

Hidden in plain sight: *Borrelia burgdorferi* and the extracellular matrix

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***Borrelia burgdorferi*, the tick-transmitted etiologic agent of Lyme borreliosis, can colonize and persist in multiple tissue sites despite vigorous host immune responses. The extracellular matrix appears to provide a protective niche for the spirochete. Recent studies in mice suggest that *B. burgdorferi* interacts in various ways with collagen and its associated molecules, exploiting molecular and structural features to establish microcolonial refugia. Better knowledge of the genetic and structural bases for these interactions of *B. burgdorferi* with the extracellular matrix will be required before an understanding of the persistence of *B. burgdorferi* in the tissues and development of chronic infections can be achieved.**

Chronic extracellular infection by *Borrelia burgdorferi*

Establishment and maintenance of infection in a new host is a dynamic process. Pathogen adhesion, invasion of tissues and cells, host protective responses and microbial evasion strategies all play a role in colonization. Host cells and tissues can also vary in their susceptibility, and infection can be localized to particular cells, tissues and organs rather than being uniform throughout the host. Vector-transmitted pathogens have additional constraints because these organisms colonize both invertebrate and vertebrate hosts and need to be able to survive in widely differing environments. Maintenance of tissue infection and pathology in chronic infections might also involve repeated internal cycles of acute infection from persistent niches where organisms are able to evade host immune responses [1].

Borrelia burgdorferi, the etiologic agent of Lyme borreliosis (Lyme disease), is a tick-transmitted spirochete that causes a chronic extracellular infection [2]. During the initial weeks of infection in the mouse model, growth of *B. burgdorferi* is controlled by innate immune responses (mediated by Toll-like receptor-dependent and -independent mechanisms) until adaptive immunity develops [3–5]. Despite vigorous specific immune responses, the spirochete is able to colonize multiple sites (skin, joints, heart, bladder, central nervous system) and can persist there for extended periods of time [2,6,7]. *B. burgdorferi* immune

evasion strategies include antigenic variation at the *vs* locus (summarized in [8]) and interference with complement function by various borrelial proteins [9–11]. Recent studies using the mouse model [12–15] have attempted to clarify aspects of other pathogenic mechanisms of Lyme borreliosis of relevance to human disease that involve interactions between *B. burgdorferi* and the extracellular matrix (ECM). Here we suggest that these interactions are of great importance in chronic infections with this organism.

Adaptive antibody responses in chronic borrelial infection: protective but not sterilizing

Mice infected with *B. burgdorferi* develop a persistent infection and strong T-cell independent and dependent spirocheticidal responses to *B. burgdorferi* proteins and lipoproteins (reviewed in [16,17]). Sera from these persistently infected animals contain high titers of antibodies that can passively transfer protective immunity to naïve mice [18–20]. Despite high titers of anti-*B. burgdorferi* antibodies, these immunocompetent mice remain persistently infected with recurring exacerbations and remissions of arthritis, carditis and spirochetemia; sterilizing immunity is not achieved in these animals [21]. Significantly, sera from human patients with chronic *B. burgdorferi* infections also contain high titers of circulating anti-*B. burgdorferi* antibodies [22] as well as antibodies able to provide protective immunity to naïve mice upon passive transfer [23].

The occurrence of strong antibody responses in the absence of sterilizing immunity in mice persistently infected with *B. burgdorferi* suggests that the organisms have been able to establish a niche in these animals where they are protected from the adaptive immune response.

Interaction of *B. burgdorferi* with ECM is important for chronic infection

Interaction between *B. burgdorferi* with the ECM [24], a hydrated complex of fibrous and non-fibrous proteins and proteoglycans, and specifically collagen (or its associated molecules), appears to be essential for persistence and chronic infection. Studies in non-primate and primate animal models suggest that the ECM is a protected niche for persistence of *B. burgdorferi* in the mammalian host [7,12,25]. In infected mice, *B. burgdorferi* can persist and proliferate in tendons and ligaments despite strong

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Available online 27 June 2007.

humoral immune responses to borrelial antigens [2,12,16], and can cause recurrent attacks of active arthritis and carditis [16,21]. Better knowledge of these interactions between spirochete and ECM is thus of great relevance to the pathogenesis of *B. burgdorferi* infections.

Adhesive interactions of *B. burgdorferi* with cells and tissues

B. burgdorferi can attach to the surfaces of host cells by way of P66 protein (BB0603), which binds to α II β 3 and α v β 3 integrins on platelets and endothelium [26–28]. After penetrating the walls of small arteries, the spirochetes interact with components of the ECM in the surrounding connective tissue [16,17]. Although nonspecific interactions between spirochete and ECM may be involved in this encounter [12,29], various borrelial adhesive proteins have also been identified [2,26,27,30–32]. These adhesins include DbpA (BBA24) and DbpB (BBA25), which bind to the proteoglycan decorin [30], and Bgp (BB0588) and BBK32, which bind to the glycosaminoglycans (proteoglycan polysaccharide moieties) heparan sulfate and dermatan sulfate [31,33,34]. BBK32 also binds to fibronectin, and it has been suggested that BBK32-mediated attachment to this ECM glycoprotein is involved in the pathogenesis of Lyme borreliosis [15,33,34]. Isolation of *B. burgdorferi* genetic variants differing in their expression of P66, DbpA, DbpB and BBK32 has clearly shown that they are involved in adhesion of *B. burgdorferi* to mammalian ECM or cells [15,28,34–36]. The role of these identified borrelial proteins in mediating binding of *B. burgdorferi* to the mammalian host ECM and their functional redundancy have been reviewed [27].

Type I collagen is the major protein of connective tissue ECM [24]. It is therefore a likely niche for the residence of *B. burgdorferi* in dermis, tendons, bladder and heart. Early reports suggested that the spirochete was not capable of binding directly to type I collagen, and interacted with it only through the associated decorin proteoglycan [37]. The ECM, however, is characterized not only by its molecular components, but also by the intramolecular and supramolecular architecture of these components [24]. When intact type I collagen was assembled into hydrated lattices under conditions conducive to formation of native protein fibers

and tissue-like cell behavior [38,39], it supported direct attachment, invasion and formation of microcolonies by *B. burgdorferi* (Figure 1a) [40]. These activities were characteristic of all *B. burgdorferi* strains examined, including one that lacked decorin-binding adhesins, indicating that direct interaction between spirochete and type I collagen was possible.

It is evident that interactions of *B. burgdorferi* with constituents of the ECM can be mediated by multiple borrelial proteins, both identified and unknown. The functional redundancy of these interactions suggests that such interactions are important to the ability of this organism to persist in the ECM.

Decorin-mediated adhesion and chronic infection

The role of adhesive interactions of the ECM in *B. burgdorferi* infection and persistence has been studied in the mouse model. Active and passive immunization of mice with the decorin-binding adhesin DbpA protected against infection with needle-inoculated virulent *B. burgdorferi* [41,42]. Furthermore, lack of immune clearance of *B. burgdorferi* from skin and joints was correlated with high levels of decorin expression in these tissues [25]. Decorin-deficient mice had a somewhat reduced susceptibility to developing arthritis [43], a reduced percentage of positive joint cultures during the acute phase of infection [43], and a significantly lower bacterial burden in the joints during chronic infection compared with wildtype mice [25]. However, DbpA-immunized mice were not protected against tick-inoculated virulent *B. burgdorferi* [44]. This lack of protection correlated with a lack of expression of *dbpA* in the infected tick, and suggested that DbpA only becomes available as an antigenic target some time after infection of a DpbA-immunized mouse by this route [44].

While these studies confirmed a role for decorin-binding in infection, they also indicated that colonization of ECM could occur in its absence. Moreover, because levels of *B. burgdorferi* in joints, skin and other tissues were similar in decorin-deficient and normal mice during the early, preimmune phase of infection [25], decorin, even when present, did not appear to be the primary basis for attachment and invasion of connective tissue ECM. Because

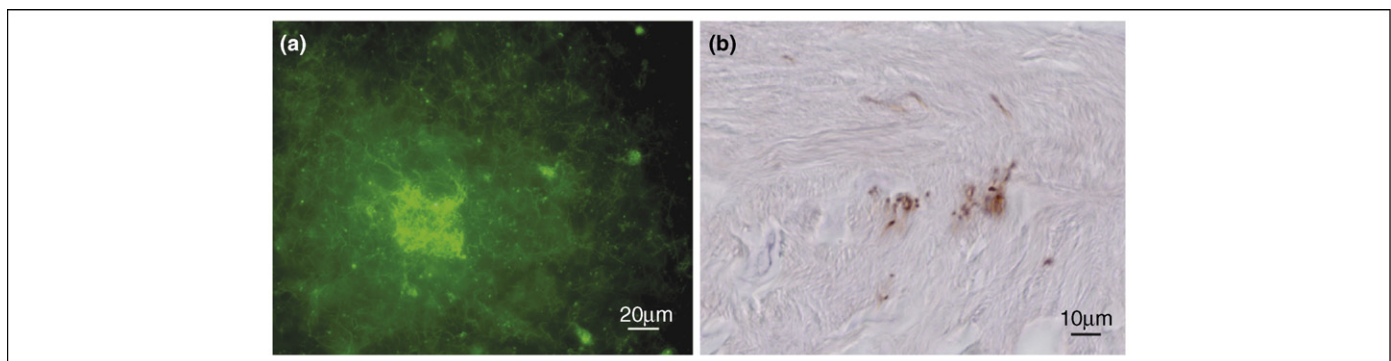


Figure 1. Microcolonies of *B. burgdorferi* in ECM *in vitro* and *in vivo*. (a) Microcolonies of *B. burgdorferi* BL206 (infectious isolate) cultured for ten days on intact type I collagen matrices in the presence of BSK-H medium containing 6% rabbit serum (acridine orange). Scale bar = 20 μ m. See [40] for details of methods. (b) Immunohistochemically detected microcolonies of *B. burgdorferi* cN40 (clonal infectious isolate) in tibiotarsal flexor tendon of a C3H-SCID mouse infected for two weeks, then immunologically reconstituted with normal lymphocytes from uninfected congenic C3H mice and necropsied four weeks after reconstitution (immunohistochemistry-Mayer's hematoxylin). Similar results were obtained with infected SCID mice following passive transfer of mouse anti-DbpA hyperimmune serum [12]. Scale bar = 10 μ m. See [12] for details of methods. Photomicrograph in Figure 1b courtesy of Dr. Stephen W. Barthold; reprinted from Figure 4D of [12] with permission of the American Society of Microbiology.

spirochete levels were significantly elevated in joints and skin (tissues with high levels of decorin expression) in wildtype mice only during the chronic immune phase of infection, it was suggested that decorin provided the bacteria with a protective niche in the ECM that fostered persistence and immune evasion [25].

ECM provides a protected niche for *B. burgdorferi* against specific antibodies

The unimpaired capacity of *B. burgdorferi* mutants to infect decorin-deficient mice might be due to the ability of the spirochete to bind to and invade collagenous connective tissue matrices independently of proteoglycans and their known adhesins [27]. While it is possible that currently unidentified proteoglycans or noncollagenous glycoprotein adhesins might be involved in the initial, acute phases of *B. burgdorferi* infection, existing data are clearly consistent with currently identified adhesins being involved in chronic borrelial infection [27]. Recent observations of Barthold *et al.* [12] are relevant in this regard.

These workers had previously found that T-cell independent anti-borrelial antibodies were involved in remission of arthritis and carditis and widespread reduction (but not elimination) of organisms in *B. burgdorferi*-infected laboratory mice [19,20,45]. Remission could also be induced by passive transfer of immune serum from chronically infected (*i.e.*, 90 days post-inoculation) wildtype or T-cell receptor (TCR)-null mice to chronically infected severe combined immunodeficiency disease (SCID) mice [19,20,45]. In an effort to determine the mechanism(s) of action involved, Barthold *et al.* [12] compared tissue spirochete numbers with histopathologic evidence of inflammation in joints and hearts of passively immunized infected SCID mice [12].

Chronically infected SCID mice treated with immune sera from wildtype or TCR-null mice had reduced numbers of tissue spirochetes and reduced inflammation in joints and hearts compared with animals receiving normal mouse serum [12]. Although both types of immune serum contained significant titers of antibody directed against decorin-binding DbpA and other outer surface proteins such as OspA [12], and passive transfer of hyperimmune mouse anti-DbpA antisera to chronically-infected SCID mice induced significant decreases in arthritis and carditis in these mice, anti-DbpA treatment was not associated with decreases in overall tissue spirochete number when compared with mice receiving anti-OspA antiserum [12]. This raises significant questions about the role of decorin in persistence of *B. burgdorferi* in connective tissues.

In contrast to the lack of change in overall tissue spirochete number in chronically infected SCID mice receiving anti-DbpA antiserum, tissue localization of immunohistologically-identified spirochetes was altered [12]. Joints of mice treated with anti-DbpA contained clusters of spirochetes suggestive of microcolonies in tendons and ligaments, whereas joints of mice treated with anti-OspA contained large numbers of spirochetes within proliferating synovium and few spirochetes within adjacent tendons and ligaments [12]. Infected SCID mice receiving naïve lymphocytes from immunocompetent mice showed similar effects (Figure 1b) [12]. Treatment of chronically infected SCID mice with anti-DbpA antiserum or immunocompetent lymphocytes also led

to elimination of spirochetes from loose connective tissue at the base of the heart and their appearance within the ECM of the aortic wall [12]. Taken as a whole, these studies suggest that disease remission, reduction of tissue spirochete burden, and protection from infection are likely to involve different borrelial tissue and cell binding capabilities including some directed at T-cell independent epitopes.

Genetic analysis of ECM adhesins in infectivity

B. burgdorferi *dbpBA* and *bbk32* adhesin deletion mutants have recently been constructed to examine the role of these gene products in infectivity in mice [13–15] and ticks [14]. Disruption of the *dbpBA* locus, which specifies the two decorin-binding adhesins, was not associated with any decrease in syringe infectivity of a high dose (10^5) of organisms in SCID mice examined one month after inoculation, compared with the infectivity of the parental *B. burgdorferi* strain [13]. Although the ability of the *B. burgdorferi* *dbpBA* mutant to infect wildtype mice was not significantly lower than that of the parental strain, its ability to infect the heart was significantly less [13]. The authors were unable to prove that this difference was due solely to deletion of the *dbpBA* locus because of inability to complement this deletion. They concluded that the *dbpBA* locus was apparently not essential for *B. burgdorferi* infection of SCID or wildtype mice although deficiency of DbpA and DbpB might decrease infectivity potential for some tissues.

Disruption of *bbk32*, which specifies the glycosaminoglycan-fibronectin adhesin, similarly had no effect on infectivity measured at three [14] or four [15] weeks after inoculation, compared with parental strains in wildtype mice syringe-infected with 10^5 organisms. Dilutional analysis of infectivity of syringe-infected mice at four weeks did reveal a small but significant tenfold decrease in infective potential in the *bbk32* mutants [15]. This decrease in syringe-inoculated infectivity of *bbk32* mutants was apparently not sufficient to interfere with transmission of *B. burgdorferi* to mice from infected ticks or with transmission to ticks from infected mice inoculated two to three weeks previously [14]. These results suggest that BBK32 is not absolutely essential for infection of mice and ticks and/or that other known or unknown borrelial adhesins can functionally replace it. The inability of Li *et al.* [14] to demonstrate any effect of the *bbk32* deletion on infectivity in mice and ticks two to three weeks after inoculation was particularly surprising because *bbk32* expression varied over the *B. burgdorferi* life-cycle: low in unfed (flat) ticks, steadily increasing during tick engorgement, and highest in the infected mouse.

The dispensability of these known borrelial decorin- and fibronectin-binding proteins for acute infectivity of *B. burgdorferi* was unexpected. Although these proteins were not essential for acute infection, they could still be important virulence factors for chronic infection. An activity in chronic infection would be consistent with the changes in expression of *bbk32* over the *B. burgdorferi* life cycle [14]. If any of these genes coded for virulence factors for chronic infection, *B. burgdorferi* *bbk32* mutants would not be expected to show a phenotype until later times after

infection (>30 days), a time-point that was not examined in any of the above studies [13–15]. In a very different chronic bacterial infection, a genetic screen for virulence mutants of *Mycobacterium tuberculosis* found that only some mutants showed decreased acute infectivity in mice [46]. Mutants of determinants associated with chronic infection such as dissemination from the initial site of colonization, longterm tissue persistence, or altered patterns of tissue pathology, were as acutely infectious as wildtype *M. tuberculosis*, and displayed a phenotype only at later times after infection [46]. Although *M. tuberculosis* is an intracellular pathogen and *B. burgdorferi* is an extracellular one, their interactions with mammalian hosts show some formal similarities [3,46] in the possibly analogous roles played by the intracellular compartments and the ECM in providing a protected niche.

To summarize, there is currently no genetic evidence for an essential role for any known ECM adhesin in chronic borreliac infection. However, none of these studies assessed mutant phenotypes during chronic infection. Furthermore, the large number and functional redundancy of known *B. burgdorferi* ECM adhesins may require multiple mutations before any phenotype is observable.

Conclusions and future perspectives

As presented in Box 1, interactions of *B. burgdorferi* with the ECM raise a range of questions regarding the relevance of these interactions for the ability of this pathogen to produce acute and chronic infections. These questions also delineate a prospective research program. *B. burgdorferi* growing in the ECM are afforded apparent protection from host immune responses resulting in their ability to persist in this niche [12]. This protection might involve down-regulation of *B. burgdorferi* antigens in response to exposure to ECM components. Alternatively, protection might result from interference with immune effectors by the ECM. This interference could involve impediments to diffusion and therefore tissue penetration of complement, antibody and lymphocytes, or interference by collagen with antibody opsonic and lytic activities. It has been shown, for example, that streptococci covered with collagen fibers are

not readily phagocytosed [47]. Similar studies need to be performed with *Borrelia*.

The resemblance of *B. burgdorferi* microcolonies in native collagen matrices (Figure 1a) [40] to those in the connective tissue of infected mice (Figure 1b) [12] suggests that this may be a microenvironment-related pattern of growth or 'ecophenotype' of *B. burgdorferi* in the ECM associated with its ability to persist in this niche. Native collagen matrices *in vitro* might be useful for analysis of the attachment of *B. burgdorferi* to ECM. Such model matrices could provide an improved *in vitro* setting for studying mutant libraries of *B. burgdorferi* differing in their expression of potential adhesins [48–50]. They would also be well suited for studying interactions of *B. burgdorferi* with components of the immune system such as antibodies and complement, or with antibiotics. The compositional uniformity of the hydrated collagen lattice makes it suitable for biophysical studies of the dynamics of spirochete motility.

Dissection of the complex suite of virulence traits necessary for the establishment of infection and persistence in the body's tissues will require the integrated use of new genetic and genomic tools, *in vitro* models of the ECM [40,51] and *in vivo* models of infection, together with a better understanding of the microarchitecture of the extracellular microenvironment and the application of concepts of virulence factors developed with other more extensively studied bacterial models of chronic infection.

Acknowledgements

This work was supported by grants R01 AI48856 and R01 AI43063 from the U.S. National Institutes of Health to F.C.C. and grant EF-0526854 from the U.S. National Science Foundation to S.A.N. We thank Clemencia Zambrano and Stephen Barthold for the micrographs in Figures 1a and 1b, respectively, Ira Schwartz for supplying *B. burgdorferi* BL206 and for a careful reading of the manuscript and helpful suggestions, and the suggestions of two anonymous reviewers.

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Box 1. Outstanding questions for future research

- Why does the strong host immune response not sterilize tissues of mice infected with *B. burgdorferi*?
- Do characterized *B. burgdorferi* adhesins P66, DbpA, DbpB, Bgp and BBK32 have a role in chronic infection caused by this bacterium?
- Are there as yet uncharacterized and redundant adhesins of *B. burgdorferi* that play a role in acute and chronic infection?
- What are the bases for the ECM being able to serve as a protected niche for chronic infection with *B. burgdorferi*?
- What are the interactions of *B. burgdorferi* in the ECM with antibodies, complement, immune cells and antibiotics?
- Is the ECM able to influence the metabolism of *B. burgdorferi* and modulate gene expression of new adhesins and immunoprotective antigens?
- To what extent is *B. burgdorferi* in the ECM metabolically active?
- Is the ECM a source of reinfection of other host tissues by *B. burgdorferi*?
- Can new genetic techniques and global gene expression studies dissect the role of the ECM in *B. burgdorferi* persistence?

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