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Active and Passive Immunity against *Borrelia burgdorferi* Decorin Binding Protein A (DbpA) Protects against Infection

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***Borrelia burgdorferi*, the spirochete that causes Lyme disease, binds decorin, a collagen-associated extracellular matrix proteoglycan found in the skin (the site of entry for the spirochete) and in many other tissues. Two borrelial adhesins that recognize this proteoglycan, decorin binding proteins A and B (DbpA and DbpB, respectively), have recently been identified. Infection of mice by low-dose *B. burgdorferi* challenge elicited antibodies against DbpA and DbpB that were sustained at high levels, suggesting that these antigens are expressed in vivo. Scanning immunoelectron microscopy showed that DbpA was surface accessible on intact borreliae. Passive administration of DbpA antiserum protected mice from infection following challenge with heterologous *B. burgdorferi* sensu stricto isolates, even when serum administration was delayed for up to 4 days after challenge. DbpA is the first antigen target identified that is capable of mediating immune resolution of early, localized *B. burgdorferi* infections. DbpA immunization also protected mice from *B. burgdorferi* challenge; DbpB immunization was much less effective. DbpA antiserum inhibited in vitro growth of many *B. burgdorferi* sensu lato isolates of diverse geographic, phylogenetic, and clinical origins. In combination, these findings support a role for DbpA in the immunoprophylaxis of Lyme disease and suggest that DbpA vaccines have the potential to eliminate early-stage *B. burgdorferi* infections.**

Lyme disease (53), or Lyme borreliosis, is caused by a group of related tick-borne spirochetes classified as *Borrelia burgdorferi* sensu lato (including *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*). Although much progress has been made in the characterization of the organism, spirochetal factors responsible for infectivity, immune evasion, and disease pathogenesis remain largely obscure. The most-studied *B. burgdorferi* membrane protein is outer surface protein A (OspA), a lipoprotein antigen expressed by borreliae in resting ticks and the most abundant protein expressed in vitro by most *B. burgdorferi* sensu lato isolates (2, 32). Experimental OspA vaccines have demonstrated efficacy in several animal models (11, 19, 24, 33), and OspA vaccines for human use are the subjects of current clinical evaluations (35, 36, 57).

During tick engorgment, OspA expression by borreliae diminishes (15) while expression of other proteins, exemplified by OspC, increases (51). By the time of *B. burgdorferi* transmission to hosts, spirochetes in the tick salivary glands express little or no OspA. This diminished expression of OspA appears to explain the weak early immune responses to this antigen in experimental or natural infection following tick-borne infection (26, 34, 47, 49) or following inoculation with minimal infectious doses of cultured spirochetes (3, 47). OspA antibodies are sometimes detected in later stages of infection (34, 50), implying that this antigen may be reexpressed by at least some spirochetes during infection. For these reasons OspA-specific antibodies are ineffective in eliminating infection when they are administered after infection is established by syringe inoculation (18, 48) or tick bite (15). Additionally, OspA immunity is circumvented by challenges of in vivo-adapted borreliae in the form of transplants of skin from infected donors into OspA-immunized mice (7). OspA immunization has been shown to mediate killing of spirochetes directly in the midgut of feeding ticks and therefore has a primary mode of action at the vector stage (15, 24). To be efficacious, OspA vaccines must elicit protective levels of antibody that must be maintained throughout periods of tick exposure in order to block spirochete transmission from the vector.

Vaccines against pathogens other than *Borrelia* are often based on in vivo-expressed antigens that boost anamnestic responses upon infection, potentiate the action of immune effector cells and complement, and inhibit key virulence mechanisms. Many laboratories have examined the vaccine potential of *B. burgdorferi* antigens that are immunogenic during infection and therefore presumably expressed in vivo. OspC is expressed during infection (37) and elicits protective immunity in rodents (27, 40, 42). However, OspC-immunized mice appear not to be protected against challenge with heterologous *B. burgdorferi* isolates (41), and in some cases OspC immunization failed to protect mice against challenge with even homologous *B. burgdorferi* isolates (6, 13). OspE and OspF are immunogenic during infection, but OspF elicits only partial protection against tick-borne or low-dose (10^2 borreliae) intradermal challenge and OspE is ineffective as a protective immunogen (38). Other immunogenic in vivo-expressed antigens that have been evaluated as targets for protection, including 41-kDa flagellin, P30, P39, P55, P83, IpLA-7, the OspE homolog P21, and the OspF homolog pG, have thus far failed to show efficacy in the prevention of infection (14, 14a, 17, 20, 27, 42, 59, 60). Identification of in vivo-expressed *B. burgdorferi* antigens that are capable of eliciting broadly protective immune responses has remained elusive.

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In mammalian hosts, *B. burgdorferi* infection is initiated when the tick vector deposits the spirochetes into the dermis during feeding. At both initial and later stages of infection, *B. burgdorferi* is commonly found in association with collagen fibers in the extracellular matrix (5, 58). Recent findings (29) suggest that colonization of these collagenous tissues may be mediated by spirochetal surface adhesins binding specifically to the collagen-associated proteoglycan decorin (12). These adhesins were expressed at low-to-moderate copy numbers on cultured borreliae (29). Genes for two adhesins of *B. burgdorferi* recognizing decorin, lipoproteins with apparent masses of 18 to 20 kDa called decorin binding proteins A and B (DbpA and DbpB, respectively), have been partially characterized (28). In the current study, we examined the immunogenicity of DbpA and DbpB during infection, examined their accessibility to antibodies and efficacy as potential vaccine candidates, and evaluated the serological conservation of these proteins.

(This work was presented, in part, at the 14th annual meeting on Modern Approaches to the Control of Infectious Diseases, 9 to 13 September 1996, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., and in a preliminary report [10a] resulting from that meeting.)

MATERIALS AND METHODS

***B. burgdorferi* isolates.** Isolates of *B. burgdorferi* sensu lato were donated by the laboratories of A. Barbour, S. Barthold, R. Johnson, J. Leong, and S. Norris or from the Rocky Mountain Laboratories Microscopy Branch collection. The biologic and geographic origins of these isolates are listed in Table 1. The molecular typing method described by Postic et al. (39) was used to confirm the phylogenetic designation of each of these isolates. Spirochetes were propagated in tightly closed containers at 34°C in modified Barbour-Stoenner-Kelly (BSKII) medium (1) overlaid with a 5% O_2–5% CO_2–90% N_2 gas mixture. Cell densities of these cultures were determined by dark-field microscopy at a magnification of ×400. Batches of BSKII were qualified for spirochete propagation and infection testing by confirming that they supported the growth of 1 to 5 cells of isolate B31. Infectivity of the *B. burgdorferi* sensu lato isolates for mice was assessed as described below. The following isolates used for immunologic protection studies were determined to have the median infectious dose (ID_{50}) values indicated: B31, 6×10^1; Sh-2-82, 6×10^2; N40, 3×10^2; 297, 3×10^3; 25015, 6×10^1; HB19, 3×10^3; and CA-3-87, 3×10^1.

Expression and purification of recombinant immunogens. Several recombinant forms of DbpA, schematically represented in Fig. 1, were expressed, purified, and used for immunologic analyses and immunizations. Molecular cloning was accomplished by standard methods (46). A library (a gift of Robin Isaacs) of approximately 2-kb *Sau*3A I fragments of total genomic DNA from a low-passage culture of *B. burgdorferi* 297 was constructed in Lambda Zap II (Stratagene Cloning Systems, La Jolla, Calif.), and plaques were plated and blotted onto nitrocellulose membranes by standard methods (46). Phage was recovered from plaques binding digoxigenin-conjugated decorin (29), and *B. burgdorferi* DNA inserts were excised as clones in the phagemid vector pBluescript II (pBsII) SK(−) according to the manufacturer's protocol. One of these clones, recombinant plasmid pBG26 (ATCC 69791), harbored a 2.5-kb *B. burgdorferi* DNA insert containing two 561-bp open reading frames (28). Both of these two genes expressed products with decorin binding activity and were subsequently named *dbpA* (GenBank accession no. U75866) and *dbpB* (GenBank accession no. U75867). The nucleotide sequences of *dbpA* and *dbpB* shared 50% identity. Single homologs of the *dbpA* and *dbpB* genes have been identified recently in the whole genome sequence of *B. burgdorferi* B31, residing on the linear plasmid lp54, which also contains the *ospAB* operon (25a). The B31 homologs share 94 and 100% similarity, respectively, with the original *dbpA* and *dbpB* genes from *B. burgdorferi* 297. Recombinant clone pBG29 was constructed from pBG26 by *Hin*dIII partial digestion, with deletion of the 5′ half of the *dbpB* gene and retaining full-length *dbpA* and expression of its product. $DbpA_{297}$ was purified from *Escherichia coli* JM101/pBG29 by affinity chromatography on decorin-conjugated Sepharose. Decorin purified (12) from fetal bovine skin, a kind gift of L. C. Rosenberg (Montefiore Medical Center, New York, N.Y.), was covalently linked to CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. A 1-liter culture of JM101/pBG29 in LB medium (46) plus 100 μg of ampicillin per ml was shaken until an A_{600} value of 0.6 to 0.8 was reached, isopropylthiogalactoside (IPTG) was added to 0.2 mM, and shaking was continued for 2 to 3 h. Cells were suspended in 10 ml of phosphate-buffered saline (PBS) and lysed in a French pressure cell, and the soluble fraction was collected following centrifugation at 200,000 × *g*. The supernatant fluid was passed through a 0.45-μm-pore-size filter, and 5 ml of the filtrate was passed through a 2-ml decorin-Sepharose column that bound the DbpA. After the column was washed with 20 ml of PBS, DbpA was eluted from the column with 1 M NaCl and recovered at >90% homogeneity as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). DbpA remained soluble after dialysis against PBS. Amino-terminal sequencing showed that this soluble DbpA retained the leader peptide and was the precursor form (pre-$DbpA_{297}$) of the mature lipoprotein.

Posttranslational modification may influence the immunogenicity of some antigens such as OspA (16, 33, 54, 57). Because posttranslational processing of pre-$DbpA_{297}$ expressed by JM101/pBG29 appeared to be inefficient, we constructed new vectors, pT7Lpp1 and pT7Lpp2, capable of expressing proteins as fusions with leader peptides derived from the abundant *E. coli* lipoprotein Lpp in order to increase posttranslational processing efficiency. Others had previously shown that a similar Lpp leader fusion strategy was effective for OspA (30). To construct pT7Lpp1 the *Nde*I-*Eco*RV fragment of pET-30b (Novagen, Inc., Madison, Wis.) was replaced with a synthetic DNA fragment created by annealing the oligonucleotides 5′-TATGAAAGCTACTAAACTGGTACTGGGCGCGGTA ATCCTGGGTTCTACTCTGCTGGCAGCATGCGATCAGAT-3′ and 5′-ATC TGATCGCATGCTGCCAGCAGAGTAGAACCCAGGATTACCGCGCCCA GTACCAGTTTAGTAGCTTTCA-3′ encoding MKATKLVLGAVILGSTLLA ACDQ, a modified version of the 5′ end of the *lpp* gene (GenBank accession no. V00302). A second vector, pT7Lpp2, was created in a similar manner with the oligonucleotides 5′-TATGAAAGCTACTAAACTGGTACTGGGCGCGGTA ATCCTGGGTTCTACTCTGCTGGCAGGTTGCTCCTCGAT-3′ and 5′-ATC GAGGAGCAACCTGCCAGCAGAGTAGAACCCAGGATTACCGCGCCCA GTACCAGTTTAGTAGCTTTCA-3′, encoding the exact sequence of the amino-terminal end of Lpp, MKATKLVLGAVILGSTLLAGCSS. DNA encoding the entire sequence (28) of the mature $DbpA_{297}$ protein (GenBank accession no. U75866), after the cysteine at the site of posttranslational modification, was amplified from *B. burgdorferi* 297 template DNA by PCR with oligonucleotide primers 5′-CCGGATCCCGGACTAACAGGAGCAACAAAAATC-3′ (the added *Bam*HI site is underlined) and 5′-TGGTCTAAGCTTTTGAGTTGCAT ATAAAAATGG-3′ (the added *Hin*dIII site is underlined). After digestion of PCR products and vector with *Bam*HI and *Hin*dIII, the *dbpA* gene fragment was ligated into pT7Lpp1 yielding pMSH24. This plasmid expresses chimeric lipoprotein Lpp1:$DbpA_{297}$ that differs from the natural sequence by the vector-added residues DQISDP between the amino-terminal C and the natural G at $DbpA_{297}$ position +2 following processing of the leader peptide. *E. coli* BL21(DE3)/pLysS (55) was transformed with pT7Lpp1 (negative control for protein expression) and pMSH24.

BL21(DE3)/pLysS/pMSH24 was grown at 37°C in LB plus 50 μg of kanamycin per ml and 20 μg of chloramphenicol per ml to mid-exponential phase, protein expression was induced by the addition of IPTG to 1.0 mM, and after an additional 2 h of growth, the cells were harvested by centrifugation. The cell paste was suspended in cold PBS at ~0.25 g (wet weight) of Triton X-114 per ml (10% [vol/vol] in PBS) was added to 2%, and the cells were lysed by sonication in an ice bath. After gentle overnight agitation at 4°C, insoluble material was removed by centrifugation at 100,000 × *g*, and the supernatant was decanted. Cloud point extraction (9) of the Triton X-114 supernatant enriched for the Lpp1:$DbpA_{297}$ in the detergent phase. This detergent phase was diluted 20-fold with 20 mM NaH_2PO_4 buffer (PB), pH 7.4, containing 1% (wt/vol) 3-[(3-cholamidopropyl)dimethylammonio]-1 propane-sulfonate (CHAPS) and loaded onto a DEAE Sepharose Fast Flow (FF) column. The flowthrough containing Lpp1:$DbpA_{297}$ was adjusted to pH 4.0 and applied to an SP Sepharose FF column. After the mixture was washed with column buffer (PB [pH 4.0]–1% CHAPS), application of a NaCl step gradient in column buffer resulted in elution of bound Lpp1:$DbpA_{297}$ from the column at the 0.5 M NaCl step. Lpp1:$DbpA_{297}$-containing fractions were adjusted to neutral pH and concentrated in a Prodicon (Spectrum Medical Industries, Inc., Houston, Tex.) vacuum concentrator against PBS–0.1% CHAPS. Lpp1:$DbpA_{297}$ was recovered at >95% purity.

$DbpA_{N40}$ was expressed from pT7Lpp2 as a chimeric lipoprotein with the addition of a vector-encoded carboxy-terminal extension VDKLAAA LEHHHHHH to facilitate purification by immobilized metal-affinity chromatography. DNA encoding the entire sequence (44) of the mature $DbpA_{N40}$ protein after the cysteine at the site of posttranslational modification was amplified from *B. burgdorferi* N40 template DNA by PCR with oligonucleotide primers 5′-CC GGATCCCGGATTAAAAGGAGAAACAAA-3′ (the added *Bam*HI site is underlined) and 5′-CTGTCTAAGCTTAGTCGACGTTATTTTTGCATTTTT C-3′ (the added *Hin*dIII and *Sal*I sites are underlined), digested with *Bam*HI and *Sal*I, and ligated into the comparable sites of pT7Lpp2 to yield plasmid pWCR129. A Triton X-114 extract of BL21(DE3)/pLysS/pWCR129 was made as described above and applied to a Ni^{2+}-charged ToyoPearl AF-Chelate-650M (TosoHaas, Montgomeryville, Pa.) column equilibrated with PBS–1.0% CHAPS. Lpp2:$DbpA_{N40}$His was eluted from the column with a 0 to 200 mM gradient of L-histidine in PBS-CHAPS. After concentration, Lpp2:$DbpA_{N40}$His purity was estimated at ~90%.

$DbpA_{297}$, $DbpA_{B31}$, and $DbpB_{297}$ were expressed in *E. coli* M15/pREP4 from the vector pQE30 (Qiagen, Inc., Santa Clarita, Calif.) as amino-terminal fusions with the vector-encoded peptide MRGSHHHHHHGS and purified essentially according to Qiagen protocols. Cloning by PCR amplification, expression, and purification of His-$DbpA_{297}$ and His-$DbpB_{297}$ (28) will be described elsewhere. The gene fragment encoding $DbpA_{B31}$ was PCR amplified with the same primers used for construction of His-$DbpA_{297}$. The first 10 codons of *dbpA* and all 20 codons of the *dbpB* leader peptide were deleted in these constructions.

TABLE 1. Evaluation of diverse *B. burgdorferi* sensu lato isolates for growth inhibition by rabbit preDbpA$_{297}$ and OspA$_{B31}$ antisera, for immunoblot reactivity, and for decorin binding activity

Borrelia isolate	Source and origin[a]	Decorin binding activity[d]	DbpA antiserum		OspA antiserum	
			Growth inhibition end point	Immunoblot reactivity[d]	Growth inhibition end point	Immunoblot reactivity[d]
B. burgdorferi sensu stricto						
B31	*I. scapularis*, New York	+[b]	5,120	++	51,200	++
297	CSF, New York	+	5,120	++	51,200	++
Sh-2-82	*I. scapularis*, New York	++	5,120	++	51,200	++
N40	*I. scapularis*, New York	++[b]	12,800	++	51,200	++
JD1	*I. scapularis*, Massachusetts	++[b]	800	++	1,600	++
HB19	Blood, United States	±	<50	±	100	++
3028	Human pus, Texas	+[b]	50[c]	++	1,600	++
G39/40	*I. scapularis*, Connecticut	±	100	±	3,200	++
LP4	Skin (EM), Connecticut	+[b]	800	++	<50	++
LP5	Skin (EM), Connecticut	+[b]	800	++	<50	++
LP7	Skin (EM), Connecticut	+[b]	400	++	<50	++
NCH-1	Skin, United States	+[b]	50[c]	++	100	++
ZS7	*I. ricinus*, Germany	++	<50	+	400	++
H11	Blood, Italy	+	200	++	400	++
CA-3-87	*I. pacificus*, California	±	<50	−	1,600	++
FRED	Human, Missouri	−	1,600	+	3,200	++
HBNC	Blood, California	±	3,200	±	3,200	++
B. afzelii						
PGau	Skin (ACA), Germany	++	50[c]	++	50	++
ACA I	Skin (ACA), Sweden	+	<50	±	<50	++
M7	*I. persulcatus*, China	±	1,600	+	<50	++
IPF	*I. persulcatus*, Japan	−	1,600	±	200	++
BO23	Skin, Germany	++	50[c]	++	<50	+
ECM-1	Skin (EM), Sweden	++	50[c]	++	100	+
B. garinii						
PBr	CSF, Germany	++	12,800	+	<50	++
PBi	CSF, Germany	++	800	−	<50	++
B4 91	Skin, Norway	++	<100	−	<50	++
G2.22	CSF, Germany	++	<50	−	<50	++
Ip90	*I. persulcatus*, Russia	+	<50	±	<50	++
IP89	*I. persulcatus*, Russia	++	<50	−	<50	+
2226	*I. persulcatus*, China	+	200	±	<50	++
Fuji P1	*I. persulcatus*, Japan	++	100	++	<50	++
20047	*I. ricinus*, France	++	50[c]	±	<50	++
B. japonica HO14	*I. ovatus*, Japan	+	<50	±	50	+
Group 25015 25015	*I. scapularis*, United States	−	10	±	6,400	++
B. andersonii 21038	*I. dentatus*, United States	++	1,600	+	<50	++

[a] Isolates were derived from patients with Lyme disease or *Ixodes* tick species. CSF, cerebrospinal fluid; EM, erythema migrans; ACA, acrodermatitis chronicum atrophicans.
[b] Two decorin binding bands at 18 to 20 kDa.
[c] Partial reduction in the number of cells at lowest dilution tested.
[d] Reactivities were scored on a scale from "−" to "++," with "−" representing background reactivity comparable to background obtained with nonimmune serum or immune serum against an irrelevant antigen and "++" representing the strongest specific signal observed. For decorin blotting, "−" represented reactivity comparable to background obtained in the absence of digoxigenin-conjugated decorin.

For expression of lipoprotein OspA, plasmid pSO3 was constructed by subcloning the *Nco*I-*Bcl*I fragment from pMV251 (54), containing the full-length *B. burgdorferi* B31 *ospA* gene, into the *Nco*I and *Bam*HI sites of pET-3d (55). After growth in LB plus 100 μg of ampicillin per ml and 20 μg of chloramphenicol per ml, lysis, and fractionation of BL21(DE3)/pLysS/pSO3 through the cloud point extraction step were carried out essentially as for BL21(DE3)/pLysS/pMSH24 described above. The detergent phase containing lipoprotein OspA$_{B31}$ was diluted approximately twofold to a final concentration of 50 mM citrate–10 mM EDTA–15 mM CHAPS (pH 4.2), and applied to an SP Sepharose FF column. Elution of the column with a linear pH gradient to pH 5.7 allowed recovery of lipoprotein OspA$_{B31}$ at ~90% homogeneity.

Negative-control immunogens for vaccination studies of mice were prepared by making extracts of each recombinant *E. coli* host that were comparable to those used for chromatography of the Dbp recombinant proteins expressed in these host strains. These were soluble fractions from lysates of JM101/pBsII and M15/pREP4 and a detergent-phase extract of BL21(DE3)/pLysS/pT7Lpp1.

Recombinant P39 protein was produced to evaluate seroconversion of mice following *B. burgdorferi* inoculation or challenge. Oligonucleotide primers 5′-ATGGATCCGAGTGGTAAAGGTAGTCTTGGGAGC-3′ (the added *Bam*HI site is underlined) and 5′-AGAGAAGCTTAGTCGACAATAAATTCTTTAAGAAACTTCTC-3′ (the added *Hin*dIII and *Sal*I sites are underlined) were designed based on the sequence (GenBank accession no. L24194) of the *B. burgdorferi* Sh-2-82 *bmpA* gene encoding P39 and used to amplify the entire mature region of the P39 protein from *B. burgdorferi* B31 template DNA by PCR. After digestion with *Bam*HI and *Hin*dIII, the P39$_{B31}$ gene fragment was cloned into the same two sites of plasmid pGMal-c, a derivative of the maltose binding protein (MBP) fusion protein expression vector pMal-c (New England Biolabs, Beverly, Mass.) with a replacement of the multiple cloning site from pMV261

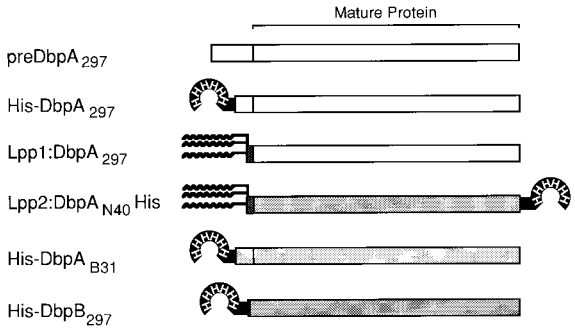

FIG. 1. Diagrammatic representation of the recombinant Dbp immunogens evaluated for vaccine efficacy. All proteins included the complete DbpA or DbpB coding sequence following Cys at the site of the presumed posttranslational processing. Lpp1:$DbpA_{297}$ is a fusion of this mature DbpA protein sequence to a modified *lpp* leader peptide. Fusions of DbpA and DbpB to a $(His)_6$ tag were made within, or in place of, their respective leader peptides. Lpp2:$DbpA_{N40}$His has both a N-terminal *lpp* leader and a C-terminal $(His)_6$ fusion.

(54). After transformation of the resulting plasmid, pNKP2, into *E. coli* DH5α, MBP-$P39_{B31}$ fusion protein was expressed and purified essentially according to the manufacturer's protocols. As a negative protein control reagent, MBP was purified from DH5α/pGMAL-c in a similar manner.

Antibody reagents. Antisera were raised against recombinant preDbpA$_{297}$ and His-DbpB$_{297}$ in New Zealand White rabbits. Antisera were also generated in rabbits against recombinant *B. burgdorferi* B31 OspA lipoprotein purified from the *E. coli* clone containing plasmid pOA1 (16) and against recombinant pneumococcal surface protein A (PspA) purified from the *E. coli* clone containing plasmid pJY4306 (62). The predicted sequences of the lipoprotein OspA's expressed by plasmids pOA1 and pSO3 are identical, but their products used for mouse and rabbit immunizations, respectively, were purified by different chromatographic procedures. Each animal received a primary subcutaneous (s.c.) immunization of 200 μg of protein emulsified with complete Freund's adjuvant (CFA), followed by two or three booster injections of 100 μg with incomplete Freund's adjuvant (IFA) at 3- to 4-week intervals. Titers of rabbit sera against their homologous recombinant borrelial antigens were 128,000 to 256,000 by enzyme-linked immunosorbent assay (ELISA). Rabbit anti-His-DbpB$_{297}$ reacted weakly with pre-DbpA$_{297}$ (titer of 4,000), but reactivity of rabbit anti-pre-DbpA$_{297}$ with His-DbpB$_{297}$ was at background levels. These proteins were previously observed to have minimal serologic cross-reactivity (28) consistent with their limited sequence homology (40% amino acid identity and 56% similarity). Monoclonal antibody (MAb) H5332 (2) against OspA, an immunoglobulin G1 (IgG1), was a gift of Alan Barbour. The DbpA-specific MAb 7D2B.3G6, an IgG2b, was obtained from a cloned hybridoma of splenic B cells from preDbpA-immunized BALB/cByJ (BALB; The Jackson Laboratory, Bar Harbor, Maine) mice fused with P3X63Ag8U.1 myeloma cells by standard methods (61).

Antisera against Lpp1:DbpA$_{297}$ and OspA$_{B31}$ were also obtained, prior to challenge, from C3H/HeJ (C3H; Jackson Laboratory) mice immunized (see below) for use in protection experiments (Table 2, experiment D, groups 2 and 3) and used for scanning immunoelectron microscopy and evaluation of passive immunization.

Scanning immunoelectron microscopy. In vitro-passage-5 *B. burgdorferi* B31 was collected from 0.5 ml of a mid-log-phase culture by centrifugation for 5 min

TABLE 2. Protection of mice by active immunization with different forms of DbpA compared with that by immunization with OspA

Expt (mouse strain/ challenge isolate)	Mouse group	Immunogen[a]	No. of positive cultures/total no. tested			P39 IgG[b]	No. of mice infected/ total no.
			Bladder	Ear	Joint		
A (C3H/297)	1	His-DbpA$_{297}$	0/5	0/5	0/5	0/5	0/5
	2	Lpp1:DbpA$_{297}$	0/5	0/5	0/5	0/5	0/5
	3	OspA$_{B31}$	0/5	0/5	0/5	−	0/5
	4	PBS	5/5	5/5	4/5	+	5/5
B (C3H/N40)	1	Lpp2:DbpA$_{N40}$His	0/5	0/5	0/5	0/5	0/5
	2	OspA$_{B31}$	5/5	0/5	0/5	+	5/5
	3	*E. coli* BL21 (det.)	4/4	4/4	1/4	+	4/4[c]
	4	None	5/5	5/5	5/5	+	5/5
C[d] (BALB/B31)	1	Pre-DbpA$_{297}$	0/5	0/5	0/5	0/5	0/5
	2	OspA$_{B31}$	0/5	0/5	0/5	−	0/5
	3	*E. coli* JM101 (sol.)	5/5	5/5	5/5	ND	5/5
	4	None	5/5	5/5	5/5	+	5/5
(C3H/B31)	5	Pre-DbpA$_{297}$	5/5	0/5	0/5	5/5	5/5
	6	OspA$_{B31}$	0/5	0/5	0/5	−	0/5
	7	*E. coli* JM101 (sol.)	5/5	5/5	5/5	ND	5/5
	8	None	5/5	5/5	5/5	+	5/5
D (C3H/B31)	1	Pre-DbpA$_{297}$	5/5	0/5	0/5	5/5	5/5
	2	Lpp1:DbpA$_{297}$	5/5	0/5	0/5	5/5	5/5
	3	OspA$_{B31}$	0/5	0/5	0/5	−	0/5
	4	*E. coli* JM101 (sol.)	5/5	5/5	5/5	+	5/5
	5	*E. coli* BL21 (det.)	5/5	5/5	5/5	+	5/5
	6	None	5/5	5/5	5/5	+	5/5
E[d] (C3H/B31)	1	Pre-DbpA$_{297}$	3/5	0/5	0/5	3/5	3/5
	2	His-DbpA$_{297}$	1/5	0/5	1/5	1/5	1/5
	3	His-DbpA$_{B31}$	1/4	0/4	0/4	0/4	1/4[c]
	4	OspA$_{B31}$	0/5	0/5	0/5	0/5	0/5
	5	*E. coli* M15 (sol.)	5/5	5/5	5/5	ND	5/5
	6	None	5/5	5/5	5/5	5/5	5/5

[a] Immunogen doses were 20 μg for DbpA, 5 μg for OspA, and 5 μg for *E. coli* soluble (sol.) and detergent (det.) extracts, except 20 μg in experiment C.
[b] Presence of IgG to P39 at the time of sacrifice was determined for most groups of mice (number positive/number tested). When sera were assayed as pools, the presence or absence of P39 IgG was scored as + or −, respectively. ND, not determined.
[c] Data were not recorded for one mouse in groups B3 and E3 that died prior to challenge.
[d] Data for experiments C and E are reproduced from Vaccines '97 (10a) with permission of the publisher.

at 1,000 × *g*. Sedimented cells were gently resuspended in 0.2 ml of BSKII containing 12 μl of immune or preimmune mouse serum and incubated for 30 min at room temperature with mixing. Spirochetes were then collected by centrifugation and washed twice in BSKII. The bacteria were labeled by incubation in 0.2 ml of BSKII containing 16 μl of goat anti-mouse IgG plus IgM conjugated to 30-nm-diameter gold particles (Ted Pella, Inc., Redding, Calif.). Labeled spirochetes were collected and gently washed twice in Tyrode's buffer and allowed to settle onto 0.1% poly-1-lysine-coated coverslips. After 10 min, the buffer was replaced with fixative containing 2.5% glutaraldehyde in 0.2 M sodium cacodylate (pH 7.2). Following fixation, the coverslips were washed twice in cacodylate and postfixed in 1% osmium tetroxide in cacodylate. After being washed in water and dehydrated in ethanol, the samples were critical-point dried from ethanol through carbon dioxide, lightly sputter coated with chromium, and observed with a Hitachi S4500 field emission scanning electron microscope equipped with an ultralow-voltage backscattered electron detector (GW Electronics, Norcross, Ga.).

Immunization of mice and challenge with *B. burgdorferi*. For passive immunizations, undiluted rabbit antiserum, or antiserum diluted in PBS, was administered intraperitoneally (i.p.) to 6- to 8-week-old female C3H mice within 1 h prior to challenge (day 0) or at various times afterward. For active immunizations, C3H or BALB mice were injected i.p. at week 0 with 20 μg of DbpA or DbpB, 5 μg of OspA, 20 or 5 μg of soluble or detergent extract of *E. coli* or PBS emulsified with CFA, given a similar booster immunization in IFA at week 4, and challenged at week 6. *B. burgdorferi* spirochetes were diluted in BSKII from exponentially growing cultures, and mice were injected s.c. at the base of the tail with 0.1 ml of these dilutions (typically 10^4 borreliae unless noted otherwise). Isolates used for challenge were passaged fewer than six times in vitro. To assess infection, mice were sacrificed at 14 to 17 days postchallenge, and specimens derived from ears, urinary bladders, and tibiotarsal joints were placed in BSKII–1.4% gelatin–13 μg of amphotericin B per ml–1.5 μg of phosphomycin per ml–15 μg of rifampin per ml, and borrelial outgrowth at 2 or 3 weeks was assessed by dark-field microscopy. Mice were scored as infected when any of the three tissues were culture positive. In some instances, seroconversion for protein P39 reactivity was also used to confirm infections (see below). Median effective dilutions (ED_{50}) of passively administered antisera and ID_{50} of borreliae were assessed in groups of three C3H mice per dose and were calculated by a standard method (43). Immune serum was also obtained from C3H mice persistently infected with B31.

In vitro growth inhibition assay. A microwell antibody titration assay (45) was used to evaluate the growth inhibition properties of antisera for various isolates of *B. burgdorferi* sensu lato. Briefly, 10^5 spirochetes in 100 μl of BSKII were added to serial twofold dilutions of antisera in 100 μl of BSKII in 96-well plates, and the plates were covered and incubated at 34°C in a 5% O_2–5% CO_2–90% N_2 gas mixture for 72 h prior to quantification of borrelia growth by dark-field microscopy. The highest serum dilution showing growth inhibition of >90% reduction in the number of cells and motility was designated as the end point growth inhibition titer. Since, in most cases, antisera diluted less than 1:100 gave only partial growth inhibition, a titer of ≥100 was defined as positive inhibition.

Immunoblotting and decorin blotting. In a single-well format, samples (2 μg) of Lpp1:$DbpA_{297}$, $OspA_{B31}$, or MBP-$P39_{B31}$ were boiled in sample buffer containing 1% SDS–2.5% 2-mercaptoethanol before SDS-PAGE through 3% stacking and 12.5% acrylamide resolving gels. Gels were electroblotted onto nitrocellulose membranes, and lanes were probed with 1:50 dilutions of immune sera from infected mice or 1:2,000 dilutions of purified MAbs, followed by the appropriate secondary antibody-enzyme conjugate. Membranes used for DbpA and OspA comparative immunogenicity studies were stained with Ponceau S to confirm that equivalent amounts of purified protein had transferred following electroblotting. Immunoblot signals were detected by fluorography with ECL chemiluminescence reagents (Amersham Corp., Arlington Heights, Ill.).

Assay of decorin binding activity was performed essentially as described by Guo et al. (29). Briefly, spirochetes were harvested from culture by centrifugation, washed once with 10 mM HEPES (pH 7.4)–20 mM NaCl, and suspended to 1×10^7 to 5×10^7 cells/ml in SDS–2-mercaptoethanol sample buffer. Samples of spirochetes were subjected to SDS-PAGE as described above, electroblotted to nitrocellulose, and probed with digoxigenin-conjugated decorin (29) followed by antidigoxigenin secondary antibody-enzyme conjugate (Genius System; Boehringer Mannheim Corp., Indianapolis, Ind.). Antidigoxigenin signals were detected by fluorography following incubation of the filters with Lumi-Phos 530 (Boehringer Mannheim).

Reactivities were scored on a scale from "−" to "++," with "−" representing background reactivity comparable to background obtained with nonimmune serum or immune serum against an irrelevant antigen and "++" representing the strongest specific signal observed. For decorin blotting, "−" represented reactivity comparable to background obtained in the absence of digoxigenin-conjugated decorin.

ELISA. Wells of 96-well microtiter plates (Immulon 2; Dynatech, Chantilly, Va.) were coated with antigen by incubating 50 μl of a 1-μg/ml antigen solution in 0.1 M sodium carbonate buffer at pH 9.6. After unbound antigen was decanted, additional binding sites were blocked by incubating 200 μl of 3% nonfat milk in wash buffer (PBS–0.2% Tween 20 [pH 7.4]). After washing, duplicate serial twofold dilutions of sera in PBS–Tween 20–1% fetal bovine serum were incubated for 1 h and removed, and the wells were washed three times. The wells were incubated with horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG, as appropriate. After three washes, bound antibodies were detected with H_2O_2 and 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate) (ABTS; Kirkegaard & Perry, Gaithersburg, Md.), and A_{405} was quantified with a Molecular Devices Corp. (Menlo Park, Ca.) V_{max} plate reader. IgG levels that were less than or equal to twice the background level in serum samples from naive mice or rabbits were assigned the minimum titer of 1:100 or 1:500, respectively.

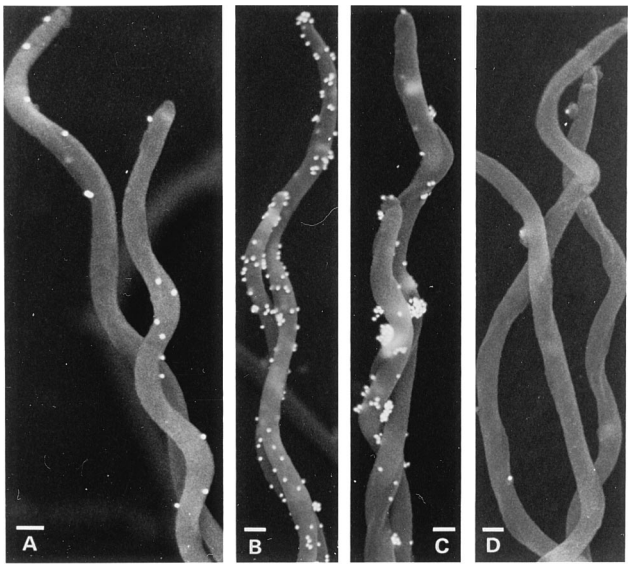

FIG. 2. Detection of surface-directed antibody labeling by scanning immunoelectron microscopy. *B. burgdorferi* B31 was incubated in BSKII plus mouse anti-DbpA (A and B), mouse anti-OspA (C), or normal mouse serum (D) prior to labeling of bound antibodies with goat anti-mouse IgG plus IgM-conjugated colloidal gold particles. Typical electron micrographs for these samples are shown except for panel B, which represents spirochetes with atypically heavy anti-DbpA labeling. Bars, 0.2 μm.

RESULTS

DbpA is surface accessible. Determination of the subcellular location of *B. burgdorferi* proteins is complicated by the chemical and mechanical fragility of the spirochetal outer membrane (13). We used scanning immunoelectron microscopy to evaluate the location of DbpA and OspA on in vitro-grown unfixed *B. burgdorferi* sensu stricto isolate B31 and to confirm the integrity of the antibody-labeled cells. Antibodies against both DbpA and OspA bound to the surface of intact borreliae (Fig. 2). Anti-DbpA labeling was uniformly scattered along the spirochete surface (Fig. 2A). Occasionally spirochetes (fewer than 1 in 10) were observed to label heavily with anti-DbpA, suggesting variability in the expression or the accessibility of this protein among cells in this population (Fig. 2B). Anti-OspA labeling was typically more dense and tended to occur in aggregates, possibly reflecting antibody cross-linking of these target proteins (Fig. 2C). The heavier labeling of OspA was consistent with analyses by SDS-PAGE indicating that in vitro OspA levels were 5- to 10-fold-higher than DbpA (29) (data not shown). Periplasmic endoflagella, which are normally contained within the spirochetal outer membrane, did not protrude from these antibody-labeled cells, leading us to tentatively conclude that these membranes were intact and that DbpA and OspA were surface exposed. Endoflagellar filaments were observed protruding from other cells that were partially disrupted (data not shown). These observations do not preclude the possibility of additional quantities of these

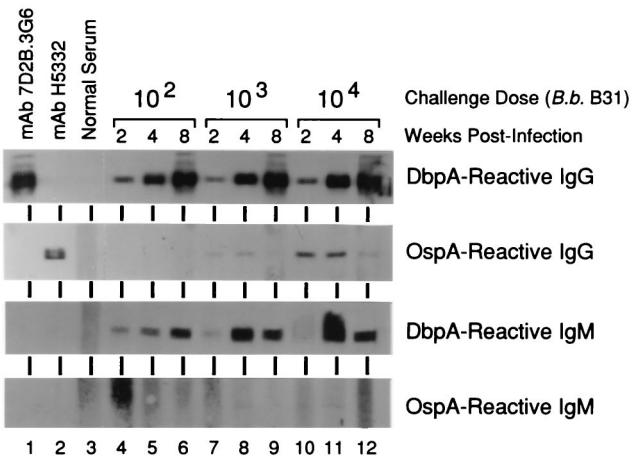

FIG. 3. Early antibody responses to DbpA and OspA elicited by low-dose challenge. Multilane immunoblot showing antibodies reactive with DbpA and OspA at weeks 2, 4, and 8 postchallenge (lanes 4 to 12) in sera pooled from mice infected by challenge with 10^2, 10^3, or 10^4 *B. burgdorferi* (*B.b.*) B31 spirochetes. Lanes: 1, anti-DbpA MAb, 1.0 μg/ml; 2, anti-OspA MAb, 0.2 μg/ml; 3, normal mouse serum. Samples for SDS-PAGE were 2 μg of purified Lpp1:$DbpA_{297}$ or $OspA_{B31}$.

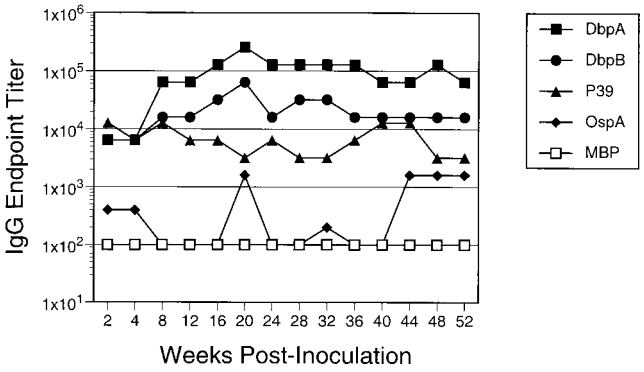

FIG. 4. Relative levels of IgG antibodies to DbpA, DbpB, and other selected borrelial antigens during chronic infection of mice. Sera were collected for a 1-year period from mice infected by challenge with 10^2 B31. Sera were pooled at each time point, and end point titers of IgG specific for each of four recombinant borrelial antigens were determined by ELISA. *E. coli* MBP, the fusion partner for recombinant P39, was used as a negative control protein.

lipoproteins at a subsurface location, as suggested by others (13).

Infection elicits strong antibody responses against DbpA and DbpB but not OspA. Dermal inoculation with low numbers of *B. burgdorferi* organisms near the ID_{50} failed to elicit antibody responses to OspA but did elicit antibodies to other proteins early in the infection (3, 47). It has been proposed that such inoculations approximate the number of borreliae delivered by tick bites (47). We examined the kinetics and level of the antibody response of C3H mice to DbpA following s.c. inoculation with 10^2, 10^3, or 10^4 B31 spirochetes. Isolate B31 (uncloned) was chosen because it was one of the most infectious isolates and could be consistently cultured from multiple tissues (including bladder, ear, and joint) of infected mice. Three of 10 mice inoculated with 10^2 B31 became infected, and all mice were infected at the higher doses. Sera from infected mice within each dose group were pooled and analyzed for immune responses to specific borrelial proteins. Antibodies reactive with DbpA were detectable by immunoblotting within 2 weeks of infection with 10^2 borreliae, while OspA-specific antibodies remained at background levels within the first 8 weeks after infection (Fig. 3). Sera from culture-negative mice in the 10^2 dose group did not react with DbpA or OspA (data not shown), indicating that the amount of these proteins in 10^2 borreliae was subimmunogenic and suggesting that DbpA immune responses were against antigens synthesized in vivo during spirochete multiplication. At inoculum doses of 10^3 and 10^4 spirochetes, early IgG responses to both DbpA and OspA were observed, but anti-DbpA responses were strongest. This was particularly evident at 8 weeks, when anti-OspA reactivity waned and responses to DbpA increased (lanes 9 and 12). Transient anti-DbpA IgM was detectable at all inoculum doses, but anti-OspA IgM responses were much weaker relative to background (lane 3) and were detectable only at inoculum doses of 10^3 and 10^4 spirochetes (lanes 8 and 11).

We extended serologic analysis to include antibody reactivity to DbpB and the lipoprotein P39 (BmpA), as well as DbpA and OspA, using an ELISA and serum pools from mice with culture-verified infection collected at intervals after inoculation with 10^2 B31 spirochetes. Others have previously shown that mice elicited antibodies to P39 when inoculated with live borreliae by syringe or tick bite but not with killed borreliae (52). At nearly every time point, the relative reactivity of antibodies against DbpA was greater than that against DbpB, which in turn was greater than antibody reactivity against P39 (Fig. 4). Antibody reactivity against OspA was near background (i.e., MBP) during early infection but rose somewhat at later times.

Passive immunization of mice with DbpA and DbpB antisera. As we initially lacked an isolate of *B. burgdorferi* 297 that was infectious for mice, isolate B31 was chosen for our first protection studies since it was found to be sensitive to killing in vitro by both preDbpA and OspA antisera (Table 1). Additionally, we sought to determine if antiserum against DbpA would passively protect mice against challenge with a heterologous *B. burgdorferi* sensu stricto isolate. Rabbit antiserum

TABLE 3. Relative potencies of rabbit pre-$DbpA_{297}$ and $OspA_{B31}$ antisera for passive protection of C3H mice from challenge with *B. burgdorferi* B31

Antiserum (0.1-ml dose)	Day(s) of antiserum administration	No. of mice infected for serum dilution of:				ED_{50}
		None (undiluted)	1:5	1:25	1:125	
Pre-$DbpA_{297}$	0	0/3	1/3	2/3	3/3	1:11.2
	0, 2	0/3	0/3	0/3	2/3	1:83.1
$OspA_{B31}$	0	0/3	1/3	3/3	ND[a]	1:7.5
	0, 2	0/3	1/3	3/3	ND	1:7.5

[a] ND, not determined.

TABLE 4. Postchallenge administration of rabbit pre-$DbpA_{297}$ antiserum aborts infection of *B. burgdorferi* B31

Expt no.[a]	Antiserum	No. of mice infected on day						
		0	2	4	5	6	7	10
1	Pre-$DbpA_{297}$	0/3	0/3	0/3	ND[b]	ND	3/3	3/3
	$OspA_{B31}$	0/3	3/3	3/3				
	PspA[c]	3/3						
	None	3/3						
2	Pre-$DbpA_{297}$	0/3	0/3	0/3	3/3	3/3	3/3	3/3
	$OspA_{B31}$	0/3	3/3	3/3				
	PspA	3/3						
	None	3/3						

[a] In experiment 1, mice were given a single passive administration of rabbit antiserum (0.1 ml i.p.) at the time of challenge (day 0) or on one of several intervals afterward. On day 17, mice were sacrificed and evaluated for infection. Data are reproduced from Vaccines '97 (10a) with permission of the publisher. In experiment 2, serum was given in two 0.05-ml doses 48 h apart, starting at the interval indicated.

[b] ND, not determined.

[c] PspA, pneumococcal surface protein A, irrelevant immunogen.

against pre-$DbpA_{297}$ and antiserum against $OspA_{B31}$ were passively administered at the time of challenge with 10^4 *B. burgdorferi* B31 spirochetes. Undiluted, both antisera protected mice from infection (Table 3; data not shown). The ELISA IgG titers of the rabbit pre-$DbpA_{297}$ and $OspA_{B31}$ antisera against their homologous recombinant antigens were comparable (~256,000). When 0.1 ml of antiserum was given at the time of challenge, the ED_{50} values of the DbpA and OspA sera were comparable (1:11.2 versus 1:7.5). A second administration of OspA serum on day 2 had no effect, whereas a second administration of DbpA antiserum was much more effective than the single treatment on day 0 (Table 3). It is noteworthy that the higher ED_{50} for the anti-$DbpA_{297}$ serum, relative to the anti-$OspA_{B31}$ serum, was obtained with the B31 isolate, which was heterologous to the $DbpA_{297}$ antigen. Passive immunization of C3H mice with rabbit His-$DbpB_{297}$ antiserum (ELISA IgG titer of 128,000) showed only partial protection against challenge with 10^4 *B. burgdorferi* B31 spirochetes (two mice protected of five) and no protection against challenge with 10^4 *B. burgdorferi* N40 spirochetes (no mice protected of five). Passive immunization against DbpB was not evaluated further in this study.

Elimination of infection by postinoculation administration of DbpA, but not OspA, antisera. Passive protection results suggested that DbpA may be accessible to antibodies for longer than OspA after challenge and that these relative changes in protein expression or accessibility happen in periods as short as 2 days. The possibility that DbpA is a target for protective antibodies after OspA is no longer accessible was addressed further by administering antisera against both of these antigens to mice at increasing intervals after challenge (Table 4). Cohorts of 30 or 36 C3H mice were inoculated with 10^4 *B. burgdorferi* B31 spirochetes, and then groups of three mice each received antisera starting at 0, 2, 4, 5, 6, 7, or 10 days later. In two separate experiments, passive transfer of rabbit pre-$DbpA_{297}$ antiserum completely protected mice from infection with *B. burgdorferi* B31, even when serum administration was delayed as long as 4 days but not 5 or more days postinoculation. $OspA_{B31}$ antisera were protective when administered at the time of challenge, but not at later times, similar to observations of others (48). Our observations showed that DbpA, unlike OspA, was a target for antibody-mediated elimination of borreliae during the early stage of infection and provided further evidence that the relative in vivo levels or accessibility of DbpA and OspA change during this period.

Active immunization of mice with DbpA protects against infection. C3H and BALB mice were actively hyperimmunized with three forms of recombinant $DbpA_{297}$ differing only at their amino termini, one form of $DbpA_{B31}$, and one form of $DbpA_{N40}$ and then challenged with both homologous and heterologous *B. burgdorferi* sensu stricto isolates (Table 2). Immunizations with DbpA or OspA preparations elicited high titers of serum IgG (ELISA end point titers were $>10^5$). C3H mice immunized with either His-$DbpA_{297}$ or Lpp1:$DbpA_{297}$ were protected from challenge with the homologous 297 isolate as judged by culture and P39 seroconversion (experiment A). C3H mice immunized with Lpp2:$DbpA_{N40}$His were protected against N40 homologous challenge (experiment B). However, $OspA_{B31}$ did not confer protection against the heterologous N40 challenge (experiment B). BALB mice immunized with pre-$DbpA_{297}$ were protected from challenge with the heterologous B31 isolate (experiment C), but protection of C3H mice from B31 challenge by immunization with the various forms of DbpA was less complete (experiments C, D, and E). Although protection of C3H mice against B31 challenge was somewhat higher in one experiment with His-tagged versions of DbpA (experiment E), more-extensive studies are required to determine whether this form of the immunogen elicits a more potent immune response in mice. Preliminary experiments comparing immunizations with 5 or 20 μg His-DbpA indicated that fewer mice were protected by the lower DbpA dose (2 of 5 protected at 5 μg versus 4 of 5 at 20 μg). Using Freund's adjuvants, we did not observe any substantial difference in immunogenicities between the acylated Lpp1:$DbpA_{297}$ and nonacylated pre-$DbpA_{297}$ and His-$DbpA_{297}$. Such differences may be more apparent with other adjuvants that are clinically relevant, as has been observed for OspA (2, 16, 33).

In almost every DbpA-immunized mouse where infection (positive bladder culture and P39 IgG) was observed, infection of ear and joint tissues was below detectable levels (Table 2). Unlike borreliae cultured from naive or sham-immunized mice, spirochetes recovered from bladders of the DbpA-immunized C3H mice were elongated and morphologically irregular, had reduced motility, and became nonviable before exceeding 10^6 cells/ml. We were unable to propagate sufficient quantities of these spirochetes to permit further physiological or genetic analyses.

As was the case with passive immunization against DbpB, only partial protection against challenge with 10^4 *B. burgdorferi* B31 spirochetes was achieved by active immunization of mice with His-$DbpB_{297}$. Three of five BALB mice were protected, but all five C3H mice immunized with His-$DbpB_{297}$ were infected at multiple sites.

Passive immunization of mice against diverse *B. burgdorferi* sensu stricto isolates. We next compared the abilities of rabbit DbpA and OspA antisera to protect C3H mice against challenge with five *B. burgdorferi* sensu stricto isolates and isolate 25015, which is classified as phylogenetically distinct by some typing methods (10, 39), to assess the conservation of protective epitope(s) on these antigens (Table 5). Antiserum against pre-$DbpA_{297}$ protected C3H mice against challenge with the three genetically heterologous *B. burgdorferi* sensu stricto isolates B31, Sh-2-82, and N40. Significantly, $DbpA_{N40}$ shares only 67% amino acid sequence identity (75% similarity) with $DbpA_{297}$ (44), demonstrating broad reactivity of rabbit DbpA antiserum. Pre-$DbpA_{297}$ antiserum gave broader protection than anti-$OspA_{B31}$ serum against these isolates (Table 5). Isolates 25015, HB19, and CA-3-87 were resistant to both passively administered pre-$DbpA_{297}$ and $OspA_{B31}$ antisera. With

TABLE 5. Comparison of pre-DbpA$_{297}$ and OspA$_{B31}$ antiserum for protection of mice against challenge with heterologous *B. burgdorferi* isolates

Expt no. and *B. burgdorferi* sensu lato challenge isolate[a]	No. of mice infected[b]		
	DbpA antiserum	OspA antiserum	No serum
1			
B31	0/5	0/5	5/5
Sh-2-82	0/5	1/5	5/5
N40	0/5	5/5	5/5
2			
B31	0/5	0/5	5/5
25015	5/5	5/5	5/5
3			
B31	0/5	0/5	5/5
HB19	5/5	5/5	5/5
CA-3-87	5/5	5/5	5/5

[a] Challenge dose with HB19 was 10^5 borreliae; the challenge dose with other isolates was 10^4.

[b] Rabbit pre-DbpA$_{297}$ or OspA$_{B31}$ antiserum (0.05 ml) was administered 1 h before challenge (day 0) and again on day 2 postchallenge. Data are reproduced from Vaccines '97 (10a) with permission of the publisher.

respect to OspA heterogeneity, the lack of cross-protection between OspA of *B. burgdorferi* sensu lato isolate 25015 and OspA from a *B. burgdorferi* sensu stricto isolate (N40) has previously been observed by others (21).

Conservation of DbpA epitope(s) binding growth-inhibitory antibodies. We next examined a large panel of *B. burgdorferi* sensu lato isolates with diverse geographic and biologic origins and representing six of the major phylogenetic groups (10, 39, 53) using a microwell-based growth inhibition assay (45) and by immunoblotting to evaluate the in vitro expression and serologic conservation of DbpA and OspA among these isolates (Table 1). To supplement immunoblotting, we also used a decorin blot assay (29) to evaluate expression of DbpA and DbpB. Sensitivities of these isolates to growth inhibition by rabbit pre-DbpA$_{297}$ or OspA$_{B31}$ antisera were assessed in parallel. Growth of 19 of the 35 *B. burgdorferi* sensu lato isolates (54%) was inhibited (defined as growth inhibitory titer of $\geq$100) by pre-DbpA$_{297}$ antiserum. This growth-inhibitory effect did not require addition of serum complement, similar to observations by others using this assay (45). Most isolates of *B. burgdorferi* sensu stricto (12 of 17 [71%]) and nearly half of the isolates of *B. afzelii* (2 of 6) and *B. garinii* (4 of 9) were inhibited by pre-DbpA$_{297}$ antiserum (range of inhibitory titers, 100 to 12,800). In some cases resistance to DbpA antiserum correlated with weak or negative reactions by immunoblotting and decorin blotting (HB19, CA-3-87, and 25015); these isolates apparently expressed little or no DbpA in vitro although PCR analysis indicated that each isolate contained a *dbpA* gene homolog (44). Some *B. garinii* isolates were inhibited weakly, or not at all, by pre-DbpA$_{297}$ antiserum but were positive by decorin blotting (PBi, B4 91, G2.22, and IP89). Isolate PBi was inhibited by pre-DbpA$_{297}$ antiserum but was negative by immunoblotting. For a few isolates (FRED and IPF), decorin binding activity appeared to be lost after SDS-PAGE, yet the isolates remained immunoreactive. Possibly differences in DbpA in vitro expression levels, in epitope structure, and in antibody accessibility, all contributed to differences in anti-DbpA inhibitory end point titers among these isolates. About half of the isolates (17 of 35 [49%]) were inhibited by OspA$_{B31}$ antiserum (range of inhibitory titers, 100 to 51,000) even though all 35 isolates were positive for OspA expression by immunoblotting. The OspA$_{B31}$ antiserum had negligible inhibitory activity against the nine *B. garinii* isolates. Thus, DbpA epitopes capable of binding growth-inhibitory antibodies appeared to be as conserved as, if not more conserved than, those of OspA. Most of the isolates (26 of 35 [74%]) were inhibited by at least one of the two antisera, suggesting that combinations of these two antigens might provide broader vaccine coverage than either one alone.

Rabbit His-DbpB$_{297}$ antiserum was also tested for in vitro growth inhibition activity. This antiserum had negligible effect on the in vitro growth of *B. burgdorferi* B31, Sh-2-82, and N40 (data not shown); therefore, this analysis was not expanded further. However, all three isolates were positive for DbpB expression by immunoblotting (data not shown), demonstrating that they expressed DbpB in vitro.

DISCUSSION

Many proteins of *B. burgdorferi*, including Osps A-F, 41-kDa flagellin, IpLA-7, P21, P30, P35, P37, P39, P55, P83, pG, and lp6.6, have been evaluated as targets for protection in animal models of Lyme borreliosis (14–17, 19–24, 27, 33, 36, 38, 40–42, 48, 54, 59, 60). Among these, only antibodies to OspA, OspB, and OspC have been shown to prevent infection completely. Combined administration of antisera against two in vivo-expressed proteins, P35 and P37, 1 day after homologous challenge also provided protection in a recent study (22) but only against 10^2, but not 10^4, spirochetes. The duration of P35/P37 target accessibility beyond day 1, or the level of serologic conservation of these antigens, was not reported. We show here that DbpA is accessible to antibodies capable of aborting infection up to 4 days after inoculation and is serologically cross-reactive among many borrelial isolates. In contrast, antibodies to OspA were ineffective at aborting established infection, as previously reported by others (48).

DbpA and DbpB are relatively minor proteins of cultured *B. burgdorferi* compared to OspA (28, 29). The antigenic stimulus provided to mice by a minimal infectious dose (10^2) of *B. burgdorferi* B31 was insufficient to elicit immune responses to these proteins in mice that failed to become infected, but persistent and high titers of DbpA and DbpB antibodies, and not OspA antibodies, were observed in culture-positive mice. These observations provide evidence that DbpA and DbpB are expressed in vivo during infection.

DbpA antibodies bound specifically to the surface of intact cultured spirochetes (Fig. 2) and were able directly to inhibit the growth of *B. burgdorferi* both in vivo and in vitro (Tables 1, 2, and 5). Similar observations were also made for OspA antibodies. However, the experiments evaluating the postchallenge protective efficacies of these antibodies were able to distinguish major differences in the expression level or surface accessibility of OspA, relative to that of DbpA, during the first 4 days after dermal inoculation of spirochetes (Tables 3 and 4). This decrease in expression or accessibility of OspA can be taken as evidence that the spirochetes are undergoing a host adaptation process at this stage of the infection. Methods capable of directly demonstrating the in vivo surface accessibility of *B. burgdorferi* outer membrane components are not currently available. The observation that *B. burgdorferi* remains sensitive to the growth-inhibitory effects of DbpA antiserum after a 4-day period of host adaptation provides evidence that DbpA, and not OspA, is surface accessible in vivo during this stage of infection. This is the first report demonstrating evidence of in vivo surface accessibility of a defined target in this manner. In order for decorin adherence (29) to play a role in

the virulence of *B. burgdorferi*, decorin binding proteins would presumably require in vivo surface exposure.

Others have shown that passive transfer of immune sera from persistently infected mice to naive mice will eliminate spirochetes in newly infected mice at 4 days postinfection but will not eliminate infection when immune serum is administered at 12 days postinfection (4). Antigenic targets for this effect have not been identified, but DbpA may be such a target. In the present study, spirochetes remained sensitive to the effects of DbpA antiserum through the first 4 days of infection in mice but became resistant to DbpA antiserum administrations at later times. Several mechanisms for this resistance are possible: (i) DbpA expression becomes down-regulated during or after infection; (ii) DbpA becomes inaccessible on the spirochete to antibodies; or (iii) spirochetes are sequestered in cells or tissues inaccessible to antibodies. A model suggesting that surface-exposed lipoproteins may be globally down-regulated in vivo by spirochetes or differentially localized during infection as part of the host adaptation process has been recently proposed (13). Our observations are consistent with this model but suggest that this may happen in a stage-specific or tissue-specific manner and that changes in lipoproteins occur in a programmed, rather than global, fashion since OspA and DbpA lipoproteins appeared to lose antibody-mediated vulnerability at different times during early infection (Table 4). In C3H mice the dissemination of *B. burgdorferi* from the site of dermal inoculation by syringe begins about 3 to 4 days postinoculation (8). This coincides with the time at which DbpA antibodies were no longer effective at aborting infection in the present studies. For instance, decorin adherence may contribute to establishing localized infection in collagen-rich connective tissues yet may be dispensable during spirochete dissemination. *B. burgdorferi* shows tropism for the skin of infected animals (5, 8), and cutaneous manifestations of human Lyme borreliosis are seen in both early and late disease (53). Immune response-mediated disease resolution has been observed in the immunocompetent mouse model of Lyme disease (3, 4) and has been interpreted to explain intermittent remissions during human Lyme disease. Anti-DbpA responses may contribute to these effects and to the control of persistent infection. The sustained anti-DbpA, as well as DbpB and P39, IgG levels in persistently infected mice may be a result of an immune stimulus by spirochetes when they periodically exit a putative immunity-privileged niche and ultimately become eliminated by the host's immune response.

Active immunization of C3H mice with DbpA conferred complete, or nearly complete, protection against challenge with 10^4 spirochetes of the isolate homologous to the immunogen. Immunization with DbpA conferred complete protection against infection following challenge with a heterologous *B. burgdorferi* sensu stricto isolate in BALB mice and in some C3H mice and appeared to limit dissemination in the remaining C3H mice. Spirochetes were recovered only from bladders, and not from ears or joints, of the partially protected DbpA-immunized mice, and these spirochetes were highly moribund. Although C3H mice hyperimmunized with $OspA_{B31}$ were protected from dermal challenge with 10^4 spirochetes of the homologous B31 isolate, OspA immunity can be overcome by challenge doses greater than 10^5 (23) (data not shown). The spirochetes recovered from OspA-immunized mice in the study by Fikrig et al. (23) were shown to be *ospA* escape mutants and remained viable and infectious, unlike the spirochetes recovered from DbpA-immunized mice. Future experiments will evaluate the disease status of DbpA-immunized mice harboring reduced numbers of spirochetes at 2 weeks postchallenge and will determine whether they eventually eliminate their infections. It is not known at this time whether the mice partially protected from infection also had reduced severity of disease or duration of symptoms.

Passive immunization with rabbit antiserum against $DbpA_{297}$ appeared to elicit more-complete and broader protection of C3H mice than active immunization. This response included protection against isolate N40 expressing DbpA having only 67% amino acid sequence identity (75% similarity) with the $DbpA_{297}$ immunogen (44). Additionally, we found that two passive immunizations of as little as 4 μl of the rabbit pre-$DbpA_{297}$ antiserum completely protected C3H mice from challenge with 10^4 *B. burgdorferi* B31 spirochetes (Table 3), but two passive immunizations of the equivalent of 4 μl of BALB or C3H mouse pre-$DbpA_{297}$ antiserum (with even higher DbpA IgG ELISA titers) did not protect naive mice from a similar challenge (data not shown). Differences in the potencies of the various sera may be due to differences in the responses of mice, which demonstrate a persistent *B. burgdorferi* infection (5, 8), and rabbits, which can clear a *B. burgdorferi* infection (25), to protective versus nonprotective epitopes or the relative avidities of the antibodies contained in the sera. We do not yet know how broad heterologous protection by DbpA antibodies may be in nonmurine species, but our in vitro results summarized in Table 1 provide encouragement.

By immunoblotting, and by blotting with tagged decorin, isolates HB19, CA-3-87, and 25015 were found to express little or no DbpA in vitro (Table 1), indicating that these borreliae had few DbpA targets at the time of challenge. Interestingly, sera from naive mice infected with HB19 or CA-3-87 reacted with purified Lpp1:$DbpA_{297}$ by immunoblotting (data not shown), suggesting that expression of *dbpA* by these isolates was up-regulated in vivo after infection began, possibly at the time when the passively administered $DbpA_{297}$ antiserum had diminished to levels below which protection may have otherwise been provided.

The high levels of antibodies to DbpB elicited during persistent infection of C3H mice suggested that, like DbpA, DbpB is also expressed in vivo (Fig. 4). However, the results of protection and in vitro growth inhibition studies suggest that DbpB is less vulnerable to antibodies or is expressed at lower levels than DbpA in vivo, or both. The DbpB sequences of the isolates examined in this study share >98% identity with $DbpB_{297}$ (44), suggesting that serologic variation is not an explanation for their lack of sensitivity to $DbpB_{297}$ antibodies. As we observed that rabbit anti-His-$DbpB_{297}$ was able to protect mice partially against a challenge with 10^4 borreliae, it appears that accessibility of DbpB to antibodies may differ in vivo and in vitro. In this regard, others have shown that antibodies from OspC-immunized mice that were protected against infection had very weak in vitro growth-inhibitory activity against the same borrelial isolate used for challenge (41, 42). These preliminary experiments suggested that DbpB is a less effective target for protection than DbpA, but additional studies are required to confirm this possibility.

Like the initial reports showing protective immunity targeting OspA (19, 48) and OspC (40), we have utilized cultured *B. burgdorferi* to demonstrate the protective effect of anti-DbpA immunity. We have also shown that cross-protection among heterologous DbpAs is feasible. The next and most critical step in the evaluation of DbpA vaccines will be to assess whether DbpA immunity will also confer protection against the tick-borne route of infection as was eventually shown for OspA and OspC (24, 27). The protective effects of an anti-DbpA immune response may extend beyond the transmission-blocking effect of an OspA immune response, which is apparently effective only against the spirochete's arthropod stage in the tick mid-

gut. If so, immune responses against DbpA may surpass the efficacy of those elicited by OspA-based vaccines by acting in concert with immune effector functions in vivo. Furthermore, mixtures of DbpA and OspA may be synergistic for efficacy. A multistage, multiantigen strategy is under consideration for vaccines against *Plasmodium falciparum* malaria, another arthropod-borne pathogen with developmental regulation of antigen expression (31, 56). This strategy may also be effective for protection against both infection and disease caused by *B. burgdorferi.*

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