

Chapter 2

Immunoseclusion and Chronic Infection by *Borrelia burgdorferi*

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2.1 Introduction

The subjects of immunoseclusion and chronic infection as pertaining to the Lyme disease pathogen, *Borrelia burgdorferi*, present challenges not only in developing suitable models for study, but also in the accurate interpretation of experimental findings in the context of human disease. The term immunoseclusion has been described as the property of a pathogen to evade host immunity by hiding itself in sites not typically afforded vasculature, also known as immunologically privileged organs or tissues. The ability of the Lyme disease bacterium to disseminate to and invade host cells and tissues may be a contributing factor for circumventing host defenses. However, any relationship between putative immunoseclusion properties of the Lyme disease agent and chronic infection has not been firmly established.

Lyme disease is the result of a tick-transmitted bacterial infection that affects thousands of people each year predominantly in North America, Europe, and Asia making it a serious public health concern. Also termed Lyme borreliosis, the infection typically begins to present clinically with the appearance of a distinctive rash at the site of the tick bite called erythema migrans (EM) and is commonly accompanied by fever, headaches, fatigue, myalgia, arthralgia, and regional lymphadenopathy (Steere 2001). If left untreated by antimicrobials, the disease can progress to a later stage with more serious manifestations including arthritis, carditis, and neurological impairment (Steere 2001). Fortunately, Lyme disease is curable in most cases with a few week (2–4) course of antibiotic treatment (Wormser et al. 2006). The disease presentation is basically similar worldwide, although there are regional

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differences due mainly to the presence of two additional major genospecies of the causative agent in Eurasia. However, because the Lyme disease agent can only be transmitted to humans by ticks of the *Ixodes ricinis* complex which exists in distinct ecological regions (Lane et al. 1991), Lyme disease is endemic, i.e., occurs in localized foci. This fact, along with the observance of the EM and support of serological tests (when warranted), greatly aids in an accurate clinical diagnosis of Lyme disease.

The bacterial culprit that causes Lyme borreliosis is *Borrelia burgdorferi*, a spirochete as defined by its characteristic corkscrew-shaped morphology (Burgdorfer et al. 1982). In addition to *B. burgdorferi*, two other genospecies cause Lyme disease in Europe and Asia, *Borrelia garinii* and *Borrelia afzelii* (Steere 2006). Collectively, the three genospecies are known as *B. burgdorferi* sensu lato. In North America, *B. burgdorferi* sensu stricto (in the strict sense) is the sole genospecies responsible for Lyme disease and is found mainly in the northeast- and northcentral-regions of the United States where the vector is *Ixodes scapularis* and the reservoirs are small vertebrates, primarily rodents (Lane et al. 1991). In nature, *B. burgdorferi* exists exclusively inside either a vertebrate host or ticks, as the organism is incapable of living freely in an external environment. Nevertheless, the pathogen thrives in an enzootic cycle passing from one reservoir host to another by way of a tick intermediate that transfers and acquires *B. burgdorferi* during feeding on the hosts. The spirochete has developed an intricate system of genetically based survival mechanisms that allow it to adapt to each of its environments, whether mammalian or tick, in order to maintain its life cycle. *B. burgdorferi* lives within the confines of each host without causing harm, however, when transmitted to humans the scenario changes and the organism becomes pathogenic when infecting this host. Because the tick that transmits *B. burgdorferi* is very small, humans quite often are unaware that they have been bitten and subsequently become infected, even though the tick feeds for 4–5 days and borreliae do not venture out of the tick until around 48 h post-attachment (Piesman 1993; Ohnishi et al. 2001). In the absence of antibiotic treatment, *B. burgdorferi* can maintain infection in either rodent or human hosts despite the elicitation of strong immune responses, both innate and adaptive, and the processes driving this evasion ability is the subject of this book. The topics covered in this chapter are:

1. Challenges in performing studies investigating dissemination, invasion, and colonization of host organs and tissues.
2. Mechanisms of immunoseclusion including the physical structures and properties of *B. burgdorferi*, extracellular localization, and intracellular invasion.
3. Host persistence and chronic infection.

This chapter provides a review and summary of the current literature regarding the properties of *B. burgdorferi* relating to immunoseclusion and chronic infection from studies performed by many research groups utilizing animal models and cell culture. Although these models are invaluable in aiding our understanding of the complex mechanisms involved in human pathogenicity, care must be utilized when extrapolating experimental results into firm conclusions regarding the pathobiology of human Lyme borreliosis. Studies exploring borrelial mechanisms of

invasion and seclusion into host tissues and cells should be regarded and interpreted in these contexts: (1) evasion of innate and/or adaptive immune responses, i.e., short-term or long-term survival or both; (2) antibiotic treated vs. untreated subjects; (3) infection in mice vs. infection in humans.

2.2 Challenges in Studying *B. burgdorferi* Host Infection, Dissemination, and Tissue Colonization

When an infected tick feeds on a mammalian host, chemical signals in the blood meal awaken the quiescent borreliae that have resided dormant as the tick overwintered. A dynamic series of gene regulation induces borrelial gene expression essential for bacterial replication and prepares *B. burgdorferi* for migration from the tick to the host during the 4–5 day feeding period (Schwan et al. 1995; Caimano et al. 2007; Boardman et al. 2008; Tokarz et al. 2004). *B. burgdorferi* are initially deposited into the host skin at the tick bite site. It is here that the organisms encounter the first stage of the host's immune attack as the innate system assaults the borreliae by complement-mediated lysis, engulfment by phagocytic cells, and induction of pro-inflammatory cytokines (Steere et al. 2004; Samuels and Radolf 2010). The inflammation that occurs in the skin results in the distinguishing EM rash. A subpopulation of *B. burgdorferi* weathers this attack and begins dissemination to other organs and tissues by hematogenous spread (Wormser 2006). After days to weeks of incubation, dissemination of the organism can result in diverse clinical manifestations such as arthritis, carditis, and neurological conditions (Steere 2001; Marques 2010), despite the production of antibodies against several *B. burgdorferi* proteins.

It is difficult to study the pathobiology of the disease in humans because of limitations in detection assays, e.g., culture reisolation and PCR, from affected tissues. *B. burgdorferi* do not replicate to high numbers in the bloodstream, therefore clinical isolation is not routine although recovery techniques from the blood of untreated patients that are EM-positive early in infection onset have improved (Wormser et al. 2005, 1998). *B. burgdorferi* recovery from skin in an EM by culturing can be accomplished and is the “gold standard” for a diagnosis of infection. Isolation of the organism from internal tissues by culture is not practical due to the extreme insensitivity of the assay and invasive biopsy procedures that would often be required to obtain tissue samples. Synovial fluid and cerebrospinal fluid (CSF) from patients have been used for study, with borrelial DNA being detected by PCR, but culture isolation of the organism from these tissues has been rare (Marques 2010; Agüero-Rosenfeld 2008). PCR as a pathogen detection assay is useful, but limited in that it proves that bacterial DNA is present, but not whether the organisms are alive or dead, and therefore not an indicator of ongoing infection. Moreover, outstanding questions remain regarding whether the DNA represents organisms that are dead or dying, or whether the organisms are viable, but uncultivable. Finally, it is not feasible or ethical to study the biological processes of the spirochete during the course of known infection in humans without administering antibiotic treatment.

Therefore, studies on *B. burgdorferi* pathogenesis and interactions with the host tissues and cells have been relegated to animal models (in vivo) and tissue culture (in vitro).

Dissemination from the site of the tick bite and subsequent colonization of tissues occurs in both humans and the murine model of infection, but there are some differences. In mice, *B. burgdorferi* migrate to and infect heart, spleen, bladder, joint, and ear tissues, but not the central nervous system (CNS) as in humans. Murine species also do not display EM or other signs of pathology commonly associated with human Lyme disease (an exception is some inbred strains of mice will develop arthritis and carditis) despite the rodents remaining chronically infected throughout their lifespan. Therefore, mouse models are useful to study chronic infection mechanisms, but may reflect the biology of borreliar persistence in reservoir hosts as seen in nature rather than as a disease model.

2.3 Properties of *B. burgdorferi* Involved in Tissue Colonization

After exposure to *B. burgdorferi* by tick bite or needle inoculation, mice remain chronically infected throughout their life which can be demonstrated by reisolation of the spirochetes from various tissues. Such persistence is consistent with *B. burgdorferi* survival within rodent reservoir hosts that is essential for the natural perpetuation of the enzootic cycle via tick vectors. An interesting biological feature is that mice remain infected despite elicitation of a strong innate and adaptive immune response against the borreliae. The antibody response in humans following *B. burgdorferi* infection parallels that seen in mice, making mice a good experimental animal model for the study of host immunity against *B. burgdorferi* infection. Throughout the infection, in both rodents and humans, antibodies against numerous *B. burgdorferi* antigens can be detected (Dressler et al. 1993; Nowalk et al. 2006). Some have been shown to be protective and/or borreliacidal (e.g., outer surface protein C [OspC] and decorin binding protein A [DbpA]) (Preac-Mursic et al. 1992; Gilmore et al. 1996; Rousselle et al. 1998; Hanson et al. 1998; Hagman et al. 1998), yet the organism is not cleared, presumably by mechanisms employed by the spirochete for immune evasion (i.e., immune suppression, antigenic variation, and variable expression of antigen genes) (Embers et al. 2004). An additional strategy proposed for evasion is the physical seclusion of the organism away from the harmful effects of the innate and adaptive humoral response. For example, it has been postulated that *B. burgdorferi* sequesters itself from antibodies by localizing in immunocompromised areas of tissues such as the extracellular matrix (ECM), collagen, and/or intracellular niches.

The ability of *B. burgdorferi* to disseminate to and interact with host ECM components and tissues is not unexpected due to the physical attributes of the organism. First, *B. burgdorferi* is a highly motile organism having an intricate network of periplasmic flagella that aid in its spread to target sites and subsequent penetration of tissue barriers (Charon et al. 1992a, b).

Second, though *B. burgdorferi* lacks the ability to produce proteases, it can bind host proteases to assist in dissemination and penetration into host tissues. Borrelial binding and activation of host plasmin, a serine protease capable of degrading ECM, has been demonstrated. This aids the organism in penetrating cell monolayers in vitro and advancing spirochetemia in the mouse model (Hu et al. 1995; Coleman et al. 1995, 1997; Klempner et al. 1995; Fuchs et al. 1994, 1996; Grab et al. 2005). *B. burgdorferi* proteins implicated in plasminogen binding include OspA, BPBP, OspC, CRASP-1, and ErpP-A and -C (Fuchs et al. 1994; Hu et al. 1997; Brissette et al. 2009a; Lagal et al. 2006; Hallstrom et al. 2010). Additionally, several studies have shown *B. burgdorferi* can induce production in vitro of host matrix metalloproteinases (MMP), enzymes produced by numerous cells that are involved in the normal restructuring of ECM (Grab et al. 2005; Behera et al. 2004; Gebbia et al. 2001; Singh et al. 2004; Zhao et al. 2007; Perides et al. 1999). However, the effect of borrelial induction of MMPs in vivo for invasion and seclusion into host tissues is not clear; instead the evidence suggests that MMP activation by *B. burgdorferi* may lead to the pathology associated with Lyme arthritis (Hu et al. 2001; Lin et al. 2001; Heilpern et al. 2009).

Third, *B. burgdorferi* synthesizes a number of proteins that function as adhesins allowing the spirochetes to adhere to host tissue and cell receptors which may then induce signals integral for cellular internalization (Coburn et al. 2005). Borrelial proteins that bind host molecules such as fibronectin, laminin, decorin, integrins, and glycosaminoglycans have been identified and are listed in Table 2.1. Studies showing that inactivation of specific adhesin protein genes does not completely abrogate either the infectious and/or adherence properties of the organism (specifically at high infection doses), indicates that *B. burgdorferi* can synthesize numerous surface localized proteins capable of infection-mediating adherence (Li et al. 2006; Seshu et al. 2006; Weening et al. 2008; Blevins et al. 2008; Shi et al. 2008; Parveen et al. 2006). Therefore, it is suspected that *B. burgdorferi* genetically regulates a complex network of protein synthesis during mammalian infection with potential functional redundancy enabling the organism to persevere in its specific tissue and/or escape the host's production of antibodies (Caimano et al. 2007; Zhang et al. 1997; Embers et al. 2007; Coutte et al. 2009; Liang et al. 2002, 2004a; Gilmore et al. 2007, 2008; Miller and Stevenson 2006). Although not completely clear whether the organism is capable of tropism for specific tissues or selectively adheres after general dissemination, it is evident that *B. burgdorferi* has the machinery to produce a variety of proteins for tissue and cellular colonization. However, questions remain relating to the fate of the borreliae following attachment to host components that enable long-term maintenance.

2.3.1 Extracellular Locales

The ECM is a complex structure of connective tissue surrounding mammalian tissues and cells, and is composed of a fibrous network of structural proteins and proteoglycans (e.g., collagen, fibronectin, decorin, and laminin). The ability of

Table 2.1 *B. burgdorferi* outer surface adhesin proteins

Adhesin	Host receptor	References
BBK32 (fibronectin binding protein)	Fibronectin Dermatan sulfate Heparin	(Probert and Johnson 1998; Fischer et al. 2006)
DbpA (decorin binding protein A)	Decorin	(Fischer et al. 2003;
DbpB (decorin binding protein B)	Glycosaminoglycans	Guo et al. 1998)
P66	Decorin	
	Integrin $\alpha_{IIb}\beta_3$	(Coburn et al. 1993; Coburn and Cugini 2003)
OspA	Integrin $\alpha_v\beta_3$	
	TrospA (tick midgut receptor)	(Pal et al. 2004)
	Evidence for binding to endothelial and neural cells	(Rupprecht et al. 2006; Comstock et al. 1993)
BmpA (Borrelia membrane protein A)	Laminin	(Verma et al. 2009)
Bgp (Borrelia GAG-binding protein)	Glycosaminoglycans	(Cluss et al. 2004; Parveen and Leong 2000)
RevA	Fibronectin	(Brisette et al. 2009b)
BBB07	Integrin $\alpha_3\beta_1$	(Behera et al. 2008)
ErpX	Laminin	(Brisette et al. 2009c)
CRASP-1	Bone morphogenic protein 2	(Hallstrom et al. 2010)
	Collagen	
	Fibronectin	
	Laminin	

bacterial pathogens to colonize host tissues and cells requires the organism to penetrate barriers such as the ECM. *B. burgdorferi* has the ability to traverse the ECM by such physical properties as motility provided by periplasmic flagella, and the ability to utilize host proteases such as plasmin(ogen) and possibly MMPs to proteolytically bore into the tissue. This capability may serve the dual purpose of allowing *B. burgdorferi* to localize in the dense structure of collagen-rich tissue or ECM to avoid immune detection.

Studies observing *B. burgdorferi* microscopically in histopathologic sections of animal ECM or collagen-rich tissues (such as tendons, joints, or cartilage) have been limited. The Barthold laboratory has shown *B. burgdorferi* in immunohistochemically stained sections of tibiotarsal synovium and tendon from experimentally infected immunocompetent and immunodeficient mice (Barthold et al. 1993, 2006; Hodzic et al. 2008). *B. burgdorferi* attachment and colonization of collagen in vitro was demonstrated by Zambrano et al. whereby they showed that following specific binding to collagen lattices, borreliae were able to replicate and form microcolonies (Zambrano et al. 2004). Liang et al. proposed that decorin-rich tissues such as skin and joints provide a “protective niche” for *B. burgdorferi* to avoid

adaptive humoral immunity by immunoseclusion due to the organism's ability to bind decorin by the surface antigens DbpA and DbpB (Liang et al. 2004b). Weening et al. extended these conclusions in a study whereby a DbpBA-deficient mutant exhibited an early colonization defect in mice, suggesting these proteins are required for resistance to the host's innate immune response (Weening et al. 2008). An independent study, however, provided evidence that although anti-DbpA antiserum can induce carditis and arthritis remission in infected mice, there was little to no effect on eliminating or reducing the numbers of borreliae in tissues (Barthold et al. 2006). These data suggested that although DbpA plays an important role in borrelial binding and colonization of ECM and host cells, there were undoubtedly other factors involved in *B. burgdorferi* tissue localization following establishment of infection. A recent review by Cabello et al. summarized the above studies, and the authors reflected on the interactive nature of *B. burgdorferi* and its adhesins with proteoglycan components of the ECM as a mechanism for the organism to establish itself within a protective habitat (Cabello et al. 2007). These authors provided a list of thought-provoking questions regarding the putative effect of *B. burgdorferi*/ECM interactions on persistent infections. Among the more interesting are: (1) What is the role, if any, for identified borrelial adhesins in persistent infections? (2) How metabolically active are *B. burgdorferi* in the ECM? (3) Does the ECM environment modulate *B. burgdorferi* gene expression to synthesize new adhesins and/or immunoprotective antigens (Cabello et al. 2007)? Other questions come to mind as well: (1) Do *B. burgdorferi* dwell and localize in connective tissues or are these locales only transient destinations as borreliae seek out host cells for colonization? (2) Does *B. burgdorferi* replicate within ECM? (3) What is the effect of *B. burgdorferi* within ECM on Lyme disease pathology? (4) Are these connective tissues resistant to permeabilization by antibiotics or antibodies thereby providing a protective site?

An interesting observation by Grab et al. indicated that *B. burgdorferi* interact with fibrocytes, fibroblast-like leukocytes that circulate in the peripheral blood. The investigators demonstrated borrelial binding to human and monkey fibrocytes in vitro and observed the organisms being taken into deep recesses of the cell membrane (Grab et al. 1999). Based on these results, they postulated a mechanism whereby *B. burgdorferi* bind to fibrocytes and are then targeted to the connective tissue. Additionally, they speculated that borreliae within the cell invaginations provide a site for immune seclusion.

Duray et al. described a human tonsillar tissue culture method in which they inoculated *B. burgdorferi* to study the invasive properties of the organism ex vivo (Duray et al. 2005). These investigators observed extracellular borreliae in the tissue and determined that the organisms were capable of replication. In addition, they described "cystic" structures formed by the borreliae and suggested they were dormant organisms that could eventually return to their spirochetal origin. More discussion on the cyst-like borreliae is provided in Sect. 2.5.

The non-human primate has been the best model to study Lyme borreliosis as it pertains to human infection. Unlike murine infection, rhesus monkeys can develop EM and a localization of *B. burgdorferi* in the CNS and peripheral nerves which mimics neuroborreliosis in humans. The CNS has been suggested as an immune

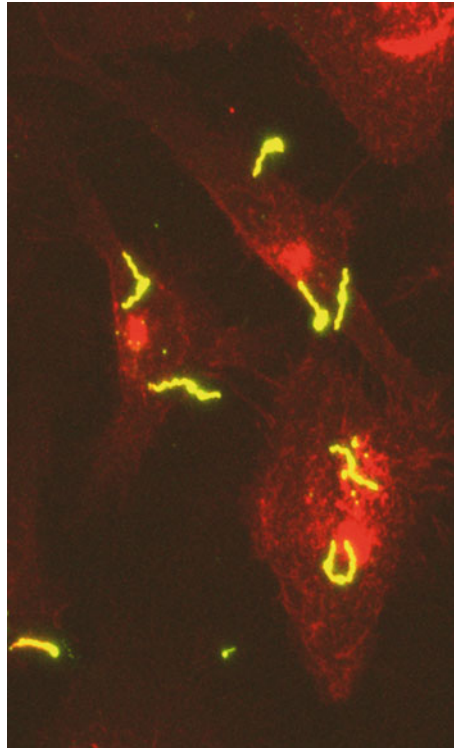
privileged organ and is therefore a potential site for *B. burgdorferi* seclusion (Embers et al. 2004). *B. burgdorferi* have been observed microscopically in the CNS of monkeys where the bacteria were found localized to leptomeninges, nerve roots, and dorsal root ganglia, as well as to the connective tissue of peripheral nerves (Cadavid et al. 2000; Roberts et al. 1995). Collectively, these studies demonstrate the propensity of *B. burgdorferi* to disseminate and localize to connective tissues of various organs within a variety of susceptible hosts.

2.3.2 Internalization by Host Cells

Host cell adherence is an initial step for host colonization and proliferation of bacterial pathogens, including *B. burgdorferi*, during infection. Numerous studies have described the interactions of *B. burgdorferi* in vitro with cells of various origins, and *B. burgdorferi* produces several outer membrane proteins that facilitate binding to host cell receptors and ligands (Table 2.1). *B. burgdorferi* have been shown to adhere to human and murine fibroblasts, endothelial cells, epithelial cells, macrophages, neuronal and glial cells, fibrocytes, and lymphocytes; also tick cells, and Vero cells (Grab et al. 1999; Sambri et al. 1993; Cinco et al. 2001; Thomas et al. 1994; Leong et al. 1998; Fischer et al. 2003; Peters and Benach 1997; Garcia-Monco et al. 1989, 1991; Szczepanski et al. 1990; Thomas and Comstock 1989; Kurtti et al. 1993; Hechemy et al. 1992; Rupprecht et al. 2006; Coburn et al. 1998; Dorward et al. 1997; Livengood et al. 2008; Chmielewski and Tylewska-Wierzbanska 2010; Wu et al. 2011; Montgomery et al. 1993). Although *B. burgdorferi* is commonly referred to as an extracellular pathogen, several investigations performed in vitro with cell culture have revealed the invasive properties of borreliae into non-professional phagocytic cells (e.g., see Figs. 2.1 and 2.2) (Hechemy et al. 1992; Chmielewski and Tylewska-Wierzbanska 2010; Wu et al. 2011; Livengood and Gilmore 2006; Ma et al. 1991; Klempner et al. 1993; Girschick et al. 1996). Related studies have demonstrated that *B. burgdorferi* can penetrate cell junctions as a putative process for dissemination (Szczepanski et al. 1990; Comstock and Thomas 1989; Grab et al. 2009). Additionally, *B. burgdorferi* are internalized by macrophages and monocytes through phagocytosis, a critical step in the innate immune response against infection (Moore et al. 2007; Cruz et al. 2008; Shin et al. 2008; Kuhlow et al. 2005). The organisms are eventually degraded within the phagolysosome of these cells, although a study by Montgomery et al. reported the occasional survivor in mouse macrophages (Montgomery et al. 1993).

The paradigm of *B. burgdorferi* as a mainly, if not exclusive, extracellular organism has primarily been due to the inability to detect intracellular spirochetes in infected tissues in vivo, either in animal models or in humans. Nevertheless, evidence from in vitro studies demonstrating the ability of *B. burgdorferi* to invade and inhabit cells internally suggests the probability that subpopulations of borreliae localize within cells during infections. Such internalization may result in host cell damage causing pathology consistent with Lyme borreliosis, or may be a mechanism for a

Fig. 2.1 Association of *Borrelia burgdorferi* with human cells in vitro. Confocal microscopy image of H4 neuroglial cells incubated with *B. burgdorferi*. Human cell plasma membranes were immunostained (red), and *B. burgdorferi* were labeled green with the images being merged whereby yellow indicates co-localization of *B. burgdorferi* with the plasma membrane. Confocal images by Jill A. Livengood



subset of the pathogen population to escape destruction from innate or adaptive host responses and/or antibiotic treatment.

Mechanisms involved in the internalization of *B. burgdorferi* into host cells post-attachment have been understudied. The first studies on borrelial adherence and invasion were performed in the late 1980s and early 1990s. A noticeable gap in this area of research ensued until recently (2006–2010) with studies by Livengood and Gilmore, Chmielewski et al., and Wu et al. reporting on borrelial invasion of eukaryotic cells (Chmielewski and Tylewska-Wierzbanska 2010; Wu et al. 2011; Livengood and Gilmore 2006). Also, studies from the Chaconas laboratory have used intravital microscopy to visualize borrelial interactions with vasculature cells in vivo (Moriarty et al. 2008; Norman et al. 2008). The Skare laboratory's research has progressed from simple intracellular observation of *B. burgdorferi* in cells to delineating mechanisms for internalized uptake. Wu et al. investigated requirements for invasion and found that β_1 -containing integrins and Src-family kinases were necessary components suggesting that *B. burgdorferi* uptake needed actin filament reorganization to gain entry. They also found that an outer surface adhesin, fibronectin binding protein BBK32, was not a requirement for the internalization process. Finally these investigators observed that *B. burgdorferi* remained viable after cellular uptake for out to 4 weeks of cocultivation (Wu et al. 2011).

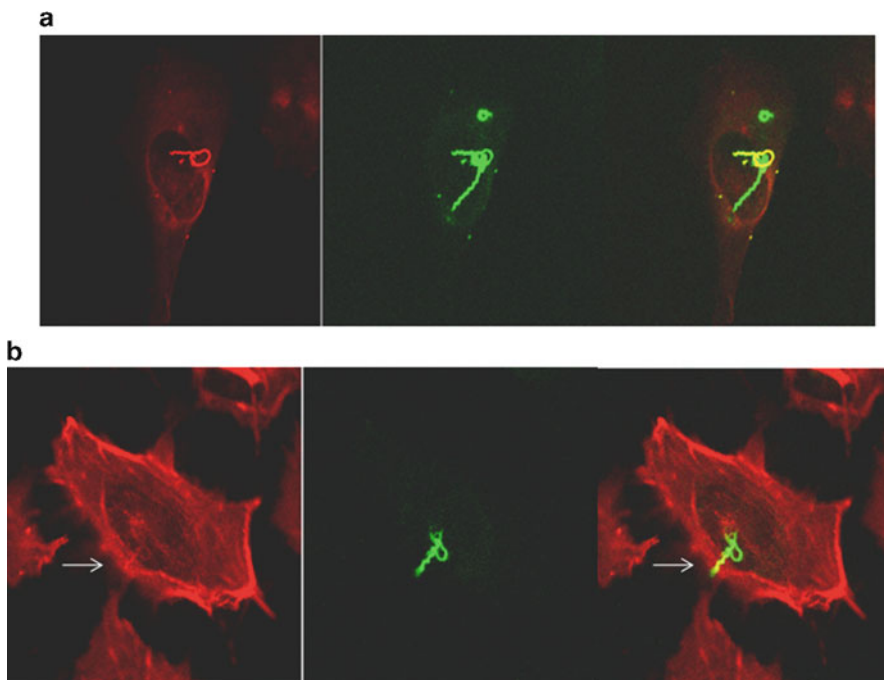


Fig. 2.2 Extra- and intracellular association of *Borrelia burgdorferi* with human cells in vitro by double immunostaining. **(a)** Human umbilical vein endothelial cells (HUVECs). **(b)** H4 neuroglial cells. *Left panels* represent extracellular spirochetes stained with a rhodamine-conjugated anti-*B. burgdorferi* antibody. *Middle panels* show the same microscopic field of spirochetes stained with a FITC-conjugated anti-*B. burgdorferi* antibody following permeabilization of fixed cells. *Right panels* show a merged image of the two stains. Yellow-appearing *B. burgdorferi* indicate extracellular organisms that have been stained with both rhodamine and FITC. Green *B. burgdorferi* indicate intracellular organisms. The arrow in **(b)** points to a portion of a spirochete that stains yellow indicating it is outside the cell; while the remainder of the organism is green indicating it is inside the cell. Confocal images by Jill A. Livengood

Many mysteries relating to borrelial invasion and intracellular existence remain with the foremost question being does *B. burgdorferi* utilize intracellular environments during any phase of in vivo infection? Other questions that warrant further investigation include: (1) Does *B. burgdorferi* replicate within host cells? (2) Does *B. burgdorferi* invasion lead to host cell damage and pathological effects to the host? (3) Is *B. burgdorferi* internalization a temporary event and do borreliae eventually exit the host cells they invade? (4) Does *B. burgdorferi* undergo differential gene expression in this environment to produce new antigens or surface components for survival? The recent studies indicated above, along with the continued work with adhesins by several investigators, signal a renewed interest in understanding more about *B. burgdorferi* adherence and invasion into eukaryotic cells and the relationship to immunoseclusion and immune evasion.

2.4 *B. burgdorferi* Persistence and Chronic Infection

Webster's dictionary defines persistent as "remaining infective for a relatively long time in a vector after an initial period of incubation"; persist is defined as "to continue to exist esp. past a usual, expected, or normal time". Webster's defines chronic as "marked by a long duration or frequent recurrence". Therefore, by these definitions, the question can be raised—does *B. burgdorferi* persist (or become persistent) within humans resulting in chronic disease? The term "chronic Lyme disease" has been used in a variety of different connotations over time and has evolved to inaccurately describe a larger array of conditions that has resulted in patient diagnoses attributing chronic subjective symptoms to persistent borrelial infections that are unsubstantiated by scientifically validated and controlled laboratory tests. Various interpretations of "chronic Lyme disease" have produced diverse viewpoints on how the Lyme disease pathogen operates to cause disease and consequently how Lyme patients should be diagnosed and treated. A review of "chronic Lyme disease" detailing the applications of the term and ramifications among the interested parties is referred (Feder et al. 2007).

It is a fact that *B. burgdorferi* produces a chronic infection in rodents in nature and in experimental animals, i.e., the spirochete will persist in the animal throughout its lifetime as evidenced by being cultured from various necropsied tissues. Despite such systemic involvement, rodents do not develop disease, consistent with the natural existence of a reservoir host necessary for borreliae to perpetuate its enzootic cycle between mammalian host and tick vector. Therefore, using the mouse as a model for infection, one must be cognizant of the key difference, i.e., mice do not get sick but people do (an exception is certain laboratory strains of inbred mice that develop arthritis during infection). Borrelial infection in mice results in both innate and adaptive immune responses including a strong antibody response. As noted previously, despite elicitation of a humoral response generating antibodies that are borreliacidal, *B. burgdorferi* is not cleared and persists within the murine host. Humans share this characteristic as antibodies against several borrelial antigens can be detected by serological assays following establishment of infection. Like mice, the antibody response in humans does not result in clearance of the organisms and resolution of the disease, therefore, without antibiotic treatment, *B. burgdorferi* can presumably persist in humans for months to years (Steere et al. 2004). In humans, a 2–4 week course of antibiotics is an effective treatment for the resolution of disease manifestations in the vast majority of patients who have been accurately diagnosed with Lyme disease, as summarized by the guidelines of the Infectious Diseases Society of America (IDSA) (Wormser et al. 2006). However, following treatment a minority of patients report subjective symptoms such as arthralgia, fatigue, and impaired cognitive function. Short-term duration of the symptoms is referred to as "post-Lyme disease symptoms", while continuation of these ailments for >6 months is referred to as "post-Lyme disease syndrome" (Feder et al. 2007). The fundamental question eliciting disagreement and controversy between Lyme disease advocates and their practitioners, and infectious disease researchers, is whether *B. burgdorferi* persists in infected

humans following antibiotic treatment, presumably by utilizing the putative seclusion strategies, to cause a chronic infection with debilitating effects.

The scientific evidence to date reports few cases whereby *B. burgdorferi* can be cultured from tissues obtained from clinically diagnosed Lyme disease patients following antibiotic treatment. Two European groups have reported culture isolation of *B. burgdorferi* *sensu lato* in humans following antibiotic treatment (Strle et al. 1996; Hunfeld et al. 2005, 2006; Preac-Mursic et al. 1989). In their study, Hunfeld et al. were able to obtain clinical reisolates from the EM site of Lyme borreliosis patients after antibiotic treatment. (The patients had EM but did not present with or develop more serious manifestations.) The investigators noted that culture reisolation is rare following the initiation of antibiotic therapy, and attributed positive cultures to treatment failure (Hunfeld et al. 2005). Their study concluded that borreliar persistence at the site of the EM was not due to acquired resistance to the antibiotic. Furthermore, these investigators pointed out that “the currently available diagnostic techniques do not reliably discriminate among possible reinfection, true endogenous relapse, and coinfection with other tick-borne pathogens. These drawbacks together with the phenomenon of resistance to therapy in individual patients undoubtedly contribute to the inconsistencies surrounding the optimal treatment regimens for Lyme borreliosis and are often misinterpreted and misused to support prolonged antibiotic treatment regimens” (Hunfeld et al. 2005, 2006).

Two separate comprehensive studies found culture-negative skin biopsy samples from EM sites following antibiotic treatment, although the patients were all culture-positive pre-treatment (Nadelman et al. 1993; Berger et al. 1992). Studies by Klempner et al. investigated the effects of antibiotic treatment on patients with a history of Lyme disease and were unable to culture *B. burgdorferi* from blood samples (12 patients) or CSF samples post-treatment (128 patients) (Wormser et al. 2006; Klempner et al. 2001). In addition, these studies evaluated blood and CSF from a total of 843 specimens and none were culture- or PCR-positive (Klempner et al. 2001; Klempner 2002). A study often cited as proof of *B. burgdorferi* surviving in patients after antibiotic treatment involved the report of positive blood cultures using a novel culture medium with Detroit tap water as an ingredient (Phillips et al. 1998). However, that study identified spirochetal forms as *B. burgdorferi* based on microscopic observation without confirmatory tests such as PCR, thereby leaving open the possibility of mistaken conclusions. Moreover, a follow-up study by an independent group sought to replicate the results by culturing in the same culture medium and found that *B. burgdorferi* were unable to grow (Marques et al. 2000). Other studies have reported similar results of *B. burgdorferi* detection, but in the end have failed to adhere to the rigorous scientific method to validate the conclusions (Wormser et al. 2006). Therefore to date, scientifically recognized proof of viable *B. burgdorferi* isolation from human patients following antimicrobial treatment is extremely rare.

A systematic approach by several researchers to investigate the existence of *B. burgdorferi* *in vivo* following antibiotic therapy has been applied to experimental animals. Details and comments are provided in a review article on this subject by Wormser and Schwartz (Wormser and Schwartz 2009). Three studies to determine whether *B. burgdorferi* persisted in mice after antibiotic therapy are deserving of

comment. Investigations by Bockenstedt et al. and Hodzic et al. employed DNA detection (by PCR), serology, histology and/or immunohistochemistry, transmission by skin allograft, and xenodiagnosis (assessment of *B. burgdorferi* acquisition by ticks after feeding on an infected animal) in addition to culture as measurement parameters for borrelial detection (Hodzic et al. 2008; Bockenstedt et al. 2002). A third study by Yrjanainen et al. evaluated borrelial persistence in antibiotic-treated mice and detected *B. burgdorferi* by culture and PCR (Yrjanainen et al. 2007). Bockenstedt et al. concluded that spirochetes could be detected in treated mice by xenodiagnosis but for a limited time following antibiotic administration. However, the spirochetes did not survive in ticks, were not able to infect new mice, were not cultivable and did not cause disease in the treated mice. The investigators surmised that the subpopulation of *B. burgdorferi* that survived were rendered avirulent and would eventually be cleared by the host's immune defenses (Bockenstedt et al. 2002). A follow-up study by Hodzic et al. investigated the possibility of borrelial persistence after an antibiotic regimen, as they had hypothesized from their previous work that spirochetes invaded collagen during long-term infection thereby minimizing the effectiveness of antimicrobials and the immune response (Hodzic et al. 2008; Barthold et al. 2006). These investigators concluded that mice remained infected with borreliae after antibiotic treatment, but the bacteria were not cultivable nor able to replicate. In their evaluation of both studies, Wormser and Schwartz pointed out that neither study considered the pharmacodynamics of the antibiotics utilized (ceftriaxone and doxycycline) in the experimental animals as compared to the administration for humans (Wormser and Schwartz 2009). How this parameter affected the outcome and conclusions of the Bockenstedt and Hodzic research is unclear. Nevertheless, both studies and the Yrjanainen et al. study provided provocative results that raise interesting questions for further study, e.g., are the subpopulation of *B. burgdorferi* that persists after treatment merely weakened organisms that will eventually be eliminated by the host or are they metabolically quiescent organisms capable of restoration to a pathogenic state? Undoubtedly, additional research in this fascinating area is awaited.

2.5 Cystic Forms

Morphological transition of *B. burgdorferi* spirochetes in response to growth in culture medium lacking serum or other suboptimal conditions has been demonstrated (Brorson and Brorson 1997; Miklossy et al. 2008; Alban et al. 2000; Murgia et al. 2002; Murgia and Cinco 2004). The changed morphological non-motile forms that result from these conditions have been termed as cysts, cystic forms, and/or cyst-like structures. The transformation of *B. burgdorferi* from motile spirochetes to these morphologically different forms is reversible when rabbit serum is added back to the growth media (Alban et al. 2000; Murgia and Cinco 2004; Brorson and Brorson 1998). These observations have led to the proposition that *B. burgdorferi* utilizes this ability to withstand and survive temporary environments of nutrient

deprivation that may occur in host tissues. It has also been speculated that these changes may allow borreliae to evade detection by the immune system. Although there is some evidence for this event in human tissue (Miklosy et al. 2008), like intracellular localization of borreliae in human cells, the existence of “cystic forms” in vivo has yet to be proven, and therefore any clinical significance has not been established. Conclusive demonstration of these structures in vivo awaits further investigations.

2.6 Summary

The ability of *B. burgdorferi* to maintain infection and cause disease within its host in the face of immune pressure is what makes the spirochete a formidable pathogen. Research has greatly improved our knowledge of the structure of the Lyme disease agent and provided new insights into the mechanisms of immune evasion. The subject of immunoseclusion, or the physical sequestering of the organism from host immunity, remains one of the more understudied areas relating to this insidious microbe. There are many unknowns in our understanding of the biology of *B. burgdorferi* as it invades and colonizes extracellularly, and likely intracellularly, within the tissues of its host. It is important to recognize that *B. burgdorferi* may utilize mechanisms to hide away from the first line of immune defense as well as the adaptive response. The spirochetes first enter the host by deposition into the skin by tick bite. The innate response can be potent in removing substantial numbers of infecting organisms and preventing further dissemination and host damage. At this early stage of infection, safe harbor into connective tissue beneath the basement membrane of dermal endothelium as well as intracellular trafficking into fibroblasts, epithelial and endothelial cells may be part of the pathogen’s portfolio for survival.

Certainly, *B. burgdorferi* is capable of persisting within mammalian hosts in the absence of treatment with antimicrobials as observed in reservoir rodents in nature and experimental animal models in the laboratory. In humans, which are not natural hosts required for *B. burgdorferi*’s enzootic cycle, we cannot conclude with certainty that the organism would persist indefinitely without treatment. As a “dead end host”, perhaps the immune system of humans is capable of eventually clearing the pathogen, albeit with resultant sequelae from the infection. More in question is the ability of the organism to persist and continue to cause disease following antibiotic treatment. To date, the evidence is overwhelming that antibiotics are effective for Lyme disease cessation without recurrence. Whether bacteriostatic or bacteriocidal, antimicrobials may not clear every individual bacterial cell, but rather renders them susceptible to the body’s immune system to “clean up” the remaining subpopulation either by reducing the invasive numbers and/or leaving them in a weakened state. Because there are no absolutes in biology, it could be argued that there may be the extremely rare instances of immunocompromised or genetically disposed individuals in which an infection could persist and cause pathology despite antimicrobial treatment. Antimicrobial treatment failure could also be a consideration for a case of persistence.

However, such hypothetical isolated cases would need to be accurately validated and separated from misinformed diagnoses in order to study and understand a scientific basis of true chronicity. As part of the immune evasion strategies for establishment and maintenance of infection, for acute and late disease, *B. burgdorferi* seemingly has the tools for physical seclusion. However, more research is needed to discover the ultimate fate of the Lyme disease pathogen after arrival to its immunosecluded destinations and how it relates to the organism's pathogenicity.

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