1 to 1.0 μ g was consistently effective and that three to four immunizations were required for full efficacy. In addition, the nodular reaction observed at the site of immunization was diminished or absent when lower doses of antigen (0.1 μ g to 1.0 μ g) were used. This reaction has been previously described when using aluminum salts as adjuvants (50). Three immunizations were still needed if monophosphoryl lipid A adjuvant was utilized instead of alum. However, it is possible that the poor protection observed with two immunizations could be due to the amount of time available for antibody response to develop (28 days for the two-immunization protocol versus 42 days for the three-immunization procedure) rather than number of immunizations.

Previous studies (34, 51) found that a single injection of *E. coli* lysate containing recombinant OspC or a two-immunization protocol of 100 μ g recombinant OspC was sufficient for protection against *B. burgdorferi* challenge. Our studies required three immunizations for full protection but used much lower immunogen doses. In addition, Gilmore et al. (51) found that, as antibody titer waned over a 1-year period, the mice were no longer protected from challenge. Our studies did not test whether a protective anamnestic response was elicited by dimeric OspC. However, since our immunogen and immunization protocol differed significantly from these authors, examination of long-term immunity induced by dimeric OspC is an important next step to explore its vaccine potential.

Similar results were obtained in terms of immunoprotection against the homologous strain when recombinant forms of OspC₂₉₇ were used, i.e., the disulfide-linked dimeric form was much more effective in protecting against infection than was the monomeric form. For OspC_{N40}, both forms of recombinant protein were effective in protecting mice from *B. burgdorferi* infection.

Interestingly, while D-OspC_{B31} generated antibodies that recognized OspC from *B. burgdorferi* 297 and N40 relatively weakly in ELISA (Fig. 4B), anti-D-OspC_{B31} (but not anti-M-OspC_{B31}) detected native OspC₂₉₇ and OspC_{N40} well on the Western blot (Fig. 4A). However, despite this recognition and the efficacy of low doses of D-OspC_{B31} against homologous challenge, the B31 antigen did not cross-protect against infection the *B. burgdorferi* strains 297 and N40. This pattern is consistent with the heterogeneity of OspC and the OspC cross-protection results obtained in prior studies (35, 37). However, D-OspC_{N40} did provide partial protection from *B. burgdorferi* 297 challenge and B31 challenge (2 of 6 animals protected in each case; Table 1). This result was unexpected, in that cross-protection was not obtained in prior OspC immunization studies. In fact, a lack of homologous protection using immunization with OspC_{N40} had been observed in a prior study by Bockenstedt et al. (37). This discrepancy may have resulted from the fact that the initial culture of N40 contained two different clones, which may differ in their immunologic properties; the clone we utilized is called cN40 (52). The unusual cross-protective activity of D-OspC_{N40} may be due to the presence of immunodominant epitopes that exhibit epitope-spreading properties. This interesting and potentially important observation will require further study to clarify the mechanism.

Ivanova et al. (53) showed that recombinant proteins of five specific OspC types (B, E, F, I, and K) could detect anti-OspC antibody from the serum of mice infected with any one of 15 different strains of *B. burgdorferi*, each of a different OspC type. The N40 OspC is of the E type, while the B31 OspC is of the A type, and the 297 OspC is of the I type (54). It is interesting to note that the partial cross-protection is observed from one of the broadly reactive OspC types.

Importantly, mice immunized with D-OspC_{B31} were also protected against tick-transmitted infection with *B. burgdorferi* B31 (Table 3), indicating that this immunization protocol is effective in preventing infection via the natural route of transmission. Further experiments with this model are ongoing and will explore the use of OspC mixtures to overcome the current limitations regarding cross-protection.

Several parameters were examined in an attempt to determine the mechanisms of heightened protection by the N-terminal dimerized form of OspC. Immunization with the monomeric and dimeric forms of OspC was found to yield similar levels of IgG subtypes, with a predominance of IgG1. In some experiments, higher total IgG or IgG1 levels were obtained with the monomeric antigen, yet the monomer-immunized mice were generally not protected against infection. Thus, the immunoprotective activity of the N-terminal dimerized OspC was not simply due to higher antibody levels or the induction of IgG1 anti-OspC antibodies. Binding inhibition studies indicated that the dimeric form of OspC presented at least one unique epitope that was not bound by antibodies induced by the monomeric form. In contrast to prior immunizations performed without adjuvant (40), sera from animals immunized with either D-OspC or M-OspC and the adjuvant alum have barely detectable levels of IgG3. In contrast, infection of unprotected mice resulted in induction of high levels of anti-OspC IgG3 (Fig. 3, upper panel). We speculate that infection and adjuvant-facilitated immunization result in differential stimulation of T cell activities and thus cytokine profiles, thereby resulting in altered patterns of IgG isotype switching. It is conceivable that infection with *B. burgdorferi* may drive the maturation of the anti-OspC-producing B lymphocytes weakly and lead to an arrest at the first IgG subclass, IgG3; indeed, OspC is expressed only during the first few weeks of mammalian infection (55, 56), which may limit immune maturation. Interestingly, the Baumgarth laboratory (57, 58) found that infection with *B. burgdorferi* leads to a strong T-independent B cell response with short-lived germinal centers and production of anti-*Borrelial* antibodies primarily of the IgM

isotype and very few of the IgG1 isotype. These results led them to speculate that *B. burgdorferi* evades clearance by altering the normal B cell response in the lymph nodes (57, 58). In any case, this differential pattern of isotype reactivity allows infected animals to be distinguished from immunized animals.

Perhaps the most revealing studies were those in which the reactivity and protective activity of monoclonal antibodies were generated by immunization of mice with D-OspC_{B31}. Hybridomas were selected that produced IgG1 antibodies reacting strongly with the immunizing antigen. The resulting monoclonal antibodies had differing reactivity with M-OspC_{B31}. Remarkably, three monoclonal antibodies that reacted strongly with D-OspC_{B31} but weakly with M-OspC_{B31} provided complete passive protection against infection of mice with the B31 strain, whereas the fourth monoclonal antibody that reacted strongly with both recombinant OspC proteins did not provide protection. These results support the concept that the antibodies to unique epitopes presented by D-OspC_{B31} are responsible for this protective activity. We hypothesize that these highly protective epitopes are present at the interface in this recombinant dimer form and mimic naturally occurring epitopes that are present in the OspC dimer on the cell surface of Lyme disease *Borrelia*.

The protective monoclonal antibodies exhibited a pattern of OspC peptide binding that is consistent with this hypothesis (Fig. 5). The B31_{loop5} peptide strongly inhibited binding of the nonprotective monoclonal antibody 2-2-H8 to the D-OspC_{B31} protein, indicating that this monoclonal antibody recognizes an epitope present in this peptide. However, none of the protective monoclonal antibodies exhibited detectable inhibition by this peptide. Therefore, these antibodies likely do not react with loop 5 in the OspC structure. Additionally, the monoclonal antibodies examined did not exhibit inhibition by a peptide corresponding to the C terminus, which has been implicated as a potentially protective antigen. It is of interest, however, that the C-terminal region of OspC is not required for tick transmission or mouse infection (59). The amino acid region(s) of OspC that forms the protective epitope(s) recognized by the passively protecting monoclonal antibodies has yet to be identified. Another future area for research would be to determine whether the protective monoclonal antibodies are OspC allele specific (as expected) or are able to cross-protect against non-B31 strains.

The enhanced protective immunogenicity of N-terminally dimerized forms of OspC provides some optimism that OspC constructs may be useful in vaccines against Lyme borreliosis. The artificial dimerization used here may stabilize a tertiary structure that contains protective epitopes found in the native OspC as expressed on the cell surface of *B. burgdorferi*. These epitopes may consist of amino acids from both OspC polypeptides (i.e., part of the interface of the dimerized proteins).

Alternatively, these dimer-specific epitopes could result from changes in the secondary structure of the two polypeptide chains arising from the protein-protein interaction; however, such secondary structure changes would be expected to be minor, given that the circular dichroism profiles of the monomer and dimer forms of these recombinant constructs are nearly identical (40).

Interestingly, OspC is believed to exist as a homodimer *in vivo*, and this interaction results in increased resistance to protease digestion (60). Crystal structures of OspC show that dimerization is mediated by interactions between alpha helices in each subunit (61, 62). In addition, dimerization results in the formation of a putative ligand binding pocket (61, 62) that contains some amino acid residues conserved across OspC phyletic types (63). The ligand binding pocket and one of the conserved residues within the pocket are required for infectivity (23). Possibly, the covalent dimerization stabilizes these important epitopes and thereby facilitates the production of protective antibodies.

Allele specificity still occurs with the disulfide-dimerized OspC constructs, but the results obtained with D-OspC_{N40} immunization suggest that the enhanced immunoprotective activity obtained may partially overcome this barrier and that specific OspC types may be able to provide some degree of cross-protection. In addition, given the lower quantity of antigen required, immunization with N-terminally dimerized OspC

antigens corresponding with multiple different OspC alleles may be a practical approach to future Lyme disease vaccine development.

MATERIALS AND METHODS

Ethics statement. All procedures involving mice were reviewed for effective experimental design and the humane treatment of animals and approved by the Animal Welfare Committee of The University of Texas Health Science Center at Houston or the Animal Use and Care Committee of the Division of Vector Borne Diseases, National Center for Emerging and Zoonotic Infectious Diseases (NCEZID), CDC.

Bacterial strains. The infectious *B. burgdorferi* strains B31-5A4 (64), N40 (52), and 297 (65) were used for animal studies. The N40 strain was the kind gift of J. D. Radolf, University of Connecticut Health Science Center, Farmington, CT, and the 297 strain was the gift of P. A. Rosa, Rocky Mountain Laboratories, Hamilton, MT. All *B. burgdorferi* strains were cultured in Barbour-Stoenner-Kelly II (BSK-II) medium at 34°C in 3% CO₂, as previously described (64). Cultures used in this study had undergone no more than two passages since thawing or clone isolation, thus minimizing the likelihood of plasmid loss. Infectivity in mice by needle inoculation was verified prior to the use of the strains in immunization studies.

Vector construction and production of recombinant proteins. Procedures for the construction of expression vectors and for expression and purification of recombinant proteins are described in the supplemental material. The PCR primers used for generating cloning fragments are described in Table S1. A schematic representation of the dimeric and monomeric forms of OspC utilized can be found in Fig. 1A.

Generation of monoclonal antibodies. Monoclonal antibodies were produced by immunization of mice with dimeric OspC from *B. burgdorferi* B31 (D-OspC_{B31}) at Eurogentec, using a standard protocol. Mouse sera and supernatants of hybridoma cultures were screened for the presence of antibodies against D-OspC_{B31}, M-OspC_{B31} (monomeric OspC from *B. burgdorferi* B31), and the peptides B31_{loop5} and B31_{Cterm} by ELISA and immunoblotting. Hybridoma clones were grown in RPMI 1640 medium supplemented with 10% (vol/vol) fetal calf serum, 1% (vol/vol) nonessential amino acids, 1 mmol/liter sodium pyruvate, and 50 μmol/liter 2-mercaptoethanol (Thermo Fisher Scientific, Germany) in standard culture flasks at 37°C and 7.5% CO₂. Antibodies from selected clones were purified by protein A-Sepharose affinity chromatography (GE Healthcare, Germany), according to the manufacturer's manual, dialyzed against phosphate-buffered saline (PBS), concentrated to 1 mg/ml, and stored at -80°C until use. For some experiments, the antibodies were biotinylated with the EZ-Link Sulfo-NHS-biotinylation kit (Thermo Fisher Scientific), as per the manufacturer's instructions.

Immunization and challenge of mice. Female C3H/HeN mice (4 to 6 weeks old) were immunized by subcutaneous injection with up to 100 μg of the recombinant OspC proteins with or without adjuvant. Immediately prior to injection, the antigens were emulsified with either Imject alum (Thermo Fisher Scientific) or 50% (vol/vol) monophosphoryl lipid A (MPL; Sigma), according to the manufacturer's instructions. Booster immunizations were administered at 2-week intervals. The amount of antigen and number of booster immunizations varied in different experiments. A typical immunization scheme is depicted in Fig. 1B. At the end of the immunization schedule, the mice were either needle inoculated with *in vitro*-cultivated *B. burgdorferi* (10⁴ cells of strain B31, clone 5A4; 10⁵ cells of strain 297; 10⁵ cells of N40) or challenged by tick bite from *B. burgdorferi* B31-infected nymphal *Ixodes scapularis* ticks. *B. burgdorferi*-infected ticks were generated as previously described (66). In the case of tick challenge, fed ticks were collected, and their infection states were analyzed by PCR. Mice were disregarded for further analysis if none of the ticks that fed on that animal were PCR positive for *B. burgdorferi* (Table S2).

Serum samples were collected from each animal prior to immunization, before each boost, at challenge, and at sacrifice. The mice were sacrificed at 2 weeks postinfection, and selected tissues (ear, inoculation site, heart, joint, and bladder) were harvested and cultured in BSK-II medium. Cultures were monitored by dark-field microscopy for the presence of spirochetes at 7, 14, and 28 days.

For passive immunization experiments, mice received a 200-μg dose of monoclonal antibody or 0.1 ml of previously frozen preimmune or immune mouse serum intraperitoneally 1 day prior to challenge with *B. burgdorferi*. Five days after inoculation, animals were given an additional 200-μg dose of monoclonal antibody or a 0.05-ml dose of preimmune or immune serum. Immune sera were derived from pooled serum collected prior to challenge from protected/D-OspC_{B31}-immunized mice or from unprotected/M-OspC_{B31}-immunized mice. Two weeks after challenge, the animals were euthanized. Blood was drawn to test for anti-OspC activity, and tissues were cultured as indicated above.

Immunoassays for the detection of murine antibodies. Ninety-six-well plates (Nunc, Germany) were coated with 100 μl of the recombinant protein at a concentration of 1 μg/ml in PBS for 2 h at 25°C, washed three times with washing buffer (0.05% [wt/vol] Tween 20 in PBS), and then blocked with blocking buffer (0.1% [wt/vol] casein in PBS) for 1 h. The success of antigen immobilization was confirmed by incubation with a murine monoclonal anti-hexahistidine tag antibody (Sigma-Aldrich, Germany) diluted 1:2,000. Experimental serum samples were diluted in sample buffer (1% [wt/vol] casein, 0.05% [wt/vol] Tween 20 in PBS) and incubated for 30 min at room temperature. After washing three times, bound antibodies were detected by incubation with anti-mouse IgG-HRP conjugate (Jackson Research, UK) diluted 1:2,000 in sample buffer, for 30 min, washed as described above, and incubated with tetramethyl benzidine (TMB) substrate (Euroimmun, Germany) for 15 min. All incubation steps were carried out at room temperature. The optical density (OD) at 450 nm was read using an automated spectrophotometer (Tecan, Germany). The reactivity of murine sera was analyzed for IgG subclasses using the same procedure, except for the use of subclass-specific conjugates diluted 1:10,000 (Jackson Research).

For liquid-phase inhibition experiments, 10 $\mu\text{g/ml}$ recombinant protein was added to the diluted sera 30 min prior to their incubation on the microplate. Percent inhibition was calculated as $100 \times [1 - (\text{Abs with inhibitor})/(\text{Abs without inhibitor})]$ (Abs, absorbance).

For the analysis of antibody reactivity to synthetic peptides (OspC B31_{loop5} [FTNKLKEKHTDLGK] and OspC B31_{Cterm} [KELTSPVVAESPCKP]), N-terminally biotinylated C-terminally carboxylated peptides (Eurogentec, Belgium) were immobilized on streptavidin-coated microplates (Euroimmun) at a concentration of 0.5 $\mu\text{g/ml}$. Blocking and sample buffers contained 0.5% (wt/vol) bovine serum albumin in PBS; all other parameters were identical to those of the protein-based ELISA. Sera were also analyzed by immunoblotting, as described by Probst et al. (40).

For the analysis of competition between murine serum antibodies and monoclonal antibodies against OspC, biotinylated monoclonal antibodies diluted in sample buffer were incubated for 30 min following the serum incubation step. Immobilized biotin was visualized using streptavidin-HRP conjugate (Sigma-Aldrich, Germany) diluted 1:5,000 in sample buffer, followed by washing and detection as described above (Fig. 6).

PepScan analysis. PepScan analysis is described in the supplemental material.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at [https://doi.org/10.1128/ CVI.00306-16](https://doi.org/10.1128/CVI.00306-16).

TEXT S1, PDF file, 0.3 MB.

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