The Early Dissemination Defect Attributed to Disruption of Decorin-Binding Proteins is Abolished in Chronic Murine Lyme Borreliosis

Denise M. Imai

D. Scott Samuels
University of Montana - Missoula, scott.samuels@umontana.edu

Sunlian Feng

Emir Hodzic

Kim Olsen

See next page for additional authors

Follow this and additional works at: http://scholarworks.umt.edu/biosci_pubs

Part of the Biology Commons

Recommended Citation
Imai, Denise M.; Samuels, D. Scott; Feng, Sunlian; Hodzic, Emir; Olsen, Kim; and Barthold, Stephen W., "The Early Dissemination Defect Attributed to Disruption of Decorin-Binding Proteins is Abolished in Chronic Murine Lyme Borreliosis" (2013). Biological Sciences Faculty Publications. Paper 59.
http://scholarworks.umt.edu/biosci_pubs/59

This Article is brought to you for free and open access by the Biological Sciences at ScholarWorks at University of Montana. It has been accepted for inclusion in Biological Sciences Faculty Publications by an authorized administrator of ScholarWorks at University of Montana. For more information, please contact scholarworks@mail.lib.umt.edu.
The Early Dissemination Defect Attributed to Disruption of Decorin-binding Proteins is Abolished in Chronic Murine Lyme Borreliosis

Denise M. Imai\textsuperscript{a}, D. Scott Samuels\textsuperscript{b}, Sunlian Feng\textsuperscript{a}, Emir Hodzic\textsuperscript{a}, Kim Olsen\textsuperscript{a}, and Stephen W. Barthold\textsuperscript{a}

Center for Comparative Medicine, Schools of Medicine and Veterinary Medicine, University of California at Davis, Davis, California, USA\textsuperscript{a} and Division of Biological Sciences, The University of Montana, Missoula, Montana, USA\textsuperscript{b}

Address correspondence to Stephen W. Barthold, swbarthold@ucdavis.edu

Running title: Dissemination defect of Dbp-deficient \textit{B. burgdorferi}
The laboratory mouse model of Lyme disease has revealed that *Borrelia burgdorferi* differentially expresses numerous outer surface proteins that influence different stages of infection (tick-borne transmission, tissue colonization, dissemination, persistence, and tick acquisition). Deletion of two such outer surface proteins, decorin-binding proteins A and B (DbpA/B), has been documented to decrease infectivity, impede early dissemination and, possibly, prevent persistence. In this study, DbpA/B-deficient spirochetes were confirmed to exhibit an early dissemination defect in immunocompetent, but not immunodeficient, mice and the defect was found to resolve with chronicity. Development of disease (arthritis and carditis) was only attenuated in the early stage of DbpA/B-deficient infection in both types of mice. Persistence of the DbpA/B-deficient spirochetes occurred in both immunocompetent and immunodeficient mice in a manner indistinguishable from wild-type spirochetes. Dissemination through the lymphatic system was evaluated as an underlying mechanism for the early dissemination defect. At 12 hours, 3 days, 7 days and 14 days post-inoculation, DbpA/B-deficient spirochetes were significantly less prevalent and in lower numbers in lymph nodes than wild-type spirochetes. However, in immunodeficient mice, deficiency of DbpA/B did not significantly decrease the prevalence or spirochete numbers in lymph nodes. Complementation of DbpA/B restored a wild-type phenotype. Thus, results indicated that deficiency of DbpA/B allows the acquired immune response to restrict early dissemination of spirochetes, which appears to be at least partially mediated through the lymphatic system.
INTRODUCTION

*Borrelia burgdorferi*, the etiologic agent of Lyme disease, utilizes a multitude of surface-exposed adhesins to bind to and interact with various components of the extracellular matrix in mammalian hosts. These adhesins include decorin-binding protein (Dbp)A, DbpB, fibronectin-binding protein (Fbp), *Borrelia* glycosaminoglycan-binding protein (Bgp), RevA, *Borrelia* membrane proteins (Bmps), ErpX, and P66. Their respective ligands include decorin, fibronectin, various glycosaminoglycans, laminin and $\alpha_{IIb}\beta_3$ integrin (1, 2, 3, 4, 5, 6, 7). This is by no means a completely inclusive list (8); for example, a yet unidentified borrelial adhesion binds directly to native type I collagen (9) and thus far, ligands for BmpD and members of the OspF family have not been characterized (10). However, the interactions of adhesins and ligands, particularly DbpA/B and decorin, appear to play an important role during all stages of infection. DbpA and DbpB are encoded in a bicistronic operon (*dbpBA*) on plasmid lp54 of the prototype *B. burgdorferi* B31 strain (11) and were two of the first borrelial adhesins identified (6, 12, 13, 14). These 19-kDa and 20-kDa proteins, respectively, are encoded by and expressed within *B. burgdorferi* sensu stricto strains and also many *B. burgdorferi* sensu lato strains, albeit as heterogeneous homologs (12, 15, 16, 17). Expression is upregulated in the mammalian host after tick-borne infection (18) and DbpA and DbpB are highly antigenic during infection (14, 19, 20). Based on mRNA levels, DbpA and DbpB continue to be expressed throughout chronic infection (12, 14, 18, 19). In comparison to DbpB, DbpA has been established as the more crucial adhesin in the context of pathogenesis, eliciting stronger protective immunity (12, 14) and, on its own, restoring a wild-type phenotype to DbpA/B-deficient mutant *B. burgdorferi* (21, 22).
In the laboratory mouse model, DbpA and DbpB have been implicated in the establishment of infection, dissemination, tissue colonization, persistence, and tick acquisition/transmission. Disruption of DbpA and DbpB, while nonessential to initial infection (23), will increase the infectious dose (21, 24, 25), decrease total spirochete tissue burdens (25), decrease recovery of spirochetes from tissues distant to the inoculation site (21, 23, 25) and decrease efficiency of tick acquisition/transmission (24). None of the aforementioned studies addressed the influence of DbpA and DbpB disruption on disease development or persistence.

The early dissemination defect of DbpA/B-deficient mutants, represented by decreased recovery of spirochetes from tissues distant to the inoculation site (21, 23, 25), seems to be a key to understanding the role of decorin-binding proteins in Lyme borreliosis. With the genetic disruption or absence of these adhesins, spirochetes may be unable to travel by conventional routes or access important microenvironmental niches, and, thus, manifest their altered dissemination phenotype. Although the extracellular matrix (ECM) is important in B. burgdorferi dissemination, as evidenced by direct dissemination through connective tissue (26, 27, 28, 29), spirochetes utilize alternate means to disseminate as well, including bacteremia (19, 29, 30, 31). In addition, a relatively unexplored means of dissemination is through lymphatics, as draining lymph nodes are often culture-positive sooner than any other tissues proximal to the inoculation site (20, 25, 32). Few molecular mechanisms that enable the lymphatic route of dissemination have been proposed, but they probably involve the interaction between adhesins and ligands. For example, fibronectin-binding protein, glycosaminoglycans and fibronectin facilitate microvascular interactions observed by intravital microscopy in infected mice (31) and both VlsE and OspC were implicated by phage display for in vivo adherence to vascular endothelium (10), which is likely to include lymphatic vessels as well.
The present study concurs with previous studies, in that decorin-binding proteins influence the early stages of infection (dissemination and tissue colonization). These early differences are unique to immunocompetent mice and are abolished in the chronic stage of infection. Results also demonstrate that decorin-binding proteins influence disease severity. We propose that the mechanism of influence pertains to the restricted routes by which spirochetes lacking dbpBA are able to disseminate, including lymphatic dissemination.

MATERIALS AND METHODS

Borrelial strains and mutagenesis. *B. burgdorferi* sensu stricto strain B31-A3, a low-passage infectious clonal isolate of B31-MI, the prototype B31 strain utilized for genome sequencing (33, 34), was utilized as both the wild-type control and the parental strain for genetic manipulation (35). The dbpBA operon was disrupted by insertion of *flgBp-aadA* (36) by electroporation of competent B31-A3 as previously described (37) and selection in 50 µg/ml streptomycin, which yielded the B31-ΔdbpBA deletion mutant. All *B. burgdorferi* strains were cultivated in liquid modified Barbour-Stoenner-Kelly (BSKII) medium supplemented with 6% normal rabbit serum (38). For isolation of transformants, *B. burgdorferi* was cultured on semisolid gelatin-free BSKII medium supplemented with 1.7% dissolved agarose plus the appropriate antibiotic (37).

The dbpBA operon was genetically reconstituted in the B31-ΔdbpBA mutant by allelic exchange recombination yielding the B31-dbpBA+ complement. The shuttle vector pBSV2G, containing a gentamicin resistance cassette (35) was utilized to create the construct in which the dbpBA operon was incorporated. One 1649-bp long fragment of B31 DNA, including the dbpBA operon, the promoter region from -266 to -1, and the terminator region after the stop codon from...
1528 to 1649, was amplified by PCR with forward primer P1FBamHI (5′-TCGTGGGATCCCAAGCCAGATTGCATAGC-3′) and reverse primer P7RPstI (5′-TCGTGCTGTGATTATCGGGCGAAGAG-3′). Both pBSV2G and the amplicon were double digested with BamHI and PstI, ligated together and sequenced to ensure the correct orientation of the dbpBA operon. The construct was electroporated into B31-ΔdbpBA mutants, and successful complements were selected with gentamicin (40 μg/ml). Six complemented mutants were obtained, and confirmed by PCR for the presence of the dbpBA operon and gentamicin marker, as well as the absence of the streptomycin marker. Plasmid profiling confirmed that all six complemented mutants contained the plasmids lp25, lp28-1, lp54, cp26 and cp32, which are required for infectivity (39).

For construction of suicide vectors and general gene cloning, Escherichia coli strain TOP10F′ (Invitrogen, Inc., CA) was utilized and grown in lysogeny broth (LB) broth under aerobic conditions at 37°C. Transformed E. coli were cultured in LB medium with 50 μg/ml spectinomycin or 5 μg/ml gentamicin.

Mice and infections. Specific-pathogen-free, 3 to 5 week old female C3H/HeN (C3H), C3H.C-Prkdcscid/AcrSmnHsd (C3H-scid) and IcrTac:ICR-Prkdcscid (Swiss-scid) mice were acquired from Frederick Cancer Research Center (Frederick, MD), Harlan Sprague Dawley, Inc. (Indianapolis, IN) and Taconic Farms, Inc. (Hudson, NY), respectively. Pregnant outbred Crl:CD1(ICR) mice were acquired from Charles River Laboratories (Hollister, CA). Mice were killed by carbon dioxide narcosis and cardiac exsanguination. Specific isolates of the borrelial mutants, B31-ΔdbpBA and B31-ΔdbpBA+ were confirmed as infectious to infant ICR mice at all inoculation doses from 10⁴ to 10⁷ (data not shown). Any individual C3H, C3H-scid or Swiss-scid mouse, in the experiments included herein, that could not be confirmed as infected (neither PCR-
positive nor culture-positive) was excluded from data analysis.

**PCR.** DNA was extracted from tissue samples using DNeasy tissue kits, according to the manufacturer’s instructions (QIAGEN, Valencia, CA). Samples were analyzed by quantitative PCR (qPCR) using optimized assays for *flaB* and *dbpA*, as previously described (18). Three oligonucleotides, two primers and an internal Taqman probe, for the *flaB* (18) and the *dbpA* genes were used. Primers DbpAB31-247F (5′-GCGAGCTACTACAGTGCGAAA-3′) and DbpAB31-444R (5′-TTTCAAGCCTCCCTTGAGCTGTA-3′) were created to amplify a 198-bp fragment of *dbpA* DNA. The internal probe DbpAB31-316P (5′-GTGAAACAGGTAGCATATCAGAAAATTCAT-3′) contained 5′ 6-carboxy fluorescein reporter dye and 3′ 6-carboxy-tetramethyl rhodamine quencher dye. Quantification of gene copies was based on absolute standard curves prepared using plasmid standards (18). Target gene copy numbers were expressed as copy number per mg of tissue weight or per μl blood. In addition, DNA extracted from positive cultures and DNA from tissue samples were used to verify *B. burgdorferi* genotypes recovered from infected mice.

**Histology.** Tissues were fixed in 10% neutral-buffered formalin, paraffin-embedded, routinely processed and stained with hematoxylin and eosin. Limbs were decalcified prior to processing. Tissue sections were blindly examined and graded for the presence of inflammation. The presence of arthritis in each mouse was determined by examination of knees and tibiotarsi. Sagittal sections through the heart, including sections of great vessels (aorta), were examined for the presence of carditis, as described previously (40, 41). Tibiotarsal arthritis severity was scored on a scale of 0 (no histologic evidence of inflammation) to 3 (severe), as described previously (42).
Enzyme-linked immunosorbent assay. Ninety-six well plates were coated with 1 μg/ml B. burgdorferi B31 whole cell lysates in carbonate coating buffer (pH 9.6), as described previously (12). Antibody binding was recognized by a secondary alkaline phosphatase-conjugated goat anti-mouse IgH+L antibody, diluted at 1:5000 (Jackson ImmunoResearch Laboratories Inc., West Grove, PA). Immunoreactivity was revealed using 1 mg/ml phosphate substrate (Sigma-Aldrich, St. Louis, MO) in diethanolamine buffer and optical density values were measured at 405nm on a kinetic microplate reader (Molecular Devices, Sunnyvale, CA), as described previously (41). Individual serum samples were titrated in three-fold dilutions (starting at 1:300). Samples were tested in duplicate, and each assay included uninfected mouse serum as a negative control and 90-day B31-infected mouse serum as a positive control.

Infection, dissemination/colonization, and persistence experiments. Mice were infected by subdermal inoculation of 10^5 to 10^6 mid-log phase B. burgdorferi B31-A3, B31-ΔdbpBA, and/or B31-ΔdbpBA+ in 0.1 ml BSKII culture medium on the dorsal thoracic midline. Subsets from each group were necropsied at 14, 28, 42, 60 and/or 90 days post-inoculation. Sub-inoculation site and urinary bladder tissues were aseptically collected for culture, as previously described (43). Tissues collected for DNA extraction and qPCR included: skin, sub-inoculation site, heart base, ventricular muscle, quadriceps muscle and left tibiotarsus. Tissues collected for histology included: heart base, left knee and right rear limb. Hearts were bisected along the longitudinal axis to provide samples for both DNA extraction and histology.

Lymphatic dissemination experiment. Groups of C3H mice were infected by subdermal inoculation of 10^5 mid-log phase B. burgdorferi B31-A3, B31-ΔdbpBA, and/or B31-ΔdbpBA+ in 0.1 ml BSKII culture medium in the skin of the right lateral thigh. Four mice from each group were necropsied at 12 hours, 3 days, 7 days and 14 days post-inoculation. Right and
left inguinal lymph nodes, spleen and urinary bladder were aseptically collected for culture. Both right and left inguinal, popliteal, lumbar, and axial lymph nodes were collected for DNA extraction. Inguinal lymph nodes were bisected to provide samples for both culture and DNA extraction. Extra-lymphatic tissues, including skin at the inoculation site, heart base, and right tibiotarsus, were collected for DNA extraction. To evaluate lymphatic dissemination in the absence of acquired immunity, the experiment was repeated in Swiss-scid mice.

**Statistics.** Analyses were performed using Fisher’s exact test for differences, independent samples t-test or two-way analysis of variance, followed by post-hoc pair-wise comparisons (Tukey’s HSD test) (PASW Statistics v. 18.0 and Prism v. 5, GraphPad software). Calculated P values ≤ 0.05 were considered significant.

**RESULTS**

*Borrelia burgdorferi* deficient in DbpA and DbpB lacks an early dissemination defect in immunodeficient mice, but exhibits attenuated disease development. The dissemination and pathogenic capabilities of the B31-ΔdbpBA mutant compared to wild-type B31-A3 was initially evaluated in immunodeficient mice. Groups of 4 C3H-scid mice inoculated with 10^6 B31-ΔdbpBA or B31-A3 were necropsied at 28 days post-inoculation. Sub-inoculation site and urinary bladder from all mice in both B31-ΔdbpBA and B31-A3-inoculated groups were culture-positive and there were no statistical differences in tissue spirochete burdens by flaB qPCR between groups (data not shown). B31-ΔdbpBA-inoculated C3H-scid mice developed both arthritis and carditis (Table 1), but the severity of tibiotarsal inflammation was attenuated in the B31-ΔdbpBA infection (0.8 mean severity score ± 0.2 SEM) compared to the wild-type B31-A3 infection (2.9 ± 0.1) (P = 0.03). Carditis was milder and in equal prevalence in the B31-ΔdbpBA-
inoculated C3H-scid mice compared to mice infected with B31-A3. Therefore, when unrestricted by acquired immunity, B31-ΔdbpBA retained the ability to disseminate and colonize distant tissues and was pathogenic, but despite the presence of equal copy numbers of spirochetes in tissue compared to wild type, B31-ΔdbpBA elicited less inflammation both hearts and joints. In the above experiment and similar studies by others in immunodeficient mice (24, 25), 1 month (28-30 days) post-inoculation was the maximum experiment duration for evaluating infections utilizing DbpA/B-deficient spirochetes. In order to evaluate the capability of B31-ΔdbpBA to persist in immunodeficient mice, we extended the duration to 90 days. Groups of 12 C3H-scid mice were inoculated with 10^6 B31-ΔdbpBA or B31-A3 and subsets of 4 mice per group were necropsied at 14 days, 60 days and 90 days post-inoculation. Sub-inoculation sites and urinary bladders from all mice were culture-positive at all intervals and in both groups. Copy numbers of flaB DNA in sub-inoculation site, heart base, ventricle, quadriceps muscle and tibiotarsal tissues were not significantly different between B31-ΔdbpBA and wild-type B31-A3-inoculated mice at any interval (Fig. 1). The severity of tibiotarsal arthritis and carditis similarly was indistinguishable between B31-ΔdbpBA and wild-type B31-A3-inoculated mice at 60 and 90 days post-inoculation (Table 1). The qPCR and histology data confirmed that in immunodeficient mice, B31-ΔdbpBA spirochetes can disseminate to distant tissues, proliferate therein to an equal degree, incite inflammation and persist in a manner similar to wild-type spirochetes.

The early dissemination defect of dbpBA-deficient spirochetes in immunocompetent mice is abolished in the chronic stage of infection and is rescued by complementation. To evaluate whether similar spirochete tissue dissemination, persistence and disease development would occur with B31-ΔdbpBA infection in immunocompetent mice, groups of 15 C3H mice
were inoculated with $10^5$ B31-$\Delta dbpBA$ or B31-A3. Five mice from each group were necropsied at 14, 28 and 42 days post-inoculation. Fewer culture-positive tissues, and fewer positive mice, were identified in the B31-$\Delta dbpBA$-inoculated mice compared to wild type at day 14 and day 28, but by day 42, numbers of culture-positive tissues and numbers of culture-positive mice increased until differences between B31-$\Delta dbpBA$ and B31-A3 infections were diminished (Table 2).

Similarly, at day 14, tissue spirochete burdens were undetectable in multiple tissues, including sub-inoculation site, heart base, ventricular muscle, quadriceps muscle and tibiotarsus (all $P = 0.0079$) in B31-$\Delta dbpBA$-infected mice compared to wild type (Fig. 2). At day 28, spirochete tissue burdens in heart base ($P = 0.034$) and ventricular muscle ($P = 0.033$) were significantly lower in B31-$\Delta dbpBA$-infected mice compared to wild type. However, by day 42 post-inoculation, qPCR tissue burdens were equivalent in both groups. No inflammation was observed on day 28 and only minimal carditis (0.1 ± 0.1; 1 out of 4 mice) and mild arthritis (0.4 ± 0.2; 2 out of 4 mice) was observed at day 42 in B31-$\Delta dbpBA$-inoculated mice (Table 1). By contrast, in the wild type-inoculated mice at day 28, there was statistically significantly greater carditis (1.0 ± 0.0; 5 out of 5 mice; $P < 0.05$) and a mild arthritis (0.2 ± 0.2; 1 out of 5 mice). At day 42, there was a trend towards slightly more severe and more prevalent disease with mild carditis (0.6 ± 0.2) and mild to moderate arthritis (0.9 ± 0.3) in 4 out of 5 mice. Results demonstrated that B31-$\Delta dbpBA$ spirochetes retained the capacity to infect, disseminate, and persist in immunocompetent mice, and eventually attain equal levels of tissue burdens and disease, but were delayed and initially only able to induce attenuated disease.

The duration of infection in immunocompetent mice was next extended to 90 days post-inoculation in order to fully evaluate the capability of the DbpA/B-deficient mutant to persist.
The complemented mutant B31-dbpBA+ was included in the experiment to evaluate whether genetic complementation could rescue the phenotype of the DbpA/B-deficient mutant. Groups of 12 C3H mice were inoculated with $10^6$ B31-ΔdbpBA, B31-dbpBA+, or B31-A3. Subsets of 4 mice were necropsied at 14 days, 60 days and 90 days post-inoculation. In mice inoculated with B31-ΔdbpBA, there were notably fewer culture and/or qPCR-positive mice (1/4) and minimal or no detectable spirochete tissue burdens in B31-ΔdbpBA-infected mice at day 14 compared to both wild-type B31 or B31-dbpBA+-infected mice (Fig. 3). At subsequent intervals (day 60 and 90), 3/4 and 4/4 B31-ΔdbpBA+-inoculated mice were culture and/or qPCR-positive and the level of spirochete tissue burden (Fig. 4) and severity of arthritis and carditis (Table 1) was not significantly different from B31-A3-inoculated mice. All B31-A3 and B31-dbpBA+-inoculated mice were positive at 14, 60 and 90 days and tissue spirochete burdens in B31-dbpBA+-inoculated mice were either not statistically different or were not significantly less than wild-type B31-A3 (day 14 shown in Fig. 3). Similarly, the severity of arthritis and carditis was not significantly different between B31-A3 and B31-dbpBA+-inoculated mice on day 60 and 90 (data not shown). The appropriate infecting *B. burgdorferi* genotypes (wild type, mutant, complemented mutant) were confirmed among isolates from each mouse group at necropsy. Thus, DbpA/B-deficient spirochetes, despite their early dissemination defect, were capable of persistence and inducing disease in immunocompetent C3H mice, and complementation of the mutant restored the early dissemination phenotype.

The early dissemination defect is dependent on the presence of an acquired immune response. The *flaB* qPCR data from the above experiments were combined to evaluate spirochete dissemination and colonization kinetics from day 14 to day 90 post-inoculation in immunocompetent C3H mice compared to immunodeficient C3H-scid mice (Fig. 4). Heart base
and tibiotarsal results were focused upon because these two tissues are distant from the inoculation site and are often poorly colonized by DbpA/B-deficient spirochetes, due to and representative of the dissemination defect (21, 25). Serology from the above immunocompetent C3H mouse experiments was also combined to evaluate the acquired immune response between DbpA/B-deficient and wild type-inoculated mice. In C3H-\textit{scid} mice, no significant differences were observed in tissue spirochete burdens in heart base (Fig. 4A) or tibiotarsus (Fig. 4B) between the B31-\textit{ΔdbpBA} mutant and wild-type B31-A3. In contrast, B31-\textit{ΔdbpBA} tissue spirochete burdens in C3H mice were markedly lower to absent compared to wild type at early time points (day 14 and day 28), but these differences were abolished by day 42 post-inoculation. Despite a continuous rise in \textit{B. burgdorferi}-specific antibody titer in mice inoculated with both genotypes, differences between the titers in B31-\textit{ΔdbpBA} and wild type infections were not abolished after day 42 and remained statistically significantly greater in the wild type-inoculated mice and in the B31-\textit{ΔdbpBA}-inoculated mice (Fig. 5).

\textit{dbpBA}-deficiency prevents early dissemination though the lymphatic system.

Regional lymph nodes have been reported to become rapidly culture-positive following infection (by needle-inoculation, tick transmission and tissue graft) during infection with wild-type as well as DbpA/B-deficient \textit{B. burgdorferi} (20, 25, 32). One study reported that distant lymph nodes in mice infected with wild-type \textit{B. burgdorferi} became progressively culture-positive over time, in the order of their proximity to the inoculation site (32). The same study concluded that spirochetes were in fact within lymph nodes, rather than in the surrounding connective tissue, by identifying morphologically intact spirochetes in subcapsular sinuses (32). In another study, in mice inoculated with DbpA/B-deficient spirochetes, spirochetes were frequently cultured from lymph nodes at 12 hours and 2 and 3 weeks post-inoculation (25). Based on these observations,
both wild-type and DbpA/B-deficient spirochetes appeared to be able to enter into, survive
within, and potentially migrate through the lymphatic system. This is in contrast to the observed
dissemination defect in DbpA/B-deficient spirochetes where heart and joint (tissues that should
be accessible by hematogenous or direct routes of dissemination) are less frequently colonized
by DbpA/B-deficient spirochetes (21, 25) than by wild-type spirochetes. Based on these
observations, we postulated that the lymphatic dissemination route might be utilized by
spirochetes lacking DbpA/B more readily than other routes.

To investigate this possibility, we determined the prevalence of wild-type B31-A3, B31-
\( \Delta dbpBA \) mutant, and B31-\( dbpBA+ \) complemented spirochetes within lymph nodes, both
proximal and distal to the inoculation site, and at multiple intervals (0.5, 3, 7 and 14 days) during
early infection by culture and qPCR for \( flaB \) DNA. Any animal that was neither culture nor \( flaB \)
qPCR-positive was considered uninfected and dropped from the data set. Both right and left
sides from each pair of lymph nodes (popliteal, inguinal, lumbar, and axillary) were evaluated
and if either one or both sides were qPCR or culture-positive, then the pair of lymph nodes was
considered positive (Table 3). Initially, we inoculated mice asymmetrically in the right hind limb
to evaluate any influence of proximity but the effect of side (right vs. left) was negligible and
therefore, each pair of lymph nodes was combined as a unit of evaluation.

At the earliest time points, qPCR-positive lymph nodes were identified in mice infected
with all three \( B. burgdorferi \) genotypes within hours after inoculation (day 0.5), but the same
lymph nodes were universally negative at the following time point (day 3), suggesting drainage
of DNA, but not viable spirochetes, from the inoculum. At day 7, the number of positive lymph
nodes from B31-\( \Delta dbpBA \)-inoculated mice was significantly lower (\( P < 0.0001 \)) than the number
of positive lymph nodes in wild type-inoculated mice. At day 14, the number of positive lymph
nodes from B31-ΔdbpBA-inoculated mice was significantly lower (P < 0.0001) than from both wild type and complemented mutant infections. Similarly, at day 7 and day 14, spirochete tissue burdens in lymph nodes from B31-ΔdbpBA-inoculated mice (10,572 mean copy no. flaB DNA per mg tissue ± 10,536 SEM; 225 ± 0.0) were lower, though not significantly, than wild type (45,904 ± 19,596; 38,995 ± 12,279).

Though there was a trend towards greater numbers of PCR-positive tissues in B31-ΔdbpBA extra-lymphatic tissues (skin, tibiotarsus and heart base) than in lymph nodes, only on day 3 was the difference significant (P = 0.0211). Otherwise, there were significantly fewer PCR-positive extra-lymphatic tissues from B31-ΔdbpBA-inoculated mice than in wild type-infected mice at the later time points (day 7 P < 0.0001, day 14 P = 0.0062) (Table 3). At day 7 and day 14, spirochete tissue burdens in extra-lymphatic tissues from B31-ΔdbpBA-inoculated mice (25 ± 4; 54,037 ± 49,271) were lower, though not significantly, than wild type (7,381,000 ± 6,459,000 vs. 103,140 ± 60,179). Based on culture, viable spirochetes could be recovered from the lymphatic system and extra-lymphatic tissue (urinary bladder) earliest in B31-A3-inoculated mice (day 7), followed by the B31-dbpBA+-inoculated mice (day 14) but were not recovered from B31-ΔdbpBA-inoculated mice at any interval (Table 3). Therefore, the early dissemination defect of DbpA/B-deficient spirochetes in immunocompetent C3H mice was characterized by minimal presence in lymph nodes, ii) greater presence in extra-lymphatic tissues, and iii) an overall lower spirochete tissue burden in lymph nodes and extra-lymphatic tissues when compared to wild type. These data demonstrate that the lymphatic route is not a dominant means of dissemination/migration utilized by DbpA/B-deficient spirochetes.

**Early exclusion of dbpBA-deficient spirochetes from the lymphatic system requires an acquired immune response.** Results indicated that the early dissemination defect of B31-
ΔdbpBA spirochetes occurs only in C3H, but not C3H-scid mice. Therefore, we next sought to determine if an acquired immune response is necessary to exclude B31-ΔdbpBA spirochetes from lymphatic dissemination. To investigate this possibility, we intended to repeat the previous experiment in congenic C3H-scid mice; however, C3H-scid mice became unavailable due to elimination of this mouse strain by the vendor. Therefore, the prevalence and tissue burdens of wild-type, mutant and complemented spirochetes within lymph nodes and extra-lymphatic tissues during the early stage of infection was repeated in equally susceptible Swiss-scid mice.

Culture and PCR-positive lymph nodes were identified in B31-ΔdbpBA-inoculated scid mice within hours after inoculation (day 0.5) (Table 4). By day 7, the number of positive lymph nodes from B31-ΔdbpBA-inoculated scid mice was significantly fewer (P < 0.0001) than the number of positive lymph nodes in wild type and B31-dbBBA+-inoculated scid mice. However, by day 14, significant differences between the numbers of positive lymph nodes in wild type, B31-ΔdbpBA or B31-dbBBA+-inoculated scid mice were no longer apparent and spirochete tissue burdens in lymph nodes from B31-ΔdbpBA-inoculated scid mice (2,352 ± 701) were not significantly different than wild type (33,497 ± 11,578) and B31-dbBBA+ (35,938 ± 10,355). At this same time point, the number of positive lymph nodes was significantly greater in scid mice inoculated with B31-ΔdbpBA (P < 0.0001) than in similarly inoculated C3H mice. No significant differences were observed between the number of positive lymph nodes and extra-lymphatic tissues in B31-ΔdbpBA-inoculated scid mice. Viable spirochetes could be recovered from the lymphatic system and extra-lymphatic tissues earliest in B31-A3-inoculated scid mice (day 3), followed by the B31-dbBBA+-inoculated scid mice (day 7) and B31-ΔdbpBA-inoculated scid mice (day 14) (Table 4). In summary, DbpA/B-deficient spirochetes in immunodeficient Swiss-scid mice were not excluded from the lymphatic route of dissemination.
DISCUSSION

The role of individual borrelial ECM adhesins is a common theme of investigation, given the importance of ECM to the lifecycle and pathogenesis of B. burgdorferi (44). Though adhesins may be necessary to a specific stage in borreliosis, no single adhesin has been shown to be absolutely essential. For instance, several studies have independently documented that deletion of dbpBA attenuates but does not abolish infectivity of B. burgdorferi (21, 23, 24).

Similarly, deletion of other adhesins has not been sufficient to alter the course of initial infection. Disruption of Bgp led to an uninterrupted infectious phenotype in immunodeficient mice after 2 weeks post-inoculation (45) and deletion of fibronectin-binding protein did not alter infection in immunocompetent mice at 3 weeks (46), although the median infectious dose was increased (47). Deletion of another adhesin, P66, resulted in loss of in vitro spirochetal attachment to the ligand integrin αvβ3 (48) and loss of infectivity in both immunocompetent and immunodeficient mice, with retention of the ability to infect ticks and survive in in vivo dialysis membrane chambers (49). Therefore, lack of any single adhesin may not be essential but, as we and others have demonstrated, may influence pathogenicity by altering the course of infection, by changing the ability to disseminate, colonize, cause disease, or persist.

While not necessary to establish infection in immunocompetent mice (23), deletion of dbpBA was reported to decrease infectivity (21, 24), display a dissemination defect (21, 23, 25) and potentially, alter the ability to persist (25). In this study, we confirmed that DhpA/B-deficient spirochetes manifested an early dissemination defect, but we demonstrated that the defect resolved with chronicity (after day 28 post-inoculation) and that persistence occurred in a manner indistinguishable from wild-type spirochetes. Furthermore, we demonstrated, for the first
time, that deletion of DbpA/B resulted in early attenuation of disease development and prevented early dissemination and colonization within the lymphatic system. We propose that one mechanism by which the early dissemination defect of DbpA/B-deficient spirochetes occurs is restriction of lymphatic dissemination through which, by comparison, wild-type spirochetes can rapidly migrate.

As unlikely as it may seem for an organism dedicated to immune evasion and persistence, there is abundant evidence that *B. burgdorferi* spirochetes actively migrate within the lymphatic system. Lymph nodes are rapidly and consistently culture-positive in both acute and chronic stages of infection (20, 25), become progressively culture-positive in order of proximity to the inoculation site (20), and morphologically intact spirochetes have been identified in subcapsular sinuses of regional lymph nodes (20). Indeed, a recent study found that the direct presence of viable (in contrast to non-viable) spirochetes in lymph nodes deceptively stimulates an atypical immune response that may actually favor survival of spirochetes during early infection (50). In the current study, we provide additional evidence for migration of wild-type spirochetes through the lymphatic system, and demonstrate the diminished ability of DbpA/B-deficient spirochetes to do likewise. Taken together, the lymphatic system appears to be a route of dissemination for *B. burgdorferi*, and DbpA and DbpB may be important for that behavior.

Based on data presented in this study and by Weening et al. (24), DbpA/B-deficient spirochetes can gain initial and sporadic access to the lymphatic system, but we postulate that the inability to maintain access and migrate therein essentially results in exclusion that coincides with the repeatedly documented early dissemination defect. Involvement of the acquired immune response is strongly implicated as only in immunocompetent mice has the dissemination defect been observed (21, 23, 25) and notably, only in immunocompetent mice have we observed...
exclusion from the lymphatic system.

The importance of the acquired immune response, B cell and antibody-mediated immunity in particular, to disease resolution and spirochete reduction in the host is well established (41, 51, 52, 53). How this clears or prevents access of DbpA/B-deficient spirochetes to lymphatics is perplexing because these genetically manipulated spirochetes lack one of the more immunogenic antigens, DbpA (12, 14). Without a vulnerable target, one might expect DbpA/B-deficient spirochetes to escape immune pressure; however, based on our observations, this is incorrect. We showed that the acquired immune response to DbpA/B-deficient spirochetes (by *B. burgdorferi*-specific serum titer) remains significantly lower than the wild-type immune response to wild type (Fig. 5) despite equilibration of tissue spirochete burdens to a wild-type level (Fig. 4C and 4D). This reduced immune response remains capable of excluding DbpA/B-deficient spirochetes from the lymphatics, at least within the early stages of infection.

Several mechanisms that would prevent lymphatic dissemination of DbpA/B-deficient spirochetes in immunocompetent mice are possible: i) DbpA/B-deficient spirochetes have increased vulnerability to antibody clearance within lymphatics, ii) DbpA/B-deficient spirochetes have increased vulnerability to non-antibody-mediated clearance within lymphatics, or iii) lymphatics become inaccessible to DbpA/B-deficient spirochetes after the initial establishment of infection. Our observations are more consistent with the first two possibilities since involvement of the acquired immune response is implicated. If DbpA/B-deficient spirochetes are more vulnerable to antibody clearance, then increased exposure to IgM could account for the greater susceptibility. IgM dominates the anti-borrelial immune response (50) and though it may be too large and unwieldy to penetrate collagenous tissues, it is present in blood and lymph (54).

The caveat remains that evidence exists to refute the hypothesis that steric hindrance alone
prevents the antibody response from targeting spirochetes embedded in collagen (55, 56). As for non-antibody-mediated clearance, recent investigations into invariant natural killer T (iNKT) cells are reminders that there are alternate immune mechanisms to consider (57, 58). For instance, disruption of the phagocyte (macrophage or Kupffer cell)-iNKT cell interaction results in diminished IFN-γ production, decreased phagocytic clearance, and increased bacterial loads (57) and dissemination (58).

Similarly, the exact mechanism by which the DbpB/A-deficient spirochetes maintain the capability to incite inflammation despite the absence of a strongly immunogenic antigen is speculative at best. Only during the earlier stage of infection (day 28) was there a statistically significant difference in severity of arthritis (in C3Hscid mice) or carditis (in C3H mice) between B31-ΔdbpBA and wild type-inoculated mice. However, in C3H mice, there was a slight attenuation in disease severity in B31-ΔdbpBA extending to day 60. Relative tissue spirochete burdens are not sufficient to explain the difference in disease severity since attenuation of disease in B31-ΔdbpBA-inoculated mice extends past the point (day 42) of equilibration between genotypes (Fig. 4C and 4D). Rapidity of dissemination to and colonization of a site of predilection for inflammation (heart base or tibiotarsus) may be an alternate possible explanation for the initially attenuated inflammation associated with B31-ΔdbpBA spirochetes. For example, in the earlier time points (<14 days), histologically evident inflammation often lags behind the wave of directly disseminating wild-type spirochetes in immunodeficient C3Hscid mice (D. M. Imai, unpublished).

In summary, we demonstrated and confirmed that disruption of dbpBA results in an early dissemination defect that is dependent on the presence of acquired immunity, resolves with chronicity of infection, and appears to reflect restricted migration through the lymphatic system.
We confirmed that deficiency in dbpBA does not diminish the ability to infect, to cause disease or to persist. The counterintuitive dispensability of DbpA and DbpB, immunodominant (19, 12, 20) but potentially protective (19, 12, 59) outer surface proteins that afford the ability to disseminate in the face of acquired immunity, is only one indication of the complexity of the borrelial pathogen-host relationship.

ACKNOWLEDGMENTS

We thank Dr. Patricia Rosa for providing the B. burgdorferi B31-A3 strain and Kevin Holden, Beth Todd and Edlin Escobar for technical assistance. This work was supported by NIAID grants R01-AI26815 (SWB), T32-AI06055 and T32-OD011147 (DMI), and R01-AI051486 (DSS).

REFERENCES


25


58. Lee WY, Moriarty TJ, Wong CH, Zhou H, Strieter RM, van Rooijen N, Chaconas

Table 1. The inflammation associated with B31-ΔdbpBA B. burgdorferi infection is not significantly different from inflammation associated with wild-type B. burgdorferi infection after day 28 post-inoculation, in either immunodeficient or immunocompetent mice. More severe inflammation does not absolutely correspond with a significantly greater spirochete tissue burden.

<table>
<thead>
<tr>
<th>Mouse strain Isolate</th>
<th>Day</th>
<th>Tibiotarsus</th>
<th>Heartbase</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No. spirochetes&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Prevalence&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>T/B cell-deficient</td>
<td>28</td>
<td>2.39E+04</td>
<td>8/9</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>5.55E+03</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>3.19E+04</td>
<td>4/4</td>
</tr>
<tr>
<td>wild type</td>
<td>28</td>
<td>3.81E+04</td>
<td>8/8</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>5.68E+04</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>3.00E+01</td>
<td>4/4</td>
</tr>
<tr>
<td>Immuno-competent</td>
<td>28</td>
<td>5.69E+02</td>
<td>0/4</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>6.17E+04</td>
<td>2/4</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>ND</td>
<td>3/4</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>1.45E+02</td>
<td>3/4</td>
</tr>
<tr>
<td>wild type</td>
<td>28</td>
<td>2.59E+04</td>
<td>1/5</td>
</tr>
</tbody>
</table>
a No. of spirochetes in respective tissues represented as mean copy no. *fluB* per mg tissue.
b No. of mice/Total no. of mice.
c Mean severity + SEM
d,h Differences in arthritis severity are statistically significantly different (all *P* values < 0.05) but differences in spirochete tissue burdens are not statistically significant.
e Arthritis severity is significantly different (*P* < 0.05) and corresponds with significantly greater tissue spirochete burden (*P* = 0.007).
f Arthritis severity is significantly different (*P* < 0.05).
g,h Carditis severity is significantly different (*P* < 0.05) and corresponds with significantly greater tissue spirochete burden (*P* = 0.0005).
h Carditis severity is significantly different (*P* < 0.05) and corresponds with significantly greater tissue spirochete burden (*P* = 0.002).
i Carditis severity is significantly different (*P* < 0.05) and corresponds with significantly greater tissue spirochete burden (*P* = 0.003).
Table 2: Viable, cultivable spirochetes lacking dbpBA are recovered from tissue in increasing frequency over time in immunocompetent C3H mice.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Day</th>
<th>No. positive cultures / total no.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Sub-inoc site</td>
</tr>
<tr>
<td>ΔdbpBA</td>
<td>14</td>
<td>2/5</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>4/5</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>4/5</td>
</tr>
<tr>
<td>wild type</td>
<td>14</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>5/5</td>
</tr>
<tr>
<td></td>
<td>42</td>
<td>5/5</td>
</tr>
</tbody>
</table>

* In 3 of the 4 positive cultures, spirochetes were observed only rarely.

Table 3: Dbp-deficiency prevents the recovery of spirochetes from the lymphatic system in the early stage of infection in immunocompetent laboratory mice. Complementation of dbpBA recovers the wild-type phenotype.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Day</th>
<th>flaB PCR (culture)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Polpiteal^</td>
</tr>
<tr>
<td>wild type</td>
<td>0.5</td>
<td>2/4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>1/3</td>
</tr>
<tr>
<td>ΔdbpBA</td>
<td>0.5</td>
<td>4/4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>2/4</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>1/3</td>
</tr>
<tr>
<td>dbpBA+</td>
<td>0.5</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0/2</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>1/1</td>
</tr>
</tbody>
</table>

* No. pos/total

^ Includes both right and left-sided nodes.
† Extralymphatic tissues collected for PCR included skin, heart base, and tibiotarsus. Extralymphatic tissues collected for culture included spleen and urinary bladder.

\(^{a,b}\) Prevalence of \textit{flaB} DNA in lymph nodes from ∆dbpBA infected mice is significantly lower \((P < 0.0001\) by Fisher’s exact test) than in wild type infected mice.

\(^c\) Prevalence of \textit{flaB} DNA in lymph nodes from ∆dbpBA infected mice is significantly lower \((P < 0.0001)\) than in \(dbpBA^+\) (complemented mutant) infected mice.

\(^x\) The number of PCR-positive extralymphatic tissues from ∆dbpBA infected mice are significantly fewer \((P < 0.0001)\) than in wild type infected mice.

\(^y\) The number of PCR-positive extralymphatic tissues from ∆dbpBA infected mice are significantly fewer \((P = 0.0062)\) than in wild type infected mice.

**Table 4:** Dbp-deficiency decreases but does not prevent spirochetes from utilizing the lymphatic system in the early stage of infection in immunodeficient laboratory mice.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Day</th>
<th>Popliteal^</th>
<th>Inguinal</th>
<th>Lumbar</th>
<th>Axillary</th>
<th>Extraln†</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>0.5</td>
<td>0/4</td>
<td>0/4 (0/4)</td>
<td>0/4</td>
<td>1/4</td>
<td>5/12 (0/4)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>2/4</td>
<td>1/4 (1/4)</td>
<td>0/4</td>
<td>1/4</td>
<td>5/12 (0/4)</td>
</tr>
<tr>
<td>(\gamma^{a,x})</td>
<td>4/4</td>
<td>4/4 (4/4)</td>
<td>4/4</td>
<td>4/4</td>
<td></td>
<td>12/12 (3/4)</td>
</tr>
<tr>
<td>∆dbpBA</td>
<td>0.5</td>
<td>4/4</td>
<td>0/4 (4/4)</td>
<td>1/4</td>
<td>4/4</td>
<td>7/12 (0/4)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>(\gamma^{a,b,x})</td>
<td>0/4</td>
<td>0/4 (0/4)</td>
<td>2/4</td>
<td>0/4</td>
<td></td>
<td>5/12 (0/4)</td>
</tr>
<tr>
<td>14(^c)</td>
<td>3/3</td>
<td>3/3 (3/3)</td>
<td>3/3</td>
<td>3/3</td>
<td></td>
<td>9/9 (3/3)</td>
</tr>
<tr>
<td>(dbpBA^+)</td>
<td>0.5</td>
<td>1/4</td>
<td>0/4 (0/4)</td>
<td>0/4</td>
<td>0/4</td>
<td>4/12 (0/4)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>3/4</td>
<td>1/4 (0/4)</td>
<td>0/4</td>
<td>0/4</td>
<td>3/12 (0/4)</td>
</tr>
<tr>
<td>(\gamma^{b,x})</td>
<td>4/4</td>
<td>3/4 (4/4)</td>
<td>4/4</td>
<td>4/4</td>
<td></td>
<td>12/12 (0/4)</td>
</tr>
</tbody>
</table>

\(*\) No. pos/Total
^ Includes both right and left-sided nodes.
† Extralymphatic tissues collected for PCR included skin, heart base, and tibiotarsus. ExtraLN tissues collected for culture included spleen and urinary bladder.

^ Prevalence of flaB DNA in lymph nodes from ΔdbpBA infected mice is significantly lower ($P < 0.0001$ by Fisher’s exact test) than in wild type infected mice.

^ Prevalence of flaB DNA in lymph nodes from ΔdbpBA infected mice is significantly lower ($P < 0.0001$ by Fisher’s exact test) than in dbpBA+ infected mice.

^ All lymph nodes from wild type, ΔdbpBA, and dbpBA+(complemented mutant) infected mice are positive for flaB DNA and therefore, could not be analyzed by Fisher’s exact test.

^ The number of PCR-positive extralymphatic tissues from ΔdbpBA infected mice is significantly fewer (all $P = 0.0046$) than in wild type and dbpBA+ infected mice.
FIGURE LEGENDS.

FIG. 1. DbpA/B are not essential for dissemination, colonization or persistence in immunodeficient mice. B. burgdorferi flaB DNA per mg tissue weight (mean ± SEM) in sub-inoculation site (A), heart base (B), ventricle (C), tibiotarsus (D) and quadriceps muscle (E) from C3H-scid mice inoculated with B31-ΔdbpBA (white bars) compared to wild-type B31-A3 (black bars) at 14 days, 60 days and 90 days post-inoculation. No significant differences observed.

FIG. 2. Early defects in dissemination and colonization, attributed to the disruption of DbpA/B, are not observed in the chronic stages of Lyme borreliosis in immunocompetent mice. B. burgdorferi flaB DNA per mg tissue weight (mean ± SEM) in tissues from C3H mice inoculated with B31-ΔdbpBA (white bars) compared to wild-type B31-A3 (black bars) at 14 days, 28 days, and 42 days post-inoculation (*, all P < 0.034).

FIG. 3. Complementation of the dbpBA-deficient mutant restores a wild-type phenotype. B. burgdorferi flaB DNA per mg tissue weight (mean ± SEM) in tissues from C3H mice inoculated with B31-ΔdbpBA (white bars) compared to the complemented mutant B31-dbPBA+ (gray bars) and wild-type B31-A3 (black bars) (*, P ≤ 0.03).

FIG. 4. The early dissemination defect is dependent on an acquired immune response. B. burgdorferi flaB DNA per mg tissue weight (mean ± SEM) in heart base (A) and tibiotarsus (B) from C3H-scid mice and heart base (C) and tibiotarsus (D) from C3H mice at days 14, 28, 42, 60, and 90 post-inoculation. Mice were inoculated with B31-ΔdbpBA (white circles) or wild-type
B31-A3 (black circles). Each data point represents 4 to 9 mice from 2 separate experiments (*, $P \leq 0.035$).

**FIG. 5.** *Borrelia burgdorferi*-specific antibody titers steadily rise over time, regardless of borrelial genotype, but remain significantly greater in mice inoculated with wild-type spirochetes compared to mice inoculated with DbpA/B-deficient spirochetes. Mice were inoculated with B31-ΔdbpBA (white circles) or wild-type B31-A3 (black circles). Each data point represent mean reciprocal dilutions ± SEM of 4 to 5 mice from 2 separate experiments (*, $P = 0.006$, $P = 0.05$, $P < 0.001$, respectively).
A

Copy no. fNBS per mg sub-IN site

$\Delta dbpBA$

wild type

<table>
<thead>
<tr>
<th></th>
<th>d14</th>
<th>d28</th>
<th>d42</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta dbpBA$</td>
<td>$10^2$</td>
<td>$10^2$</td>
<td>*</td>
</tr>
<tr>
<td>wild type</td>
<td>$10^6$</td>
<td>$10^4$</td>
<td>$10^7$</td>
</tr>
</tbody>
</table>
Copy no. *flaB* per mg tibiotarsus

- **14d**: *
- **40d**: *
- **60d**: *

D
Day 14

Copy no. flaB per mg tissue

ΔdbpBA
wild type
dbpBA+

salM
heparin
ventricle
epiglottis
quadriceps

* * * *