INFECTION AND IMMUNITY, Nov. 2000, p. 6457–6460 Vol. 68, No. 11
0019-9567/00/$04.00+0
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NOTES

Evidence for Vaccine Synergy between *Borrelia burgdorferi* Decorin Binding Protein A and Outer Surface Protein A in the Mouse Model of Lyme Borreliosis

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Received 14 April 2000/Returned for modification 12 June 2000/Accepted 24 August 2000

Mice immunized with either the predominantly vector-stage lipoprotein outer surface protein A (OspA) or the in vivo-expressed lipoprotein decorin binding protein A (DbpA) are protected against *Borrelia burgdorferi* challenge. DbpA-OspA combinations protected against 100-fold-higher challenge doses than did either single-antigen vaccine and conferred significant protection against heterologous *B. burgdorferi*, *B. garinii*, and *B. afzelii* isolates, suggesting that there is synergy between these two immunogens.

Lyme disease (20), or Lyme borreliosis, is a tick-borne illness of humans and domestic animals caused by at least three antigenically diverse species of spirochetes (*Borrelia burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii*) classified collectively as *B. burgdorferi* sensu lato. Recent clinical trials showed that monovalent recombinant subunit vaccines composed of the *B. burgdorferi* outer surface protein A (OspA) lipoprotein were efficacious through two Lyme disease transmission seasons (19, 21).

At the start of feeding, *B. burgdorferi* spirochetes in ticks are highly vulnerable to OspA antibodies imbibed from immunized hosts (9), but after adaptation to the mammalian host environment following natural or experimental inoculation, most spirochetes down-regulate OspA expression and become resistant to OspA antibodies (3, 5, 9, 14). Protection from tick-borne transmission of *B. burgdorferi* appears to depend on OspA immunization achieving a critical threshold level of circulating antibodies prior to the tick bite (8).

The addition of mammalian-host-stage antigens may extend the duration, or enhance the level, of protective efficacy of transmission-blocking OspA vaccines against tick-borne Lyme borreliosis. Decorin binding protein A (DbpA) is another *B. burgdorferi* surface-exposed lipoprotein that has shown vaccine efficacy against experimental infection in the mouse model (5, 10, 13, 14). *B. burgdorferi* continues to express DbpA, but not OspA, after dermal inoculation and remains vulnerable to DbpA antibodies during the early stages of local and disseminating infection in mice (5, 14); additionally, DbpA is immunogenic during human Lyme disease (6). OspC (25) and other antigens (1, 11) on mammalian-host-adapted *B. burgdorferi* also represent potential targets for protective or disease-resolving antibodies. We compared the protective efficacies of DbpA and OspA, singly and in combination, against dermal challenge of mice as a first step in the evaluation of second-generation DbpA-OspA combination vaccines for Lyme disease.

The recombinant fusion lipoproteins Lpp2:$OspA_{N40}$ ($OspA_{N40}$) and Lpp2:$DbpA_{N40}(His)_6$ ($DbpA_{N40}$) were used as vaccine antigens and had been previously described (5, 14). A detergent extract of *Escherichia coli* membrane proteins (5) was used as a negative-control antigen preparation.

In one vaccine experiment, four groups of 20 female 7-week-old C3H/HeJ mice (The Jackson Laboratory, Bar Harbor, Maine) were immunized by intraperitoneal injection of 10 μg of $DbpA_{N40}$, 10 μg of $OspA_{N40}$, 5 μg of $DbpA_{N40}$ plus 5 μg of $OspA_{N40}$, or 2.5 μg of *E. coli* protein extract with complete Freund's adjuvant and then, 4 weeks later, were given a second immunization of protein in incomplete Freund's adjuvant. At week 6, five of the mice in each immunization group were challenged by subcutaneous injection, into the dorsolateral thorax, of cloned *B. burgdorferi* N40 (2) from an exponentially growing culture diluted with BSKII medium (14) to give escalating doses of 10^3, 10^4, 10^5, or 10^6 spirochetes in 0.1-ml volumes. Other experiments used vaccines prepared by adsorbing antigens to the aluminum hydroxide adjuvant Alhydrogel (Superfos Biosector, Kvistgård, Denmark). Mice were immunized by subcutaneous injection of 0.1 ml of vaccine at weeks 0, 4, and 8 and challenged with spirochetes at week 10. A challenge dose of 10^4 was used for *B. burgdorferi* N40 and Sh-2-82; a dose of 10^5 was used for *B. garinii* G25 and *B. afzelii* IPF. The median infective doses for these isolates were determined to be approximately 3×10^2 for N40 (14), 6×10^2 for Sh-2-82 (14), 3×10^3 for G25, and 2×10^4 for IPF. Two weeks after challenge, the mice were killed by CO_2 asphyxiation and samples of the inoculation site skin, blood, ear, urinary bladder, and both tibiotarsal joints were cultured in BSKII plus antibiotics to detect spirochetal infection (14).

An enzyme-linked immunosorbent assay was used as previously described (14) to determine the prechallenge DbpA and OspA immunoglobulin G (IgG) endpoint titers of antisera from individual mice and antisera pooled from mice within each immunization group. The borreliacidal activity of the prechallenge antisera was determined, in a microtiter plate format, as the dilution of antiserum from each individual mouse giving 50% growth inhibition by a [^{3}H]adenine metabolic labeling method (15) or as the dilution of pooled antiserum giving a >90% reduction in spirochete numbers (14).

Antigen expression by the spirochetes was evaluated by a direct immunofluorescence assay using combined $DbpA_{N40}$- and $OspA_{B31}$ (14)-specific purified rabbit polyclonal IgG antibodies conjugated with the fluorochromes Alexa 546 and Alexa

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TABLE 1. Comparison of the immunogenicities and in vitro potencies of antisera from mice vaccinated with $DbpA_{N40}$ and $OspA_{N40}$, singly and in combination, and their protective efficacies against challenge with escalating *B. burgdorferi* N40 doses

Immunogen(s) (dose)	Antiserum geometric mean titer[a]			No. of mice protected/total no. challenged at challenge dose:			
	DbpA IgG	OspA IgG	Causing 50% growth inhibition	10^3	10^4	10^5	10^6
DbpA (10 μg)	1,910,852	500	2,263	0/5[b]	0/5[b]	2/5	2/5
OspA (10 μg)	966	4,389,984	67,559	0/5[b]	1/5[b]	3/5	5/5
DbpA (5 μg) + OspA (5 μg)	2,048,000	4,389,984	53,006	0/5[b]	0/5[b]	0/5[b]	0/5[b]
E. coli extract (2.5 μg)	3,364	27,858	<50[c]	5/5	5/5	5/5	5/5

[a] Values are the geometric means of data for the 20 mice in each immunization group.
[b] $P < 0.05$ versus controls (Fisher's exact test).
[c] No inhibition at lowest dilution tested (1:50).

488, respectively, according to the manufacturer's protocol (Molecular Probes, Inc., Eugene, Oreg.). Double-labeled slides were viewed at 1,000× magnification, using a Nikon E600 epifluorescence microscope (Nikon, Melville, N.Y.), and images were acquired with a Sony DKC-5000 digital photo camera (Sony Electronics, Inc., Park Ridge, N.J.).

DbpA and OspA in combination protect against higher *B. burgdorferi* challenge doses than single-antigen vaccines. The possibility that dual immunity to both DbpA and OspA could provide more effective protection than either single antigen alone was addressed in two complementary ways. First, we attempted to exceed the *B. burgdorferi* challenge dose at which the single antigens provided protection, and second, we asked whether DbpA-OspA combinations provided protection at a lower vaccine dose that was ineffective for either single immunogen. Nearly all mice immunized with 10 μg of either $DbpA_{N40}$ or $OspA_{N40}$ were protected from challenge with 10^3 or 10^4 spirochetes (Table 1), as expected from our earlier observations (14). At a challenge dose of 10^5 or 10^6 spirochetes, mice immunized with $DbpA_{N40}$ or $OspA_{N40}$ alone were protected only partially or not at all. In contrast, all mice immunized with the combined $DbpA_{N40}$-$OspA_{N40}$ vaccine (5 μg of each antigen) were protected against even the highest challenge dose. The 10^6 challenge inoculum is at least 3,000 times higher than the median infectious dose for this *B. burgdorferi* strain (14). At each challenge dose, all mice vaccinated with the *E. coli* extract were infected, with at least three of the five tissues tested being culture positive for *B. burgdorferi*. The antisera from all mice immunized with either $DbpA_{N40}$ or $OspA_{N40}$, or the combination of both proteins, inhibited the in vitro growth of *B. burgdorferi* N40, and antisera from *E. coli*-immunized mice were not borreliacidal. The in vitro killing potency of the antisera from $DbpA_{N40}$-immunized mice was about 20-fold lower than that of $OspA_{N40}$, probably because DbpA is expressed at much lower levels than OspA in vitro (5, 14). Interestingly, the potency of $OspA_{N40}$ antisera was not significantly different from that of the combined vaccine ($P = 0.43$, Student's two-tailed t test) in this in vitro assay. Our observations clearly showed that the in vitro and in vivo potencies of $DbpA_{N40}$ and $OspA_{N40}$ antibodies were divergent and that surrogate in vitro assays were inadequate to predict the relative effectiveness of single-antigen and combined-antigen vaccines. The enhanced in vivo activity of $DbpA_{N40}$ antibodies is likely due to prolonged or increased vulnerability of the spirochetes to $DbpA_{N40}$ antibodies (5, 14), the potentiating effects of immune effector functions toward $DbpA_{N40}$ but not $OspA_{N40}$ antibodies, or a combination of the two effects.

DbpA and OspA in combination are more effective than single-antigen vaccines against heterologous *B. burgdorferi* sensu lato isolates. Next, mice were immunized with single-antigen or $DbpA_{N40}$-$OspA_{N40}$ combination vaccines formulated with Alhydrogel, an adjuvant approved for use in humans. Antisera from mice immunized with either 1.0- or 10-μg doses inhibited the in vitro growth of *B. burgdorferi* N40, and again, $DbpA_{N40}$ antiserum was less potent for killing in vitro than antisera against $OspA_{N40}$ or the $DbpA_{N40}$-$OspA_{N40}$ combination vaccine (Table 2). Mice were well protected against challenge with a 10^4 dose of the homologous *B. burgdorferi* N40 strain when immunized with the 10-μg dose regimen of $DbpA_{N40}$ (9 of 10), $OspA_{N40}$ (10 of 10), or the $DbpA_{N40}$-$OspA_{N40}$ combination vaccine (10 of 10). However, the $DbpA_{N40}$-$OspA_{N40}$ combination vaccine also elicited significant protection (8 of 10) at the 1.0-μg dose, a level at which the single-antigen vaccines were only partially

TABLE 2. Comparison of the relative protective efficacies of immunizations with $DbpA_{N40}$ and $OspA_{N40}$, singly and in combination and at either of two doses, against challenge with homologous and heterologous *B. burgdorferi* sensu lato isolates

Immunogen(s)	Dose (μg)	Result with challenge isolate:							
		B. burgdorferi N40		*B. burgdorferi* Sh-2-82[a]		*B. garinii* G25[a]		*B. afzelii* IPF[a]	
		Growth inhibition titer[b]	Infection prevalence	Growth inhibition titer[b]	Infection prevalence	Growth inhibition titer[b]	Infection prevalence	Growth inhibition titer[b]	Infection prevalence
DbpA	10	1,600	1/10[c]	1,600	10/10	200	6/10[d]	800	7/10
	1	200	6/10[d]	200	10/10	ND[e]	8/10	ND	9/10
OspA	10	12,800	0/10[c]	12,800	6/10[d]	800	5/10[d]	200	10/10
	1	3,200	7/10	3,200	9/10	ND	7/10	ND	9/10
DbpA + OspA	5 + 5	6,400	0/10[c]	12,800	0/10[c]	3,200	2/10[c]	800	4/10[d]
	0.5 + 0.5	3,200	2/10[c]	3,200	0/10[c]	ND	3/10[c]	ND	7/10
E. coli	2.5	<50	10/10	<50	10/10	<100	10/10	<50	9/10

[a] Sequence identities for DbpA versus N40: Sh-2-82, 66.5%; G25, 53.3%; IPF, 33.7%. Sequence identities for OspA versus N40: Sh-2-82, 99.6%; G25, 80.7%.
[b] Values are the means of duplicate determinations on antisera pooled from the 10 mice within each immunization group. The growth inhibition titer is the antiserum dilution factor for a >90% reduction of the spirochete number.
[c] $P < 0.01$ versus control (Fisher's exact test).
[d] $P < 0.05$ versus control (Fisher's exact test).
[e] ND, not determined.

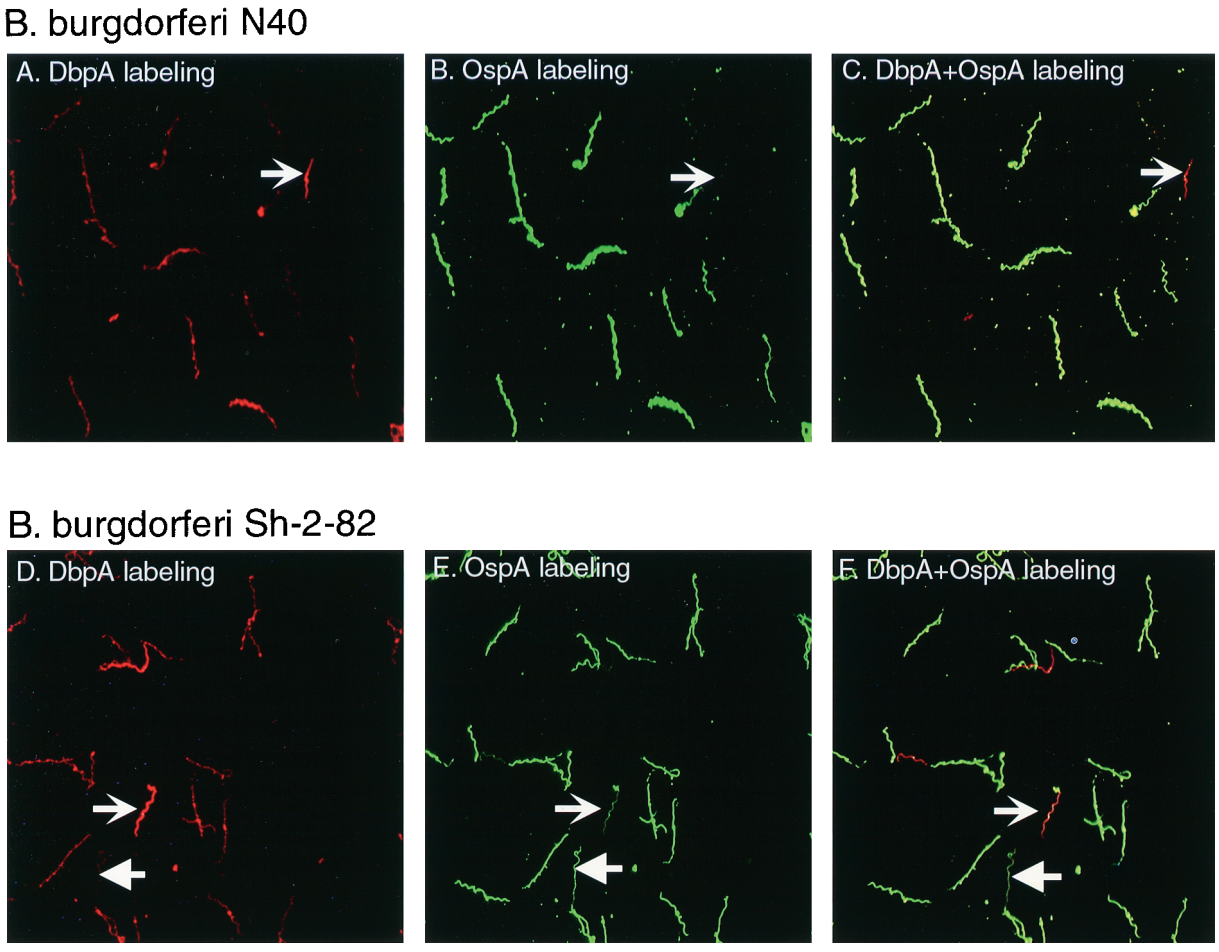

FIG. 1. Detection of *B. burgdorferi* phenotypic variants deficient in DbpA or OspA by direct immunofluorescence assay. *B. burgdorferi* N40 and Sh-2-82 spirochetes from standard cultures were fixed to microscope slides and double labeled with a combination of two fluorochrome-conjugated IgGs, prepared against DbpA and OspA. Staining of a representative microscopic field for *B. burgdorferi* N40 (A to C) and for *B. burgdorferi* Sh-2-82 (D to F) is shown. Images were acquired alternately with the green filter (A and D) (DbpA labeling), the blue filter (B and E) (OspA labeling), or the triple-band-pass filter (C and F) (DbpA and OspA labeling). Leftward-pointing arrows indicate representative DbpA-deficient spirochetes, and rightward-pointing arrows indicate representative OspA-deficient spirochetes.

protective. Nearly identical results were also obtained with a 0.1-μg dose and with adjuvant-free formulations of these antigens, demonstrating the intrinsic immunogenicity of the recombinant lipoproteins (data not shown).

We next examined the relative efficacies of single-antigen and combination vaccines formulated with Alhydrogel against heterologous challenge with *B. burgdorferi* sensu stricto Sh-2-82, *B. garinii* G25, and *B. afzelii* IPF, isolates that express DbpA and OspA proteins whose sequences are substantially divergent (12, 17, 18, 22) (Table 2) from those of the $DbpA_{N40}$ and $OspA_{N40}$ immunogens. The $DbpA_{N40}$-$OspA_{N40}$ combinations were more effective against all these heterologous challenges than single-antigen vaccines (Table 2). Only partial protection (6 of 10) at the 10-μg dose was achieved against the most divergent isolate, *B. afzelii* IPF, but this reached significance ($P = 0.027$).

In each vaccination experiment, we consistently observed that the various formulations of the $DbpA_{N40}$-$OspA_{N40}$ combination (Freund's, Alhydrogel, or adjuvant free) were more effective than the same dose of either single antigen. Since the single-antigen and combined-antigen vaccines were compared at doses of equivalent total mass, the improved effectiveness of the $DbpA_{N40}$-$OspA_{N40}$ combination was not merely due to an additive effect of the two immunogens but rather provided evidence of vaccine synergy between DbpA and OspA in this model.

Heterogeneity of OspA and DbpA expression by *B. burgdorferi* may limit efficacy of single-antigen vaccines. Cultures of cloned *B. burgdorferi* N40 and uncloned *B. burgdorferi* Sh-2-82 were found to be heterogeneous for DbpA and OspA expression when double labeled and examined by direct immunofluorescence microscopy (Fig. 1). One percent of *B. burgdorferi* N40 spirochetes appeared to express little or no OspA, and 2 to 3% expressed little or no DbpA. For *B. burgdorferi* Sh-2-82, the OspA and DbpA variants represented approximately 9 and 11% of the population, respectively. It is likely that phenotypic heterogeneity in the inoculum contributed to the limited efficacy of the single-antigen vaccines (Tables 1 and 2), particularly for *B. burgdorferi* Sh-2-82. This phenotypic heterogeneity may be relevant to tick-transmitted *B. burgdorferi*, since one recent study reported that spirochetes in salivary glands of feeding ticks expressed host-stage OspC predominantly but some still expressed OspA (7).

OspC is another in vivo-expressed *B. burgdorferi* antigen that has been evaluated with OspA as a combination vaccine. In that study (4), the addition of OspC did not improve upon the vaccine efficacy of OspA alone, but it was also shown that OspC immunization was ineffective against challenge with this particular *B. burgdorferi* strain (N40). The serological heterogeneity of OspC (23, 24) complicates vaccine design, and thus far only strain-specific protection has been reported with this immunogen (16).

We have shown that a vaccine combining the predominantly vector-stage OspA and the mammalian-host-stage DbpA is superior to either antigen alone against experimental *B. burgdorferi* challenge of mice. The enhanced efficacy of the $DbpA_{N40}$-

$OspA_{N40}$ combination was not merely due to the additive mass of the two immunogens but appeared to be mediated, at least in part, by sustained vulnerability of the spirochetes to DbpA antibodies during early disseminating infection (5, 14). We recognize that dermal injections of cultured spirochetes do not recapitulate the *B. burgdorferi* inoculum delivered by the natural route of infection, and tick challenge studies are ultimately required to validate Lyme disease vaccine candidates. The effectiveness of combined DbpA-OspA immunity against experimental infection now provides the impetus for performing studies, possibly of a more complicated nature, using the natural route of infection. Given the several possible mechanisms of complementary interactions between DbpA and OspA immune responses, DbpA-OspA combinations may have a role as second-generation Lyme disease vaccines. Vaccinees receiving DbpA-OspA may also benefit from an anamnestic immune response to DbpA upon natural infection that is unlikely to occur with OspA alone.

We thank Alan Barbour, Stephen Barthold, and Russell Johnson for providing *Borrelia* isolates. We thank Christine Bachy and Christine Fazenbaker for technical assistance and Luis Branco for advice on fluorescence microscopy. We also thank Scott Koenig and Syd Johnson for helpful discussions and for critical review of the manuscript, and we are grateful to Donni Leach for assistance in its preparation.

ADDENDUM

While the manuscript was being reviewed, a paper by Hagman et al. (13a) reporting that DbpA immunity was ineffective at preventing tick-borne transmission of *B. burgdorferi* infection to mice was published. These authors suggest that DbpA is a host stage antigen but is not a target for protective antibodies, a theory that conflicts with our earlier reports (5, 14). Differences in the potencies of the DbpA immunogens used in the two studies may be a factor contributing to this apparent discordance. Hagman et al. used a recombinant cytosolically expressed form of DbpA, lacking posttranslational modifications, that conferred only partial protection against experimental *B. burgdorferi* challenge (13) even with much higher vaccine doses (20 to 50 μg in Freund's adjuvant) than those that were effective for our acylated and secreted DbpA in the present study. We have found that conformational epitopes contribute substantially to DbpA immunity (N. D. Ulbrandt, N. K. Patel, and M. S. Hanson, unpublished data), as has been reported for other *B. burgdorferi* vaccine antigens (9a, 11a).

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Editor: R. N. Moore