Review

Survival strategies of *Borrelia burgdorferi*, the etiologic agent of Lyme disease

Monica E. Embers, Ramesh Ramamoorthy, Mario T. Philipp *

Division of Bacteriology and Parasitology, Tulane National Primate Research Center, Tulane University Health Sciences Center, 18703 Three Rivers Road, Covington, LA 70433, USA

Abstract

To fight, flee or hide are the imperatives of long-term survival by an infectious microbe. Active immune suppression, induction of immune tolerance, phase and antigenic variation, intracellular seclusion, and incursion into immune privileged sites are examples of survival strategies of persistent pathogens. Here we critically review the supporting evidence for possible stratagems utilized by *Borrelia burgdorferi*, the spirochete that causes Lyme disease, to persist in the mammalian host.
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1. Introduction

Borrelia burgdorferi sensu lato, the etiologic agent of Lyme disease (or Lyme borreliosis), is transmitted to a mammalian host by any of several species of *Ixodes* ticks. The progression of Lyme borreliosis is divided into early localized, early disseminated, and late stages. The disease usually begins with a characteristic skin lesion, erythema migrans (EM) at the site of the tick bite. After several days or weeks, the spirochetes typically spread hematogenously, and patients may develop early-disseminated disease with dermatologic, cardiac, neurologic, and rheumatologic involvement. Late-stage disease can present chiefly as arthritis and/or neurological impairment [1]. During and following transmission, *B. burgdorferi* spirochetes encounter many levels of host defense, yet they are uniquely adapted to fend off those defenses and prolong their own survival. In this review, the resources potentially available to *B. burgdorferi* for escape of immune clearance by the host will be described. These involve active immune suppression of both the innate and adaptive arms of the immune system, immune evasion by virtue of phase and antigenic variation, and physical seclusion (see Fig. 1).

2. Active immune suppression

2.1. Complement inhibition

One of the earliest challenges that infectious spirochetes must face is the complement system of pathogen opsonization. The alternative (antibody independent) complement pathway may be classified as innate immunity and utilizes the deposition of specific proteins and their cleaved products to summon phagocytes and erode cell membranes of the infectious agent. Host cell surfaces possess complement control proteins or have the ability to bind complement inhibitory factors, so as to avoid complement-mediated destruction. Many bacteria are able to use similar mechanisms. Specifically, *B. burgdorferi* has been shown to bind factor H and/or FHL-1/reconectin host complement inhibitory proteins through a family of surface-exposed lipoproteins (OspE, p21, ErpA, and ErpP) [2,3], effectively recruiting the host proteins to the spirochete's own cell surface. Separately reported, other factor H-binding proteins from *B. burgdorferi* were identified and named BbCRASP-1, -2, and -3 [4,5]. Although complement resistance has not been demonstrated as a direct consequence of factor H binding, serum resistance

Proposed Mechanisms of *Borrelia burgdorferi* Persistence

Active Immune Suppression

Immune Evasion

Innate:
-complement inhibition
-induction of anti-inflamatory cytokines
-tolerization of monocytes

Adaptive:
-induction of anti-inflammatory cytokines
- tolerization of lymphocytes
-complement inhibition
-sequestration of antibodies in immune complexes

Phase and antigenic variation:
-gene conversion
 mutation and recombination
-variable expression of antigens

Physical seclusion:
 intracellular
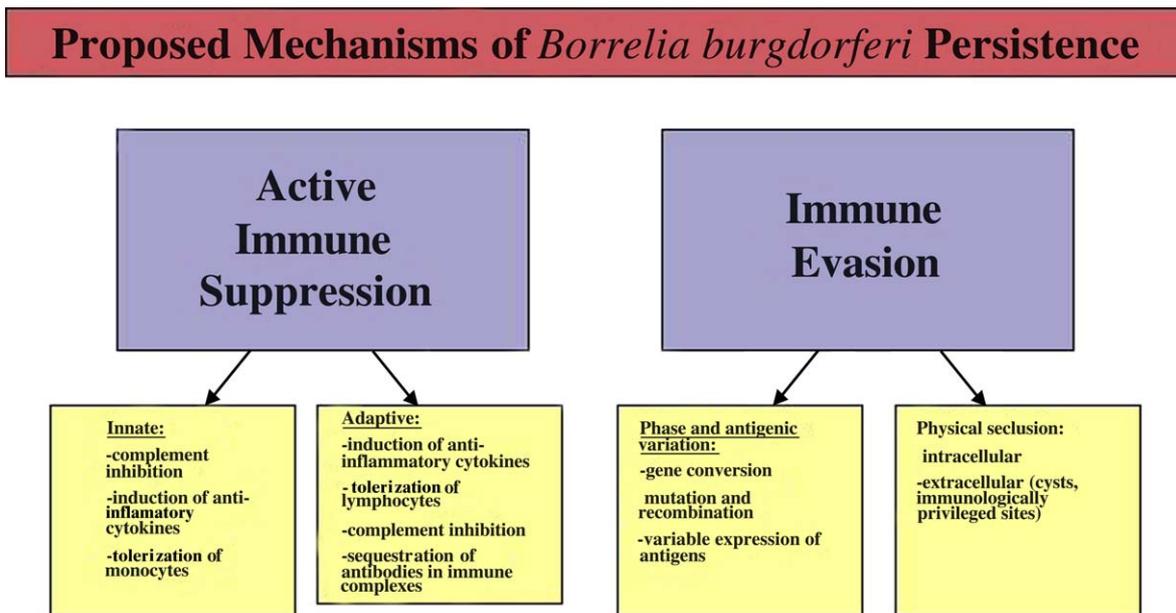-extracellular (cysts, immunologically privileged sites)

Fig. 1. Proposed mechanisms of *Borrelia burgdorferi* persistence.

and serum sensitivity in three different *B. burgdorferi* sensu lato genospecies (*B. garinii, B. afzelii* and *B. burgdorferi* sensu stricto) was correlated with the ability of those species to bind factor H [5,6]. The probable mechanism of complement resistance is the inactivation of a central component of the complement cascade, C3b. The ability to inactivate C3b has likewise been demonstrated for serum-resistant strains versus those that are sensitive to serum-mediated killing [7]. The known borrelial factor H-binding proteins are encoded by plasmid-borne paralogous *erp* (*ospE/F*-related) genes whose expression is enhanced upon tick feeding [3], when the spirochetes migrate from the arthropod vector to the mammalian host. Binding of factor H appears to be common in the *B. burgdorferi* genospecies, occurs through C-terminal conserved motifs in the exposed region(s) of Erp proteins, and correlates well with resistance to serum-mediated killing over a broad range of mammalian hosts [7–9]. While the role of specified components of *B. burgdorferi* in complement resistance and subsequent persistence is difficult to demonstrate in vivo, the role of the central complement component C3 in combating early spirochete infection has been assessed by a C3-deficient mouse model [10]. The ordinarily disease-resistant C57BL/6 mice, and C3 (–/–) C57BL/6 mice were infected with *B. burgdorferi* and examined for both disease pathology and spirochetal dissemination. In this study, tissue-specific increases in spirochete load early in infection, along with moderately higher joint disease pathology were recorded for the C3 (–/–) mice compared with the wild-type counterpart. Evidently, factors other than C3 inactivation also must be involved in the ability of these organisms to elude the host's innate defense mechanisms.

A recent report indicates that *B. burgdorferi* may produce a protein that mimics the widely expressed host complement regulatory protein CD59 (protectin). CD59 is known to inhibit formation of the membrane attack complex (MAC) that comprises the final step in complement-mediated cellular destruction. A molecule with antigenic similarity to host CD59 and with the ability to preferentially bind pore-forming C9 MAC molecules was identified within complement-resistant *Borrelia* species [11]. This activity was also correlated with susceptibility to complement-mediated killing in different *Borrelia* genospecies, and the use of blocking anti-CD59 antibody was shown to render spirochetes susceptible to complement killing. Such in vitro studies illustrate another possible mechanism of complement resistance in vivo. An interesting example of co-evolution between a parasite and its vector is the production of anti-complement salivary proteins by the tick; these proteins likely assist both vector and spirochete at the earliest point of infection. Complement inhibitory proteins have been isolated from the saliva of *Ixodes* ticks and characterized as such [12,13].

2.2. Induction of anti-inflammatory cytokines

Cell-mediated immune responses of the host may be actively mired by *B. burgdorferi*. An example of this phenomenon is the capability of this pathogen to induce the production of the anti-inflammatory cytokine interleukin 10 (IL-10). Produced both by lymphocytes and monocytes/macrophages, IL-10 is a key (negative) regulator of inflammatory cytokine release and/or function. The demonstration that *B. burgdorferi* stimulated production of IL-10 in uninfected human and rhesus monkey peripheral blood mononuclear cells (PBMCs) was a valuable development in understanding how the spirochete could mitigate the ability of an early inflammatory response to resolve infection [14]. That spirochetal lipoproteins were the perpetrators of such activity also became apparent [15]. The dual role of IL-10 in Lyme borreliosis pathogenesis has been characterized by the utili-

zation of disease-susceptible C3H/HeN and disease-resistant C57BL/6 mice. Arthritis severity is significantly more pronounced in C3H/HeN mice than in their C57BL/6 counterparts, although spirochete burden in the joints is comparable between the two strains [16]. An increased production of IL-10 by macrophages of disease-resistant mice emerged as a probable factor in this phenomenon. Indeed, increased arthritis severity and decreased spirochete numbers were evident within the joints of infected IL-10-deficient C57BL/6 mice [16]. Therefore, the induction of IL-10 by *B. burgdorferi* apparently must fall within a delicate range so as to inhibit host defense and regulate arthritis severity.

B. burgdorferi, or its components, may induce hyporesponsiveness in human PBMCs, as demonstrated by decreased tumor necrosis factor-alpha (TNF-α) and interferon gamma (IFN-γ) cytokine release [17]. This tolerization was shown for healthy donor PBMCs exposed to *B. burgdorferi* lysates and was also noted in the comparison between cytokines released from blood cells of borreliosis patients and those of uninfected patient controls. This occurrence appears to require toll-like receptor-2 and probably results largely from modulation of IL-10 production [18]. In mice, modulation of pro-inflammatory cytokine production by IL-10 in lymph node cells also has been documented and associated with disease susceptibility. IL-10 was shown to be significantly more efficient in downregulation of pro-inflammatory cytokine production in C57BL/6 disease-resistant mice than in disease-susceptible animals [19]. These studies suggested that disease susceptibility may be influenced more by lymphocyte hyporesponsiveness to IL-10 than by sheer production of this cytokine (by lymph node cells) in the presence of *B. burgdorferi* [19,20].

2.3. Immune complexes

B. burgdorferi may also tie up host antibodies by releasing soluble antigens, allowing for the formation of immune complexes (antigen:antibody aggregates). This strategy could effectively prevent the opsonization of live spirochetes in vivo and is claimed to affect the sensitivity of diagnostics based on the detection of specific antibodies. The notion that this may occur was tested in the blood of apparently seronegative patients with Lyme disease symptoms and in the cerebrospinal fluid of neuroborreliosis patients [21,22]. In both studies, complexed immunoglobulin was detected in the serum of most of the Lyme borreliosis patients tested and in patients who had already tested positive for infection by other diagnostic methods (95% and 69%, respectively). These results were corroborated in a larger study (168 Lyme patients and 148 healthy controls), where immune complexes were detected in >95% of patients with EM, or in patients with neurologic Lyme borreliosis who lacked detectable EM [23]. No complexes were detected in patients who had been treated successfully for Lyme borreliosis. The reactivity of antibodies found in such complexes against *B. burgdorferi* antigens was shown in these and other studies. The demon-

stration that spirochetal antigens are in fact contained within the complexes presented a necessary addition to these reports [24]. A serologic test for the detection of immune complexes in seronegative patients suspect of having Lyme disease was consequently developed [25]. Surprisingly, although serum antibodies to outer surface protein A (OspA) traditionally have not been detected in early stages of Lyme disease, presumably due to downregulation of OspA expression during initial infection, OspA antigen:antibody complexes have been identified early in experimental animal infections with *B. burgdorferi* [26].

3. Immune evasion

3.1. Phase and antigenic variation

Phase variation may be defined as the "on and off" switching of phenotype expression, usually occurring at a high frequency and resulting in a heterogeneous population [27]. Phase variation may be random, programmed, or modulated by environmental cues. Antigenic variation, which has been described as a form of "successive phase variation" [27], encompasses programmed changes in protein structure that lead to variation in protein antigenicity. The significance of this mechanism lies in these proteins being known, or reasonably assumed, to be the targets of protective immune responses. *B. burgdorferi* is known to utilize several phase-variation mechanisms that may contribute to evasion, including combinatorial diversity from variable region cassettes, mutation, recombination and selective (on–off) expression of genes encoding antigenic proteins.

Borrelia species utilize mechanisms of antigenic variation that are similar in some respects to the gene switching events of the variable surface glycoprotein (VSG) that enable trypanosomes to escape host immunity (reviewed in [28]). Through a process of duplicative gene transposition, any one of some 10^3 antigenically variable *vsg* genes may be expressed following insertion into a site of active transcription. A dense layer of this VSG protein comprises the surface coat of trypanosome parasites. Spirochetal gene switching elements were first identified in the relapsing fever agent, *Borrelia hermsii* and were designated *vmp*, for variable membrane protein [29]. Antigenic variation in the *vmp* genes of *B. hermsii* also involves duplicative gene transposition, but additional diversity may be generated by segmental gene conversions that utilize as templates *vmp* pseudogenes located upstream from the expressed *vmp* gene. Similar genetic elements were later identified in *B. burgdorferi* and labeled *vls* (for *vmp*-like sequences) [30]. It is now known that *B. burgdorferi* possesses a 28-kilobase linear plasmid that encodes 15 silent *vls* cassettes adjacent to a transcriptionally active expression site (*vlsE*) [30]. Unlike the trypanosomes and *B. hermsii*, only portions of the *vls* cassettes in *B. burgdorferi* recombine with segments of a central domain of the expressed *vlsE* gene to generate antigenic diversity. The *vlsE*

central domain contains multiple interspersed variable regions, and recombination involving these variable segments results in the expression of antigenically diverse VlsE proteins over time on the spirochete's outer surface.

The contribution of VlsE antigenic variation to evasion of host antibody responses and subsequent persistence has been difficult to discern, primarily as a result of (a) immunodominance of a conserved region and (b) limitations of in vitro analyses. Demonstration of decreased antigenicity comparing VlsE of a clonal isolate with VlsE variants generated in vivo over 28 days of infection provided the first evidence that this genetic mechanism does result in antigenic variation [30]. In this study, a correlation was drawn between the presence of linear plasmid 28-1 (that contains the *vls* locus) and infectivity. This notion was tested further by showing that lp28-1-deficient spirochetes could persist in severe combined immunodeficient (SCID) mice but were cleared from immunocompetent mice [31]. This demonstrated that the inability of spirochetes lacking this plasmid to persist in the host was likely a direct result of host borreliacidal antibody effectiveness. Although the *vls* locus is present on this plasmid, other *cis* genetic factors may dictate or contribute to the non-persistent phenotype of lp28-1-deficient spirochetes.

Several studies have indicated that VlsE antigenicity is in fact dominated by epitopes located within invariant regions. One such invariant region (designated IR_6) has been utilized in a peptide-based enzyme-linked immunosorbent assay that exhibits high diagnostic sensitivity and specificity for exposure to *B. burgdorferi* sensu lato [32]. Interestingly, although IR_6 is exposed, albeit sparingly [33], on the surface of the VlsE protein, it is not exposed on the spirochetal surface [34] and is therefore inaccessible to IR_6-specific antibodies. VlsE-dominant epitopes may divert the immune response from other regions of VlsE that are capable of eliciting protective antibodies [34]. To show immune reactivity of variable domains, experiments involving preabsorption of immune serum with recombinant VlsE protein variants have been performed [35]. When antibodies reactive to invariant epitopes were removed, the differential antigenicity of variant VlsE lipoprotein molecules was revealed.

Diversity, at the genetic level, of several *B. burgdorferi* surface proteins has been detected among different strains and isolates. A recent comparative sequence analysis of two proteins belonging to a paralogous *B. burgdorferi* gene family concluded that the poorly immunogenic protein BapA was quite stable among isolates, whereas the more immunogenic EppA protein showed evidence of considerable sequence diversity among isolates [36]. Other examples of genetic heterogeneity within genes of *B. burgdorferi* surface antigens include decorin-binding protein A (*dbpA*) and the *mlp* (for "multicopy lipoprotein") genes [37,38].

A well-characterized example of genetic variability and alterations introduced throughout the course of infection is that of the outer surface protein E (*ospE*) genes [39]. Sequence analyses of cloned spirochetes derived from mice infected for a 3-month period with a clonal isolate of *B. burg-*

dorferi revealed multiple polymorphisms in *ospE* alleles [40]. The noted genetic changes may have arisen by several mechanisms, including mutation and/or recombination, with the insertion or deletion of DNA segments between *ospE* alleles. The evolution of antibody responses to several OspE variants was subsequently demonstrated [40]. Outer surface protein C (OspC) also has been shown to exhibit immunological heterogeneity. Analyses of the *ospC* sequences from different strains of *B. burgdorferi* have revealed both molecular polymorphism and antigenic diversity [41]. However, in contrast to *ospE*, the *ospC* gene appears to remain stable throughout the course of infection [42]. It is thus not surprising that with many variants in nature [43], infection with *B. burgdorferi* generally does not confer protection to subsequent infection, especially in patients treated for EM [1].

Certain findings have prompted conjecture over immune evasion strategy by the capacity of *B. burgdorferi* to regulate the expression of antigenic molecules in vivo and to limit the exposure of such lipoprotein molecules on the bacterial outer membrane. In the case of OspC, the antibody-mediated selection of spirochetes lacking expression of this surface antigen was demonstrated as a plausible mechanism for immune escape [44]. Coincident with the generation of OspC-specific antibodies, only OspC (mRNA) non-expressers could be detected in the skin of mice at 2 weeks post-infection. The demonstration of OspC expression throughout infection of SCID mice, coupled with an absence of OspC expression in SCID mice passively immunized with anti-OspC antibodies, defined a probable role for the regulation of surface antigen expression in *B. burgdorferi* escape from host borreliacidal antibodies. OspC may not be the only lipoprotein involved in this phenomenon.

A broad gene array analysis of the mRNA expression of 137 putative lipoprotein genes revealed two phases of expression [45]. Within 10 days after infection, generally prior to the generation of significant antibody titers, most (116 of 137) lipoprotein genes were transcribed. In the second phase, between 17 and 30 days post-infection, transcription was downregulated for most of these genes, suggesting again that immune selection may direct this apparent molecular adaptation.

One notable finding came from surface antigen labeling studies utilizing the treatment of *B. burgdorferi* spirochetes with fixative and detergent [46]. Although only small amounts of the outer surface proteins-A, -B, and -C could be detected on live spirochetes, membrane permeabilization with methanol or detergent dramatically increased the level of fluorescent staining, indicating that many molecules of these surface proteins are present, but limited amounts are actually exposed on the outer membrane surface. The authors postulated that (a) these lipoproteins are mainly subsurface constituents; (b) *B. burgdorferi* likely possesses some form of secretory apparatus for translocating the lipoproteins from the cytoplasmic membrane to the outer membrane; and (c) the antigenicity of the spirochetal cell surface may be attenuated by this limited exposure of antigens. The latter may be

influenced by immune selection pressure and location of spirochetes within the host, where the ability of antibodies to penetrate particular tissues could conceivably affect surface phenotype.

3.2. Physical seclusion

As mentioned in the introduction, following the localized phase of infection, *B. burgdorferi* may spread to multiple organs. Among these, the joints, eyes and CNS may be considered immunologically privileged organs. This is so, in part, because these sites contain extracellular fluids (synovial and cerebrospinal) that do not circulate through conventional lymphatics [47]. Sequestration of *B. burgdorferi* into such sites could, in a purely physical sense, make them less accessible to cells and molecules of the host's immune system.

It has been suggested that *B. burgdorferi* may avoid immune surveillance mechanisms altogether by seeking refuge within a host cell or by encapsulating itself inside a cyst membrane. In partial support of the former mechanism, *B. burgdorferi* has been shown to bind and invade a variety of cell types in vitro that include endothelial cells [48], fibroblasts [49], macrophages [50], Kupffer cells [51] and synovial cells [52]. Intracellular survival within human synovial cells was recorded for as long as 8 weeks [52]. However, intracellular spirochetes have not been demonstrated in vivo and it remains contentious that the in vitro observations have a true bearing on the host–spirochete interplay.

Several studies in vitro have motivated the suggestion that *B. burgdorferi* may transform into cysts in vivo [53–55]. Cyst formation in *B. burgdorferi* occurs in response to nutritional stress, driven by the lack of serum (or more accurately, fatty acids) in the growth medium. More importantly, cyst formation also has been shown to occur in body fluids such as the cerebrospinal fluid [56] and in response to the addition of β-lactam antibiotics in vitro [57]. The reasonable, yet unproven notion that an inability to resolve infection with antibiotics in some patients could result from such cyst formation may follow.

The process of transformation from cysts to spiral forms has been reported to occur in vivo [58] and to occur reversibly in vitro [54–56]. Upon replenishment of serum in the culture medium, these non-motile inert cysts revert to the motile spiral forms. Motile spiral forms have also been recovered from tissues of animals apparently inoculated with only the cyst forms of the spirochetes generated in vitro [58]. There is some evidence to suggest that such cystic or otherwise resilient forms of the spirochete may exist in vivo [59]. Physical seclusion by intracellular localization or cyst formation is particularly significant given that, in the face of apparent disease symptoms, it has been difficult or impossible to demonstrate the presence of spirochetes [60]. Intracellular dwelling and cyst formation in vivo by spirochetes are potential defense mechanisms that have yet to be demonstrated conclusively, but that are worthy of further research.

4. Discussion

Probably the most enigmatic facet of *B. burgdorferi* infection is that this organism, generally known to be an extracellular pathogen, generates a vigorous immune response in the host, particularly with regard to specific antibodies. However, that response is ordinarily insufficient. The fact that host antibodies can kill this organism in vitro [61], but not in vivo, suggests that immune evasion strategies are manifest upon particular cues specific to the host environment.

Clearly, these spirochetes are suitably adapted to avoid or retaliate against every tier of the host response. The evidence presented here encompasses only the mechanisms thought to make significant contributions to immune evasion by the Lyme borreliosis spirochete. It seems probable that no one strategy alone may be sufficient to allow survival of these organisms within the host. Targeted gene disruption in *B. burgdorferi* has been complicated by genomic instability associated with in vitro propagation [62,63] and low transformation efficiency linked to an essential plasmid and it remains [64]. These hindrances have now been overcome such that the inactivation and subsequent complementation of specific *B. burgdorferi* genes is now possible [62,65–67]. The application of these techniques to *B. burgdorferi* genes thought to encode products involved in immune evasion will allow for systematic study of these phenomena. Phenotypic analyses of such targeted mutants in vivo could provide conclusive evidence for the contributions from each of the survival strategies reviewed herein to *B. burgdorferi* persistence and Lyme disease pathogenesis.

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