Clinical and Developmental Immunology

Lyme Borreliosis: From Pathogenesis to Diagnosis and Treatment

Guest Editors: Joanna Zajkowska, Piotr Lewczuk, Franc Strle, and Gerold Stanek

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Editorial Board

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 231657, 2 pages
doi:10.1155/2012/231657

Editorial

Lyme Borreliosis: From Pathogenesis to Diagnosis and Treatment

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Received 24 July 2012; Accepted 24 July 2012

Since discovery of the etiologic agent of Lyme borreliosis (Lyme disease) in 1981 by Willy Burgdorfer, marked increases in knowledge of pathogenesis based on new technologies, more precise descriptions of common and seldom encountered clinical features, and significant progress in laboratory diagnosis have occurred. Ecological studies continue to contribute to the understanding of the disease epidemiology and new genospecies of *B. burgdorferi* sensu lato (s.l.) continue to be discovered. The geographical distribution of genospecies and their vectors and hosts explains some important differences in the clinical presentation of the disease. At least 5 genospecies of *B. burgdorferi* s.l. cause the disease in Europe while *B. burgdorferi* sensu stricto (s.s.) is the only *Borrelia* species pathogenic to humans in North America. In both continents, the same issues are still unresolved, such as the mechanisms for immunoevasion by the borreliae, the diagnostic challenges of current and historic infection, lack of effective vaccination, uneven diagnostic laboratory standardisation, and unsatisfactory therapeutic management. The persistence of symptoms in Lyme borreliosis patients following recommended antibiotic therapy continues to be controversial. New diagnostic approaches indicating ongoing infection are expected in patients with objective and subjective long-term sequelae of Lyme borreliosis. There are many current studies investigating new biomarkers or attempting to optimize existing ones.

This special issue consists of articles highlighting recent advances in immunopathogenesis, diagnosis, and epidemiological approaches to Lyme borreliosis.

Lyme borreliosis is the most commonly reported tick-borne infection in Europe and North America, and despite the uneven reporting systems in different countries, it is apparent that Lyme borreliosis shows an increasing incidence. For good management, available clinical case definitions must be utilised. Speculation regarding links between *Borrelia* infection and a variety of nonspecific symptoms and disorders has resulted in over-diagnosis and over-treatment of suspected Lyme borreliosis, creating a challenge for health systems in many countries.

Arthritis is one of the main manifestations of Lyme borreliosis. A chronic, debilitating arthritis is classified as severe destructive Lyme arthritis. Antibiotic refractory Lyme arthritis (more often in the USA than in Europe) may result from *B. burgdorferi* s.l. induced autoimmunity in affected joints. This immune response might pose a potential problem in the production of a safe vaccine. Another problem is that there is no reliable medical laboratory marker for the diagnosis of Lyme arthritis. High levels of IgG antibodies to *B. burgdorferi* s.l. in serum are not sufficient for confirmation, especially in regions where there is high background seroprevalence. The excellent review article by *Erik Munson* and colleagues introduces experimental models of Lyme arthritis, clarifying the participation of mediators and molecular mechanisms of inflammation. Lessons from investigations on animal models of Lyme arthritis are required, but a universal animal model of Lyme borreliosis does not exist. It seems that the hamster model facilitates the assessment of severe destructive Lyme arthritis, while murine models allow investigation of immunopathology mechanisms.

To be protected from recognition and subsequent killing borreliae in the mammal host downregulate their surface

proteins, hide in the extracellular matrix, and use complement neutralizing proteins such as Salp, CRASPs (complement regulator-acquiring surface proteins), or ISAC/IRAC, or induce the formation of immune complexes by secreting soluble antigens. The ability of the spirochete to neutralize complement allows it to survive and determines competent animal reservoirs associated with particular *B. burgdorferi* s.l. genospecies. CRASPs, presented on the surface of the spirochete differs in pathogenic genospecies of *B.burgdorferi* s.l. (CRASP-1, CRASP-2, CRASP-3, CRASP-4 and CRASP-5). *Claudia Hammerschmidt* and colleagues analyse the contribution of CRASP-4 in mediating resistance to complement in *B. burgdorferi* s.l. and the interaction with human complement regulators, indicating that CRASP-4 plays a subordinate role.

Iris Müller and colleagues introduce a retrospective model of analysis evaluating frequency, diagnostic quality, and cost of Lyme borreliosis testing in Germany. The findings strongly suggest ongoing issues related to care for Lyme borreliosis and may help to improve future disease management.

Why some individuals develop clinical manifestations after infection with *B. burgdorferi* s.l. while others remain asymptomatic is largely unknown. *Babro Skogman* and colleagues investigated adaptive and innate immune responsiveness to *B. burgdorferi* s.l. in Borrelia-antibody-positive asymptomatic children, children with previous clinical Lyme borreliosis, and unexposed controls. Lyme borreliosis in children follows a slightly different clinical course than in adults, and the duration of symptoms is often shorter. Children seem to have a better prognosis than adults and report persisting symptoms less often. The fact that some individuals may be infected with *B. burgdorferi* s.l. without developing clinical symptoms is interesting from an immunological standpoint and could indicate a more effective immune response to the spirochete in these individuals. In the conducted study, the adaptive and innate immune responsiveness to *B. burgdorferi* s.l. was similar in borreliae-infected asymptomatic children and children with previous clinical Lyme borreliosis. So, further studies on the immunological mechanisms of importance for eradicating the spirochete effectively are necessary.

John Lazarus and colleagues consider in an excellent study whether current research methods, using either ELISA to detect seroconversion to *B. burgdorferi* s.s. antigens or PCR quantification of bacterial DNA within tissues, can accurately distinguish between an active infection and a past *B. burgdorferi* s.s. infection that was rapidly cleared by the innate responses. The results strongly indicate that both ELISA-based serological analyses and PCR-based methods can clearly distinguish between the two situations.

Acknowledgments

The guest editors would like to express grateful thanks and acknowledgments to all the authors and to the reviewers for their efforts in preparing the papers for this issue and hope that the papers reflecting the multidisciplinary progress on Lyme borreliosis will be helpful and provide the readers with inspiration for future studies.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 504215, 12 pages
doi:10.1155/2012/504215

Review Article

Hamster and Murine Models of Severe Destructive Lyme Arthritis

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Received 20 July 2011; Revised 2 October 2011; Accepted 3 October 2011

Academic Editor: Franc Strle

Arthritis is a frequent complication of infection in humans with *Borrelia burgdorferi*. Weeks to months following the onset of Lyme borreliosis, a histopathological reaction characteristic of synovitis including bone, joint, muscle, or tendon pain may occur. A subpopulation of patients may progress to a chronic, debilitating arthritis months to years after infection which has been classified as severe destructive Lyme arthritis. This arthritis involves focal bone erosion and destruction of articular cartilage. Hamsters and mice are animal models that have been utilized to study articular manifestations of Lyme borreliosis. Infection of immunocompetent LSH hamsters or C3H mice results in a transient synovitis. However, severe destructive Lyme arthritis can be induced by infecting irradiated hamsters or mice and immunocompetent *Borrelia*-vaccinated hamsters, mice, and interferon-gamma- (IFN-γ-) deficient mice with viable *B. burgdorferi*. The hamster model of severe destructive Lyme arthritis facilitates easy assessment of Lyme borreliosis vaccine preparations for deleterious effects while murine models of severe destructive Lyme arthritis allow for investigation of mechanisms of immunopathology.

1. Introduction

1.1. Lyme Borreliosis Background. Since its discovery in the mid 1970s, Lyme borreliosis has become the most prevalent tick-borne infectious illness in the United States [1]. Following a brief early infection period that can be characterized by constitutional symptoms [2] and the pathognomonic erythema migrans [3], successive stages of the illness in nontreated individuals can consist of a dissemination of the etiologic agent *Borrelia burgdorferi* to multiple organ sites and subsequent persistence of the spirochete. Consequences of this activity are immunopathologies, with afflicted individuals presenting with cardiac [4], neurological [5, 6], and musculoskeletal [7] abnormalities.

1.2. Synovitis and Severe Destructive Arthritis in Humans. In early Lyme borreliosis, afflicted individuals may experience joint, tendon, muscle, or bone pain. Subsequently, fifty to sixty percent display acute signs of arthritis, including musculoskeletal pain without objective findings [7, 8]. Histopathological analyses of synovectomies and synovial biopsies at this stage of Lyme borreliosis articular involvement have described a hyperplastic synovitis [9]. Arthritic involvement in late Lyme disease includes intermittent episodes of joint pain, with 50–60% of such individuals suffering from inflammatory arthritis with synovial effusion or pain on motion [7, 8]. Of these patients, 11% may progress to a chronic and debilitating severe destructive Lyme arthritis [7]. The increased severity of arthropathy at this stage has been characterized as a synovial hyperplasia, an infiltration of mononuclear cells into the synovium, a formation of an inflammatory exudate over the lining of synovial cells (pannus formation), a destruction of cartilage, and as an erosion of bones and joints [10, 11].

Early associations were hypothesized between the major histocompatibility complex (MHC) class II alleles HLA-DR2 and HLA-DR4 and severe destructive Lyme arthritis [12, 13]. Furthermore, individuals afflicted with a severe destructive Lyme arthritis that was refractory to antimicrobial therapy predominately showed an HLA-DR4 specificity [14]. Gross et al. [15] subsequently identified an immunodominant epitope within *B. burgdorferi* outer surface protein A (OspA) for T lymphocytes that may shoulder responsibility for the induction of treatment-resistant severe destructive Lyme arthritis. In support of this statement, Steere et al. [16] showed that the majority of patients with late-stage, antimicrobial-refractory Lyme arthritis possessed at least one of the HLA-DR molecules known to bind to this putative arthritogenic epitope of OspA while those individuals with antimicrobial-responsive arthritis possessed these molecules to a significantly less-frequent degree. Moreover, extensive investigations into identifying additional arthritogenic, T lymphocyte-binding epitopes of *B. burgdorferi* [17] yielded no more suitable candidate for further investigation into inducers of late-stage antimicrobial-refractory Lyme arthritis than the $OspA_{163–175}$ epitope. Although OspA is considered a leading candidate that induces arthritis in humans, other *B. burgdorferi* antigens, as well as intrinsic host factors and host HLA-DR alleles, may also play a role in the induction of arthritis. Such considerations provide a tremendous impetus for the elucidation of mechanisms that result in severe destructive Lyme arthritis, especially when considering that OspA or other antigens are prominent Lyme borreliosis vaccine candidates.

It is known that two clinical entities of Lyme borreliosis articular involvement occur in humans. It is critical to make the distinction between synovitis and severe destructive Lyme arthritis. This review will focus on the utility of the hamster and murine models in experimental investigations of severe destructive Lyme arthritis. Each animal model of an articular condition analogous to that which afflicts humans in early Lyme borreliosis will be discussed. This will be followed by presentation of experimental models of severe destructive Lyme arthritis. Benefits and applications of these models will subsequently be expounded upon.

2. Hamster Models of Synovitis and Severe Destructive Lyme Arthritis

2.1. Synovitis. When immunocompetent adult London School of Hygiene (LSH) hamsters were challenged in the footpad with 10^6 live *B. burgdorferi sensu stricto* isolate 297, a slight degree of tibiotarsal joint swelling was observed between 10 and 14 days after inoculation [18]. In addition, histopathological changes were demonstrated in these animals. Synovial hyperplasia with focal areas of ulceration and overlying adherent fibrin was present at one week of infection. The majority of immune cells responsive to this inoculum were neutrophilic in nature, with this infiltrate involving the synovial and periarticular structures, including the fibrous capsule, periosteum, ligaments, tendons, and tendon sheaths. Numerous spirochetes were also observed through the use of silver impregnation staining techniques.

Such histological evidence of synovitis persisted for approximately three weeks, after which both macroscopic edema and histopathological involvement waned. Following a 15-week observation of infected hamsters, histopathological examination noted that subsynovial and periarticular soft tissues contained increased fibroblasts and collagen, persistent small hypertrophic synovial villi were still evident, and spirochetes were no longer seen. No destructive bone erosion was reported. No edematous or histopathological findings were seen in hamsters challenged in the footpad with growth medium.

This model is used to investigate the innate immune response to infection with *B. burgdorferi*. Since humans develop arthritis weeks to months after infection, the hamster model of synovitis is valuable for understanding the early immune events that drive the neutrophilic response to infection, induction of cytokines, and early antigenic processing that are responsible for the later induction of adoptive immunity. Most investigations using mice, hamsters, and other animals have focused on this early stage of development of arthritis. Therefore, there is considerable debate over whether T lymphocytes are responsible for induction of arthritis. Other animal models (see the following) that focus on nonearly events strongly support a role for T lymphocytes in the induction and resolution of arthritis. Most important, the early innate immune responses responsible for the induction of synovitis are directly connected to the T lymphocyte responses that drive the more severe pathology of severe destructive Lyme arthritis.

2.2. Irradiation Model of Severe Destructive Lyme Arthritis. Adult LSH hamsters that were subjected to 550 rads of gamma radiation manifested a tibiotarsal joint swelling upon footpad challenge with 10^6 live *B. burgdorferi* 297 [18]. Edema peaked approximately ten days after challenge and was found to be significantly elevated over that of nonirradiated infected hamsters throughout the first five weeks of observation. Furthermore, behavioral changes were observed in the irradiated infected animals. These hamsters were increasingly irritable and vicious, maneuvering about their cages very slowly on their abdomens, dragging their hind legs. Analogous behavior was not observed in nonirradiated infected hamsters.

Histopathological examination of infected irradiated hamsters demonstrated a more severe and fulminate articular involvement. Greater synovial hyperplasia and hypertrophy were observed to the point that pannus formations bridging synovial spaces were noted. Neutrophilic infiltrate resulted in erosion of articular cartilage and superficial destruction of underlying bone. Increases in collagen formation and numbers of fibroblasts were shown also to result in fibrosing ankylosis, loss of alignment of the articular surfaces, and overall deformation of the joints [19]. This animal model is used to evaluate the adoptive immune responses that protect against infection. Cells or sera from

immunized or naturally infected animals are transferred to irradiated hamsters and then challenged with *B. burgdorferi* to determine their role in protection. In addition, cells and cytokines participating in preventing or augmenting the arthritis can be determined.

2.3. Immunocompetent Model of Severe Destructive Lyme Arthritis. The relevance of experimental hamsters was further established with the advent of the immunocompetent hamster model of severe destructive Lyme arthritis. When Lim et al. [20] vaccinated adult LSH hamsters with formalin-inactivated *B. bissettii* (formerly *B. burgdorferi sensu stricto* isolate C-1-11) in aluminum hydroxide adjuvant (alum), gross tibiotarsal joint edema was observed upon footpad challenge with heterologous isolates of *B. burgdorferi* or with *B. bissettii* prior to the induction of protective immunity. Edema measurements demonstrated statistically significant increases in tibiotarsal swelling over those observed in nonvaccinated hamsters challenged with multiple isolates of *B. burgdorferi*. It was also shown that arthritis could be induced using live spirochetes, heat-inactivated spirochetes, or antimicrobial-treated organisms mixed in alum. Only viable organisms, however, were required upon challenge to elicit the arthritis.

Analogous to differences observed macroscopically, histopathological examination revealed varying degrees of arthritis severity in vaccinated and nonvaccinated hamsters following challenge with *B. burgdorferi*. Erosion of articular cartilage, focal destruction of underlying bone, and chronic hyperplasia and hypertrophy characterized by pannus formation were observed in the synovia of hamsters challenged with *B. bissettii* following a five-week vaccination with *B. bissettii* in adjuvant. These animals also manifested a cellular infiltrate of neutrophils, macrophages, lymphocytes, mast cells, and plasma cells in the subsynovial and periarticular tissues. In contrast, nonvaccinated hamsters challenged with *B. bissettii* displayed an acute synovitis characterized by a cellular inflammatory infiltrate devoid of pannus formation or bone erosion. Severe destructive Lyme arthritis can also be induced using other isolates of *B. burgdorferi* mixed in alum and then infected with a homologous spirochete before borreliacidal antibody develops or with a heterologous isolate when isolate-specific borreliacidal antibody is present.

This model mimics how humans develop Lyme arthritis. Humans develop Lyme arthritis many weeks after they are infected/vaccinated by spirochetes that entered the host by the bite of an infected tick. Persistence of the spirochete or its antigens, such as OspA, in the nontreated individual would initiate a series of immunological events in the host that finally induce arthritis. By vaccinating hamsters, the immunological events responsible for the induction of arthritis are compressed from weeks into days. A major concern with the hamster model of severe destructive Lyme arthritis is the lack of immunological reagents that can be utilized to decipher the immune components responsible for the induction of arthritis.

2.3.1. Utilization of Hamster Models of Severe Destructive Lyme Arthritis to Assess Protective Immunity. A feature common to both the irradiated and immunocompetent LSH hamster models of severe destructive Lyme arthritis is their utility in characterizing and monitoring protective immunity. Schmitz et al. [21] reported that heat-inactivated *B. burgdorferi*-immune sera were able to inactivate inocula of live *B. burgdorferi* in the presence of complement in an *in vitro* assay. These specific killing antibodies were thus termed borreliacidal antibodies. To determine the protective capability of these borreliacidal antibodies, irradiated hamsters received sera through adoptive transfer and were subsequently challenged with *B. burgdorferi*. Irradiated hamsters receiving sera with low titer of borreliacidal antibody developed severe destructive Lyme arthritis while hamsters receiving sera containing significant borreliacidal activity were protected from *B. burgdorferi* infection, as well as the evocation of severe destructive Lyme arthritis. In similar fashion, Callister et al. [22] used the irradiated hamster model of severe destructive Lyme arthritis to prove that humans generate a borreliacidal response upon infection with *B. burgdorferi*. Adoptive transfer of sera from humans with clinically diagnosed Lyme borreliosis (significant borreliacidal antibody response detected *in vitro*) protected irradiated hamsters from *B. burgdorferi* infection and subsequent progression to severe destructive Lyme arthritis, while transfer of sera from healthy individuals (no borreliacidal activity detected *in vitro*) failed to protect hamsters from induction of severe destructive Lyme arthritis. Taken together, these data suggest that *in vitro* borreliacidal activity correlates with protection events *in vivo*.

Induction of these borreliacidal antibodies establishes the effectiveness of Lyme borreliosis vaccine preparations [23]. When Jobe et al. [24] actively immunized irradiated hamsters with a commercial canine whole cell Lyme borreliosis vaccine, gross tibiotarsal joint swelling, and severe destructive Lyme arthritis were induced in these animals when challenged three weeks after vaccination with live *B. burgdorferi*. Conversely, animals challenged five weeks after vaccination were protected from such manifestations. Similarly, when Lim et al. [20] vaccinated immunocompetent hamsters with formalin-inactivated *B. bissettii* in aluminum hydroxide, severe destructive Lyme arthritis was induced in these animals when challenged with a homologous isolate of *B. burgdorferi* at intervals of less than seven weeks after immunization. By contrast, hamsters challenged with *B. bissettii* seven and nine weeks after vaccination failed to manifest severe destructive Lyme arthritis. Subsequent experiments [25] revealed that hamsters vaccinated with formalin-inactivated *B. burgdorferi* isolate 50772 in aluminum hydroxide were protected against a subsequent homologous challenge at time points ranging from 5 to 18 weeks after vaccination. Severe destructive Lyme arthritis was induced in vaccinated hamsters that were challenged at intervals both earlier (10 days, 3 weeks) and later (24 weeks) than those which afforded protection. These data exemplify how irradiated and immunocompetent hamsters have been used as an *in vivo* correlate to a common concern pertaining to Lyme disease vaccine preparations that was

first observed *in vitro* [24, 26], namely, relatively short duration of protective borreliacidal antibody production. Therefore, these models can be utilized to validate the duration of protection induced in individuals receiving any future Lyme borreliosis vaccine. They may also establish the number of vaccinations required to establish long-term protection.

Irradiated and immunocompetent hamster models of severe destructive Lyme arthritis have also been implemented to investigate an additional pitfall with regard to Lyme borreliosis vaccine preparations. Seroprotectivity is an index of the ability of immune sera generated in one mammalian host to protect, via adoptive transfer, a naïve recipient animal against subsequent challenge. As an example, irradiated hamsters that were passively immunized with sera generated by the infection of hamsters with *B. burgdorferi* 297 were protected from the induction of severe destructive Lyme arthritis upon challenge with *B. burgdorferi sensu stricto* isolate B31. Conversely, passive administration of anti-*B. burgdorferi* 297 sera failed to protect irradiated hamsters from the evocation of severe destructive Lyme arthritis upon challenge with *B. bissettii* [27]. On the basis of these and other data [28], Lovrich et al. established six seroprotective groups of *B. burgdorferi sensu lato* isolates, with inherent differences in seroprotectivity being partially reflective of the worldwide distribution of *B. burgdorferi sensu lato* isolates. Immunocompetent hamsters were utilized in a similar fashion to classify isolate-specific rOspA preparations into analogous seroprotective groups [29], signaling an additional concern pertaining to any new federally licensed Lyme borreliosis vaccine preparations. These models can be easily utilized to assess the degree of cross-protection and development of a universal vaccine.

2.3.2. Utilization of Hamster Models of Severe Destructive Lyme Arthritis to Assess the Role of Cell Populations in Induction of Severe Destructive Lyme Arthritis. The findings of Lim et al. [20] were important for an additional reason. This published model of severe destructive Lyme arthritis did not require the utilization of gamma radiation. This allowed for elucidation of roles that certain cell populations play in the induction of severe destructive Lyme arthritis in the context of an immunocompetent mammalian host. Du Chateau et al. [30] reported that macrophages play a direct effector role in the induction of severe destructive Lyme arthritis. Macrophages that were obtained from vaccinated or nonvaccinated LSH hamsters, exposed *in vitro* to formalin-inactivated *B. burgdorferi*, and subsequently infused into footpads of naïve recipients induced severe destructive Lyme arthritis upon challenge with *B. burgdorferi*. Both the severity and onset of the arthritis were dependent on the number of macrophages infused into the recipient footpads. In addition, macrophages not exposed to *B. burgdorferi in vitro* failed to induce severe destructive Lyme arthritis upon *B. burgdorferi* challenge. Roles for T lymphocytes and subset populations thereof, either alone [31, 32] or in synergy with macrophages [33, 34], have been proposed in the pathology of severe destructive Lyme arthritis. However, the value of the hamster model of severe destructive Lyme arthritis in further studies of the immunoregulation of severe destructive Lyme arthritis has been constrained due to the unavailability of many hamster-specific molecular and immunological reagents.

2.3.3. Immunocompetent Model of Severe Destructive Lyme Arthritis for Assessment of Lyme Borreliosis Vaccine Preparations. An extremely practical and relatively straightforward application of the immunocompetent LSH hamster model of severe destructive Lyme arthritis involves safety assessment of Lyme borreliosis vaccine preparations. Speculation that the severe destructive Lyme arthritis was elicited in hamsters only because a whole cell component of *B. burgdorferi* was used as the immunogen was refuted by Croke et al. [35]. Vaccination of immunocompetent LSH hamsters with 30 μg of recombinant (r) OspA in alum primed these hosts for the induction of gross macroscopic edema and corresponding severe destructive Lyme arthritis upon footpad challenge with both heterologous and homologous isolates of *B. burgdorferi*. By contrast, hamsters that were vaccinated with 30 μg of rOspA in alum and not subsequently challenged with *B. burgdorferi* manifested no tibiotarsal joint edema. Intact joints and normal capsular and pericapsular soft tissue were noted upon histopathological examination. The rOspA vaccine preparation that was shown to prime hamsters for induction of deleterious effects is analogous to commercial vaccine preparations [36, 37] that were licensed by the United States Food and Drug Administration for use in humans. These data further confirm the importance of the immunocompetent hamster model of severe destructive Lyme arthritis for the determination of possible deleterious effects that commercial Lyme borreliosis vaccines may evoke in mammalian hosts. As additional support, Croke et al. [35] also demonstrated that a commercial canine rOspA vaccine prepared in alum primed hamsters for the induction of severe destructive Lyme arthritis upon challenge with *B. burgdorferi*. Obviously, this model should be utilized to evaluate vaccine preparations for adverse effects before clinical trials begin.

3. Murine Models of Synovitis and Severe Destructive Lyme Arthritis

3.1. Synovitis. In early investigations, it was determined that the strain of mouse was important for the degree of Lyme borreliosis articular involvement potentiated [38–40], which, in turn, was correlated to the MHC haplotype of the mouse. C57BL/6 mice ($H2^b$) were resistant to the induction of arthritic manifestations in spite of infection with a large inocula of *B. burgdorferi*, while BALB/c mice ($H2^d$) developed a mild articular condition. In contrast, strains of $H2^k$ haplotype mice, including AKR and C3H, manifest a Lyme arthropathy upon challenge with *B. burgdorferi*. The edema and synovitis observed in these infected mice closely resembled those of adult immunocompetent LSH hamsters challenged with *B. burgdorferi* [41, 42]. Endotoxin susceptibility differences inherent to the C3H mouse did not contribute to potentially varying degrees of arthropathy,

as endotoxin-resistant C3H/HeJ mice manifest equivalent synovitis to that of endotoxin-susceptible C3H/HeN mice [43].

Although the C3H mouse is the most frequently utilized animal model to investigate the immune mechanisms of Lyme arthritis, the model has limitations. Mice develop synovitis within days of infection. Humans do not develop synovitis within days after infection with *B. burgdorferi*. In addition, the arthritis in mice is mild and is not sustained. Moreover, most C3H mice resolve the arthritis 3 to 6 weeks after infection. Rapid resolution of arthritis also does not occur in humans infected with *B. burgdorferi*. The model is utilized to determine the early innate immune events that occur when spirochetes enter the host after being bitten by a *Borrelia*-infected tick. Therefore, this model may not mimic the immunological events that drive the induction of severe arthritis in humans.

3.2. Murine Models of Severe Destructive Lyme Arthritis

3.2.1. Immunodeficient Mouse Models. Multiple studies [44–46] have reported that challenge of SCID mice with *B. burgdorferi* induces a severe destructive Lyme arthritis. When Schaible et al. [44] challenged CB.17 SCID mice subcutaneously in the tail with *B. burgdorferi sensu stricto* isolate ZS7 or ZQ1, inoculum-dependent clinical signs of arthritis were first observed between days 7 and 20 after infection. Mice developed redness and swelling of tibiotarsal joints and were observed to have difficulty walking. Such manifestations persisted throughout the entire observation period of 87 days. Histopathological examination characterized arthritic involvement as synovial hyperplasia, infiltration of mononuclear leukocytes into inflamed synovium, and the formation of pannus with joint erosion and cartilage destruction. In contrast, normal CB.17 mice did not develop any histopathological alterations following challenge with *B. burgdorferi*. A subsequent study by Schaible et al. [45] extended observations of CB.17 SCID mice to 195 days after challenge with *B. burgdorferi* ZS7. Macroscopic manifestations of arthritis failed to resolve even after this interval and severe destructive arthritic involvement progressed from tibiotarsal joints to the metatarsal and ulnocarpal joints. Furthermore, histopathological changes were noted in the ligaments, tendons, fascia, and skeletal muscle.

Barthold et al. [46] also compared the progression of Lyme arthritis in C3H/He SCID mice to that observed in C3H/He mice. While the maximum severity and distribution of arthritis were observed at relatively early intervals after intradermal infection with *B. burgdorferi sensu stricto* isolate N40, arthritis severity progressively worsened in C3H/He SCID mice throughout a 60-day observation period. Histopathological analysis of infected C3H/He SCID mice 60 days after infection revealed very severe joint disease involving nearly all tibiotarsi, knees, elbows, and carpi examined. Intensely proliferating synoviocytes caused effacement of joints, tendons, and bursae. Obliteration of the joint cavity and losses of cartilage and bone were noted. Such characterization of histopathology is consistent with the clinical entity of severe destructive Lyme arthritis. By contrast, at a peak interval of articular involvement in C3H/He mice (between 14 and 30 days after *B. burgdorferi* challenge), tibiotarsal joints displayed moderate synovial proliferation, leukocytic infiltration, and exudation into the joint lumen. The immunodeficient mouse model may not represent the actual events that occur in humans. However, the model is important for understanding the components of the immune response involved in the induction of arthritis. Without this model, the complexity of the immune response participating in the development of synovitis would be extremely difficult to decipher.

3.2.2. Irradiated Mouse Model. Irradiation of mice followed by infection with *B. burgdorferi* can also induce severe destructive Lyme arthritis. Low-passage ($P < 5$) *B. burgdorferi* 297 was cultivated at 32°C in Barbour-Stoenner-Kelly (BSK) medium [47] containing screened lots of bovine serum albumin [48]. Two groups of three C3H/HeJ mice each were subjected to 550 rads of gamma radiation with a ^{60}Co irradiator (Picker Corporation, Cleveland, OH). Mice were then immediately anesthetized with methoxyflurane and footpads of one group of mice were infused with 0.2 mL of a 5×10^6/mL suspension of *B. burgdorferi* 297. The second group of irradiated mice was injected in the footpad with 0.2 mL of sterile BSK. A group of three C3H/HeJ mice, not subject to gamma radiation, was challenged in the footpad with 10^6 *B. burgdorferi* 297 delivered in a 0.2 mL volume.

Inflammatory responses were assessed at the macroscopic level for 20 days by carefully measuring the width and thickness of each tibiotarsal joint with a dial-type vernier caliper (Fisher Scientific, Pittsburgh, PA), combining these values, and determining mean dimensions within a given group. Tibiotarsal joint edema peaked nine days after challenge of nonirradiated mice with *B. burgdorferi* and began to wane thereafter (Figure 1). By contrast, onset of edema was slightly delayed in irradiated mice that were challenged with *B. burgdorferi*, with swelling beginning to peak 20 days after challenge (Figure 1). Both groups of mice challenged with *B. burgdorferi* manifested peak edema that was relatively equal to each other and exceeded that observed in mice injected with BSK medium.

After 21 days of observation, mice were euthanized by CO_2 asphyxiation and hind legs of all mice were amputated at the midfemur and fixed in 10% neutral buffered zinc formalin (Fisher). While peak edema measurements between infected irradiated and nonirradiated mice were similar, the degree of arthropathy was distinctly different between the two treatment groups. A severe destructive Lyme arthritis was induced in the hind legs of irradiated mice challenged with *B. burgdorferi*. This was characterized, in part, by a dense infiltration of inflammatory cells which compacted synovial spaces and by widespread erosion of articular cartilage and destruction of small bones (Figures 2(a) and 2(b)). This was in stark contrast to the histopathological response of hind legs of nonirradiated mice infected with *B. burgdorferi* which demonstrated a mild synovial hyperplasia and joint spaces free of inflammatory cells

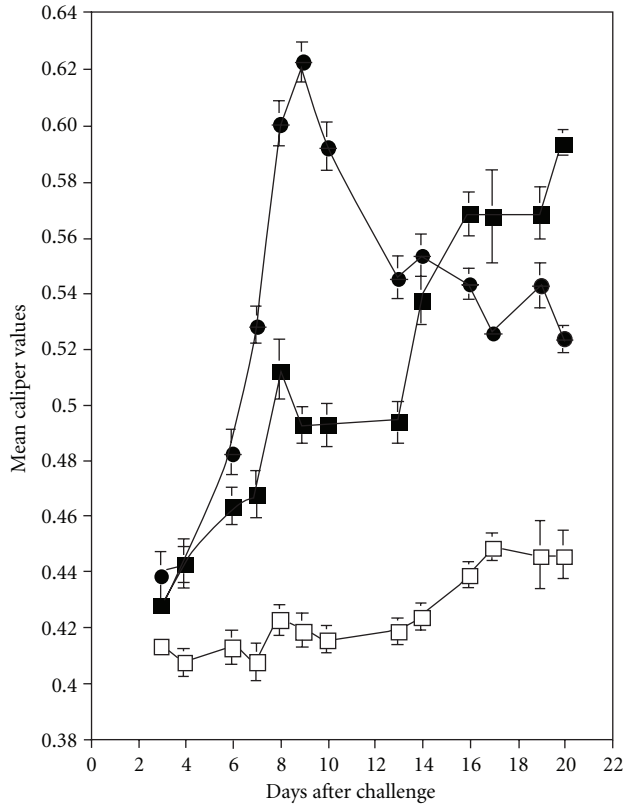

Figure 1: Swelling of the tibiotarsal joint detected in irradiated C3H/HeJ mice injected with *B. burgdorferi sensu stricto* isolate 297 (■) or BSK medium (□) and in nonirradiated C3H/HeJ mice infused with *B. burgdorferi* 297 (•).

(Figure 2(d)). Examination of histopathology from infected nonirradiated mice at an interval (day 14) closer to that at which peak macroscopic edema was observed revealed a similar mild Lyme synovitis (Figure 2(c)). Mice injected with BSK medium failed to present any articular involvement (Figure 2(e)). Although this model is infrequently utilized, it is valuable to assess the role of immune components in protection and the development of severe destructive Lyme arthritis.

3.2.3. Interferon-Gamma- (IFN-γ-) Deficient Mouse Model. Classically, a T helper lymphocyte type 1 (Th1) response picture has been painted to explain the induction of Lyme arthritis. Humans with clinically diagnosed Lyme borreliosis produce increased serum levels of the Th1 cytokine IFN-γ and decreased quantities of interleukin-(IL-) 4 when compared to healthy individuals [54]. Yang et al. [55] noted that mice susceptible to synovitis following *B. burgdorferi* infection produced increased serum levels of IgG_{2a}, an immunoglobulin isotype associated with a Th1 response. Independent groups [56, 57] reported that C3H mice produced IFN-γ following *B. burgdorferi* infection, while BALB/c mice, with an inherent variable susceptibility to synovitis, produced IL-4 following challenge with *B. burgdorferi*. Furthermore, treatment of BALB/c mice with neutralizing antibody to murine IL-4 facilitated the development of synovitis upon challenge with *B. burgdorferi*, whereas treatment of C3H mice with rIL-4 or neutralizing antibody to murine IFN-γ reduced the severity of synovitis [56–58].

On the other hand, studies have presented instances in which decreases in IFN-γ production or an inherent deficiency of IFN-γ had no impact on arthritis severity. Blockage of the B7-CD28 interaction in BALB/c mice resulted in an increase of IFN-γ production with no concomitant change in synovitis [59]. Depletion of natural killer cells in C3H/HeJ mice resulted in a loss of early IFN-γ production with no alteration of synovitis [60]. Direct evidence not supporting a role for IFN-γ in synovitis induction was presented by Brown and Reiner [61]. Upon histopathological examination of tibiotarsal joints, IFN-γ-deficient mice that were challenged with *B. burgdorferi* were shown to manifest equivalent synovitis to that observed in infected wild-type controls.

An *in vitro* neutralization model [50] supplied additional evidence in support of the hypothesis that IFN-γ does not play a significant role in the induction of articular manifestations of Lyme borreliosis. C3H/HeJ mice were immunized with formalin-inactivated *B. burgdorferi* in alum. Inguinal lymph node cells were cocultured with *B. burgdorferi* and macrophages and were treated *in vitro* with either rIFN-γ or neutralizing antibody to IFN-γ. Cultures were incubated for 24 hours at which time contents were infused into footpads of recipient C3H/HeJ mice. While three-week edema measurements did not vary significantly between recipients of immune lymph node cells treated with rIFN-γ or anti-IFN-γ, histopathological examination of hind legs infused with cells treated *in vitro* with anti-IFN-γ manifested severe destructive Lyme arthritis [50]. In contrast, both recipients of immune lymph node cells treated *in vitro* with rIFN-γ and recipients of nontreated cultures of cells obtained from vaccinated or nonvaccinated C3H/HeJ mice demonstrated synovitis.

A clearer picture of the role of IFN-γ on the development of severe destructive Lyme arthritis was shown by Christopherson et al. [51]. When C57BL/6 IFN-γ-deficient mice were vaccinated with Formalin-inactivated *B. burgdorferi* in alum, gross tibiotarsal edema and a corresponding severe destructive arthritis were induced upon footpad challenge with a heterologous isolate of *B. burgdorferi* [51]. Challenge of nonvaccinated mice produced only a mild synovitis and tibiotarsal joint measurements that were significantly less than those observed in vaccinated mice but slightly greater than those measured in IFN-γ-deficient mice inoculated with BSK medium. These data corroborated those of Brown and Reiner [61] that IFN-γ is not required for the development of arthropathy in an infection model of murine Lyme borreliosis.

The findings of Christopherson et al. [51] also suggested that inflammatory pathways other than a traditional Th1, IFN-γ-mediated response may stimulate the development of severe destructive Lyme arthritis following infection with *B. burgdorferi*. Using the IFN-γ-deficient murine model, Burchill et al. [52] demonstrated a significant role for the proinflammatory cytokine IL-17 in the induction of severe

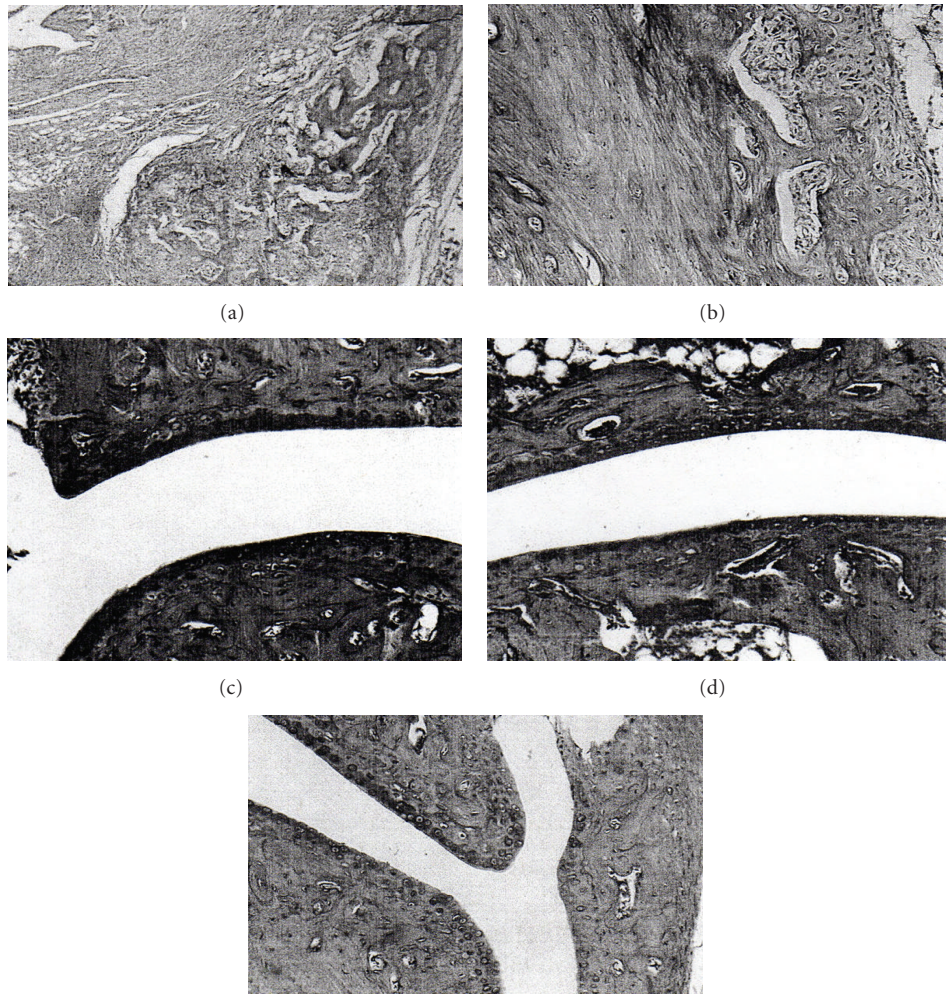

Figure 2: Histopathological responses detected in irradiated ((a) and (b)) and nonirradiated ((c) and (d)) C3H/HeJ mice injected with *B. burgdorferi sensu stricto* isolate 297. Irradiated control mice (e) were injected with BSK medium. All specimens were acquired 21 days after challenge, with the exception of panel (c), day 14. Panels (a) and (b) were viewed with a 4x or 10x objective lens, respectively.

destructive Lyme arthritis. When these mice were vaccinated and challenged three weeks later with a heterologous bacterial strain in the footpad, they developed inflammatory changes analogous to those observed by Christopherson et al. [51]. However, administration of antibodies to either IL-17 or the IL-17 receptor on the day of infection and daily thereafter for 11 days reduced the severity of hind paw edema and abrogated the development of severe destructive Lyme arthritis [52].

These findings were extended by Nardelli et al. [53] who showed that administration of anti-IL-17 to *Borrelia*-vaccinated and -infected IFN-γ-deficient C57BL/6 mice also correlated with an inordinate increase in the numbers of $CD4^+CD25^+$ T lymphocytes in local lymph nodes. Concomitant depletion of IL-17 and $CD4^+CD25^+$ T lymphocytes from these *Borrelia*-vaccinated and -infected mice resulted in massive edema of the hind paws, extensive erosion of cartilage, bone, and synovial tissue of the tibiotarsal joints, and pannus formation into the joint space, indicating that these cells played a significant role in the protection from *Borrelia*-induced arthritis mediated by neutralization of IL-17 [53]. In support of this, depletion of only $CD4^+CD25^+$ T lymphocytes from *Borrelia*-vaccinated and -infected mice resulted in inflammatory changes similar to those of *Borrelia*-vaccinated and -infected control mice [62]. Significantly, adoptive transfer of $CD4^+CD25^+$ T lymphocytes obtained from anti-IL-17-treated *Borrelia*-vaccinated and -infected mice wholly prevented histopathological changes of the hind paws [63]. This suggested that those $CD4^+CD25^+$ T lymphocytes which developed in the absence of IL-17-mediated inflammation conferred protection against the induction of arthritis. Therefore, the development of arthritis in this murine model may be attributed to the combined effects of IL-17 production and a correspondingly low presence of $CD4^+CD25^+$ T lymphocytes. These findings were obtained using IFN-γ-deficient mice, which allows investigators to determine the interaction among cytokines that directly or indirectly are affected by IFN-γ.

Table 1: Hamster and murine models important for evaluation of the pathogenesis of severe destructive Lyme arthritis.

Model	*B. burgdorferi* strains	Timing of arthritogen delivery	Result	Comments	Reference(s)
Immunocompetent hamster (challenge)	Numerous[a]	Can vary	Synovitis	Studying innate responses	[18]
Immunocompetent mouse (challenge)	Numerous[a]	Can vary	Synovitis	Studying innate responses	[38–42]
Irradiated hamster (challenge)	Numerous[a]	Following 550 rads gamma radiation	SDLA[b]	Insight into cellular components responsible for SDLA	[18, 19]
Irradiated mouse (challenge)	297	Following 550 rads gamma radiation	SDLA	Insight into cellular components responsible for SDLA	Stated in this publication
SCID mouse (challenge)	Numerous	Can vary	SDLA	Insight into cellular components responsible for SDLA	[44–46]
Immunocompetent hamster (vaccination/challenge)	Numerous[c]	Challenge prior to development of significant borreliacidal antibody titer (homologous challenge)[d]; timing of heterologous strain challenge can vary	SDLA	Insight into adoptive cellular components responsible for SDLA in an immunocompetent host	[20, 30–34]
Immunocompetent mouse (vaccination/challenge)	297	Challenge following 21 days of vaccination	SDLA	Elucidating adoptive immunological pathways of SDLA in an immunocompetent host	[49]
In vitro murine IFN-γ neutralization	297	Lymph node cells pulsed *in vitro*	SDLA	Addition of IFN-γ to lymph node cells prior to *in vitro B. burgdorferi* pulse; footpad challenge of naïve mice following cell culture transfer; Assessment of arthritogenic potential of *B. burgdorferi* immunogens	[50]
IFN-γ-deficient mouse (vaccination/challenge)	297	Challenge following 21 days of vaccination	SDLA	Elucidating immunological pathways of SDLA	[51–53]

[a]Referenced study(ies) focused on *B. burgdorferi sensu stricto* isolate 297.
[b]Severe destructive Lyme arthritis.
[c]Referenced study(ies) focused on *B. bissettii* vaccination with subsequent *B. burgdorferi sensu stricto* isolate 297 challenge.
[d]At least six seroprotective groups have been characterized ([27–29]).

Collectively, these findings not only provided potentially valuable insights into the pathogenesis of severe destructive Lyme arthritis, but also have played a significant role in elucidating immunological pathways beyond the realm of Lyme borreliosis. In support of this statement, Codolo et al. [64] showed that synovial T lymphocytes isolated from humans with Lyme arthritis secrete IL-17 upon stimulation with neutrophil activating protein A of *B. burgdorferi*. In addition, Shen et al. [65] observed the presence of Th17 cells in the synovial fluid of humans with Lyme arthritis; however, their direct role in mediating the induction or propagation of Lyme arthritis was not determined. Moreover, these early investigations of the role of IL-17 in the development of murine Lyme arthritis were used to support the seminal immunological findings of Bettelli et al. [66] that Th17 cells and $CD4^+CD25^+Foxp3^+$ regulatory T lymphocytes develop in a reciprocal manner from a common precursor cell, and that this development is governed by the presence or absence of an inflammatory environment.

3.2.4. Immunocompetent Mouse Model of Severe Destructive Lyme Arthritis. Although use of IFN-γ-deficient mice to study the role of cytokines in the development of severe destructive Lyme arthritis is valuable, severe arthritis can also be induced in C57BL/6 mice. Mice are vaccinated in the inguinal region with washed spirochetes mixed with 1% alum. Approximately three weeks after vaccination, mice are challenged with viable *B. burgdorferi*. This animal model was utilized to determine the role of IL-35 on the production of IL-17 [49].

IL-35 is a major cytokine produced by $CD4^+CD25^+$ $Foxp3^+$ regulatory T lymphocytes. Based on previous findings that administration of anti-IL-17 prevented arthritis in

Table 2: Hamster models important for evaluation of protective immunity versus endpoint of severe destructive Lyme arthritis.

Model	*B. burgdorferi* strains	Timing of immunogen delivery	Endpoint	Comments	Reference(s)
Irradiated hamster (challenge)	Numerous[a]	Following 550 rads gamma radiation	SDLA[b]	Adoptive transfer (protection) experiments	[18, 21, 22]
Irradiated hamster (vaccination/challenge)	Numerous[a]	Challenge prior to development of significant borreliacidal antibody titer (homologous challenge)[c]; timing of heterologous strain challenge can vary	SDLA	Assessment of vaccine candidates	[24]
Immunocompetent hamster (vaccination/challenge)	Numerous[d]	Challenge prior to development of significant borreliacidal antibody titer (homologous challenge)[c]; timing of heterologous strain challenge can vary	SDLA	Characterizing duration of protective immunity of vaccine candidates; Assessing Lyme borreliosis vaccine candidates for adverse events	[20, 25] [29, 35]

[a]Referenced study(ies) focused on *B. burgdorferi sensu stricto* isolate 297.
[b]Severe destructive Lyme arthritis.
[c]At least six seroprotective groups have been characterized ([27–29]).
[d]Referenced study(ies) focused on *B. bissettii* vaccination with subsequent *B. burgdorferi sensu stricto* isolate 297 challenge.

Borrelia-vaccinated and -infected mice and induced production of $CD4^+CD25^+$ T lymphocytes with immunoregulatory function, we hypothesized that IL-35 would decrease the severity of arthritis by inhibiting the production of IL-17 in *Borrelia*-vaccinated and -infected mice. When IL-35 was administered to *Borrelia*-vaccinated and -infected mice, IL-35 enhanced arthritis. This suggests that IL-35 does not play a major role in preventing the induction of arthritis. Additional investigations are needed to determine which cytokines are responsible for the induction and resolution of arthritis. These studies are important because they may lead to a therapeutic agent that may decrease the effects of arthritis. The mouse model of severe destructive Lyme arthritis yields reproducible data and hopefully the results can be applied to humans with Lyme arthritis.

4. A Unifying Hypothesis

Using the data obtained through the investigation of hamster and mouse models, as well as through analysis of human tissues *ex vivo*, a unifying hypothesis can be developed to elucidate the mechanisms by which severe destructive Lyme arthritis is induced. Central to the development of this chronic manifestation are (1) the stimulation of innate immune effectors following infection with *B. burgdorferi*, with subsequent stimulation of adaptive immune mechanisms; (2) survival and persistence of the spirochete in the face of a robust innate immune response and increasing (but eventually waning) borreliacidal antibody titers, and (3) recognition of *B. burgdorferi* or its proteins by primed T lymphocytes, with ensuing tissue-destructive effects occurring in the joint.

Upon bloodmeal acquisition by infected *Ixodes* spp., host macrophages, dendritic cells, and/or their monocyte precursors rapidly recognize lipoproteins of *B. burgdorferi* via Toll-like receptor 2. Processing of the spirochete by these cells stimulates the secretion of various cytokines, such as tumor-necrosis factor-α, interleukin-1β, and interleukin-6, [64, 67], which serve to recruit neutrophils and amplify the early inflammatory events manifesting as synovitis. Some spirochetes survive this initial innate immune defense, as well as the borreliacidal antibody response triggered by innate effectors. *B. burgdorferi* disseminates to the articular tissues, where the spirochetes or their proteins may be recognized weeks, months, or years later to activate a new wave of innate immunity as well as previously primed adaptive immune cells. Innate immune cells in the synovial tissues and/or fluid may again secrete an array of cytokines, including transforming growth factor-β, IL-6, IL-12 (among others) that polarize the T lymphocyte response toward inflammatory subsets such as Th1 and Th17 cells. In turn, the immune response would be directed away from T lymphocyte subsets, such as Th2 and regulatory T lymphocytes, which antagonize these inflammatory populations. Both Th1 and Th17 responses have been postulated to play a role in later-stage Lyme arthritis [68], which is supported by observation of these cells in the synovial fluid of humans with Lyme arthritis [65], implication of their prototypical cytokines in disease [52, 69], and recognition that these cytokines induce severe destruction in other models of arthritis [70, 71]. These inflammatory responses may persist until the antigenic stimulus is eventually cleared, at which point regulatory T lymphocytes may mediate suppression of inflammation and tissue remodeling may occur.

5. Conclusions

Selection of the proper animal model is paramount in relevant investigations of articular involvement of Lyme disease. Delivery of *B. burgdorferi* to immunocompetent LSH hamsters and C3H mice elicits a mild and transient synovitis that mimics pathology observed in individuals in early Lyme

borreliosis. However, in order to investigate the chronic destructive arthropathy with which a certain subpopulation of individuals in late Lyme borreliosis may be afflicted, one must choose either the immunocompetent hamster model of severe destructive Lyme arthritis or various murine models of severe destructive Lyme arthritis that manifest a degree of immunodeficiency (Table 1).

While murine models, with their replete armamentarium of immunological reagents, appear very promising in the future study of severe destructive Lyme arthritis, we do not intend to eulogize the hamster model of severe destructive Lyme arthritis. The immunocompetent hamster model of severe destructive Lyme arthritis can be a very advantageous system for safety assessment of Lyme borreliosis vaccine preparations (should redevelopment efforts ever come to fruition [72, 73]) due to the fact that macroscopic observation of footpad edema correlates well with the severity of histopathology (Table 2).

Murine systems of severe destructive Lyme arthritis will provide a tremendous advantage in the future elucidation of immunological mechanisms that result in severe destructive Lyme arthritis. In particular, the advent of a system in which immune cells from an immunocompetent host are programmed *in vitro* to elicit severe destructive Lyme arthritis upon subsequent transfer into a recipient immunocompetent host presents great potential in the ultimate elucidation of the mechanisms of severe destructive Lyme arthritis. In addition, this system may also allow for simultaneous assessment of Lyme borreliosis vaccine candidates (following selection of protective epitopes [74]) for production of protective borreliacidal antibodies *in vitro* [50, 75–78] as well as the deleterious capability of priming recipients for induction of severe destructive Lyme arthritis [50]. Taken together, these investigations may contribute to the production of a safe and efficacious vaccine to prevent *B. burgdorferi* infection.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 349657, 12 pages
doi:10.1155/2012/349657

Research Article

Contribution of the Infection-Associated Complement Regulator-Acquiring Surface Protein 4 (ErpC) to Complement Resistance of *Borrelia burgdorferi*

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Received 22 June 2011; Accepted 5 October 2011

Academic Editor: Franc Strle

Borrelia burgdorferi evades complement-mediated killing by interacting with complement regulators through distinct complement regulator-acquiring surface proteins (CRASPs). Here, we extend our analyses to the contribution of CRASP-4 in mediating complement resistance of *B. burgdorferi* and its interaction with human complement regulators. CRASP-4 (also known as ErpC) was immobilized onto magnetic beads and used to capture proteins from human serum. Following Western blotting, factor H (CFH), CFH-related protein 1 (CFHR1), CFHR2, and CFHR5 were identified as ligands of CRASP-4. To analyze the impact of native CRASP-4 on mediating survival of serum-sensitive cells in human serum, a *B. garinii* strain was generated that ectopically expresses CRASP-4. CRASP-4-producing bacteria bound CFHR1, CFHR2, and CFHR5 but not CFH. In addition, transformed spirochetes deposited significant amounts of lethal complement components on their surface and were susceptible to human serum, thus indicating that CRASP-4 plays a subordinate role in complement resistance of *B. burgdorferi*.

1. Introduction

Lyme borreliosis, caused by spirochetes of the *Borrelia burgdorferi* sensu lato complex, is the most prevalent vector-borne anthropozoonosis in Eurasia and the United States [1]. The ability of spirochetes to perpetuate their natural vertebrate-tick infectious cycle spirochetes requires an array of mechanisms to successfully colonize their tick vectors and rodent reservoir hosts, survive in diverse environments, and evade host innate and adaptive immune responses. Recently, it has been shown that certain genospecies resist complement-mediated killing of human serum, in particular *B. burgdorferi* sensu stricto (hereafter referred to as *B. burgdorferi*), *B. afzelii*, *B. spielmanii*, and *B. bavariensis* (formerly known as *B. garinii* OspA serotype 4 strains) [2–5]. Elucidation of the underlying molecular mechanism(s) of complement resistance among Lyme disease spirochetes revealed that binding of the host complement regulators factor H (CFH) and factor H-like protein 1 (FHL1) to the bacterial surface directly correlates with serum resistance [3, 6–10]. In contrast, *B. garinii*, *B. valaisiana*, and *B. lusitaniae* are highly susceptible to complement-mediated killing and either do not bind, or bind inadequate levels of complement regulators [2, 4, 10–12].

Complement plays a central role in the recognition and elimination of invading microorganisms [13]. Upon

activation of the initial steps of the complement cascade via the classical, alternative, or lectin pathway, a C3 convertase is generated which cleaves the central component C3 into its reactive fragments C3a and C3b. The highly reactive C3b fragment covalently binds to molecules, proteins, and nearby membranes, thereby leading to opsonization of the intruding microorganisms. This initial step is necessary for clearance of foreign microorganisms by phagocytosis, formation of the C3 convertase, and assembly of both the C5 convertase and the membrane attack complex (MAC). To protect host cell surfaces from uncontrolled and continuous activation, the complement system is well balanced and finely tuned by diverse fluid phase and membrane-anchored negative regulators [14–16]. CFH and FHL1 are the key fluid phase regulators of the human alternative pathway and act as cofactors for factor-I-mediated inactivation of C3b to iC3b, compete with factor B for binding to C3b, and finally support the dissociation (decay-accelerating activity) of the alternative pathway C3 convertase, C3bBb [16–20]. CFH is composed of 20 individually folding protein domains termed short consensus repeats (SCRs) of which the four N-terminal-located SCRs exhibit the complement regulatory activity. FHL1 is a 42 kDa glycoprotein, comprised of the seven amino-terminal SCRs of CFH plus four unique amino acids at the C-terminus [17, 20]. The human CFH family includes additional "factor H-related" proteins (CFHR), namely, CFHR1, CFHR2, CFHR3, CFHR4A, CFHR4B and CFHR5, all of which are encoded by distinct genes located in the regulators of complement activation (RCA) gene cluster on human chromosome 1 [21–23]. The C-terminal SCR domains of the CFHR proteins share high degrees of similarity to the C-terminal surface binding region of CFH, that is, SCRs 18–20 [16, 24]. The CFHR1 protein consists of five SCRs and exists in two glycosylated forms, the 37 kDa CFHR1α protein with one and the 43 kDa CFHR1β protein with two carbohydrate chains attached [25, 26]. CFHR1 is a complement regulator that blocks C5 convertase activity as well as assembly and membrane insertion of the terminal membrane attack complex [27]. CFHR2 is composed of four SCRs and is found in plasma as a nonglycosylated 24 kDa form (CFHR2) and a glycosylated 29 kDa form (CFHR2α) [28]. The function(s) of CFHR2 is as still unclear. The 65 kDa CFHR5 protein is comprised of 9 SCRs and displays cofactor activity for factor-I-mediated inactivation of C3b [29, 30]. CFHR5 also inhibits the activity of the fluid phase C3 convertase.

Lyme disease *Borreliae* camouflage themselves with host-derived complement regulators through three groups of genetically unrelated genes/proteins collectively termed *c*omplement *r*egulator-*a*cquiring *s*urface *p*roteins or "CRASPs" [3, 9, 31–35]. All investigated serum-resistant borrelial strains so far express the CRASP-1 protein in different combinations with CRASP-2, CRASP-3, CRASP-4, and/or CRASP-5. Based on the binding profile for complement regulators, CRASPs expressed by *B. burgdorferi* are divided into CFH and FHL1 binding proteins that do not bind CFHR1 (CRASP-1/CspA and CRASP-2/CspZ) and molecules that interact with CFH and CFHRs, but not FHL1 (CRASP-3/ErpP, and CRASP-4/ErpC, CRASP-5/ErpA) [9, 34, 36–39]. The potential of single CRASP-molecules in mediating complement resistance of *B. burgdorferi* s.s. is still under debate. Borrelial strains lacking functional CRASP-1 and CRASP-2 are highly susceptible to complement-mediated killing, and complementation with the respective CRASP encoding genes restores the serum-resistant phenotype [31, 40–42]. The contributions of the CFH and CFHR-binding CRASP-3 and CRASP-5 proteins in facilitating complement resistance of *Borreliae* are disputed. Heterologous production of either CRASP-3 or CRASP-5 in a *B. garinii* strain lacking all functional CRASP molecules failed to convert the serum-sensitive phenotype of the wild-type strain [39]. In contrast, Kenedy and Akins have shown that CRASP-3 and CRASP-5 produced in a CRASP-1 deletion strain lead to increased survival in human serum as compared to a serum-sensitive strain lacking CRASP-1 [43].

In the present studies, we extended our previous investigations on the CFH- and CFHR1 binding capacity of CRASP-4/ErpC protein to additional proteins derived from human serum and their contributions to convey complement resistance. To this end, a *B. garinii* strain that ectopically produced CRASP-4 was generated by transformation with a shuttle vector harboring the CRASP-4 encoding *erpC* gene, then the transformed strain was assayed for (i) the ability to bind human complement regulators, (ii) surface deposition of complement activation products, and (iii) survival in human serum. Using recombinant CRASP-4, two additional members of the human CFH protein family, CFHR2 and CFHR5, were identified as novel ligands for CRASP-4 of *B. burgdorferi* whereby CFHR2 showed stronger binding capacity for CRASP-4 as compared to CFHR1 and CFHR5. However, borrelial cells producing CRASP-4 on their surface did not bind CFH. Upon incubation in human serum, large amounts of activated complement components were deposited onto the surfaces of CRASP-4 producing cells and the bacteria did not survive. This suggests that binding of CFHR1, CFHR2, and CFHR5 is not sufficient to protect spirochetes from complement-mediated bacteriolysis once complement is activated.

2. Material and Methods

2.1. Bacterial Strains and Culture Conditions. *B. burgdorferi* strains LW2 (skin isolate, Germany), *B. garinii* isolate G1 (CSF isolate, Germany), *B. garinii* transformants G1/pKFSS1 as well as G1/pCRASP-4 were grown at 33°C for 2 to 4 days to midexponential phase (1×10^7 to 5×10^7 spirochetes/mL) as described previously [39]. *Escherichia coli* DH5α used for cloning experiments and protein expression was grown at 37°C in yeast tryptone broth, supplemented with the appropriate antibiotics.

2.2. Human Sera and Polyclonal and Monoclonal Antibodies. Normal human serum (NHS) obtained from 20 healthy human blood donors without known history of spirochetal infections was used as a source of complement regulators. The study and the respective consent documents were approved by the ethics committee at the Goethe University of Frankfurt (control number 160/10). All blood donors provided written, informed consent.

Table 1: Oligonucleotides used in this study.

Oligonucleotide	Sequence (5′–3′)[a]	Use in this work
ErpC 5nc(+) HindIII	GTTGTATGTGTTTTGAAGCTTTTAGTAATGAGCAGGGC	Cloning in pKFSS1 and amplification of *erpC*
ErpC 3nc(−) HindIII	CGATCTCTCCTGTATTTTAAGCTTCTATTTTAAATTTTTCTTAAG	Cloning in pKFSS1 and amplification of of *erpC*
aadA + NdeI	CATATGAGGGAAGCGGTGATC	Amplification of *aadA* gene
aadR + AatII	GACGTCATTATTTGCCGACTACC	Amplification of *aadA* gene
Fla6	AACACACCAGCATCGCTTTCAGGGTCT	Amplification of *flaB* gene
Fla7	TATAGATTCAAGTCTATTTTGGAAAGCACCTA	Amplification of *flaB* gene

[a]Sequences of specific restriction endonuclease recognition sites are underlined.

A polyclonal anti-CFH antiserum was utilized to detect human CFH, CFHR1, and CFHR2 (Merck Biosciences, Bad Soden, Germany and Complement Technology, Tyler, TE). Rabbit polyclonal anti-CFHR1 antibody or monoclonal antibody JHD 7.10 was used for detection of CFHR1 and CFHR2 and CFHR5 [39]. The goat anti-human C3 and C6 antibodies were purchased from Calbiochem, and the monoclonal anti-human C5b-9 antibody recognizing the MAC was obtained from Quidel (San Diego, CA). MAb L41 1C11 was used to detect the periplasmic FlaB protein. For analyzing surface-exposed CRASP-4, a rabbit polyclonal antiserum that recognizes CRASP-4 and CRASP-5 was used [44, 45].

2.3. Expression of Recombinant CFHR1, CFHR2, and CFHR5. Recombinant CFHR1 was expressed in *Spodoptera frugiperda* Sf9 insect cells infected with recombinant baculovirus. The cloning of various deletion constructs, expression, and purification have been described previously [38].

The full length CFHR2 cDNA was cloned into pPICZαB (Invitrogen), and the protein was expressed in the yeast *Pichia pastoris* strain X33 according to standard protocols [39]. The full length CFHR5 cDNA was cloned into pBSV-8His and expressed in the baculovirus system as described [46]. All expressed His-tagged recombinant proteins were purified by Ni^{2+} chelate affinity chromatography as described [46].

2.4. Expression of Recombinant CRASP-4. The construction of vector pBLS528 used for the production of amino-terminally polyhistidine-tagged CRASP-4 (ErpC) was described previously [47]. The *erpC* encoding sequence of *B. burgdorferi* strain LW2 is identical to the sequence of the *erpC* gene of *B. burgdorferi* type strain B31.

Expression of recombinant CRASP-4 protein was induced in DH5α at an OD_{600} of 0.6 by the addition of 0.2 mM IPTG. Following incubation for 4 h at room temperature, cells were centrifuged (5000 g, 20 min, 4°C) and subsequently suspended in lysis buffer (300 mM NaCl, 56 mM NaH_2PO_4 pH 8, and 10 mM Imidazole) containing 50 mg/mL lysozyme. Bacterial cells were lysed by 6 rounds of sonication for 30 sec using a Branson B-12 sonifier (Heinemann, Schwäbisch Gmünd, Germany). After centrifugation (14000 g, 20 min, 4°C), supernatants were filtered through 0,45 μm filters and stored at −20°C for later purification via affinity chromatography.

2.5. Serum Incubation with Magnetic Beads Coated with His-Tagged CRASP-4 Protein. Purified CRASP-4 (20 μg) was incubated with 50 μL of magnetic beads (Dynabeads TALON, Invitrogen Dynal AS, Oslo, Norway) for 10 min at room temperature as recommended by the manufacturer. After four wash steps with phosphate buffer (50 mM phosphate, 300 mM NaCl, 0.01% Tween 20), histidine-tagged proteins coupled onto beads were incubated with NHS for 1 h on ice. After extensive washing with phosphate buffer, bound proteins were eluted with 50 μL of 100 mM glycine-HCl (pH 2.0) for 15 min. The eluate and the last wash fraction were separated by 12.5% SDS-PAGE under nonreducing conditions followed by silver staining.

2.6. Construction of Shuttle Vectors. To allow ectopic expression of CRASP-4 by the serum-sensitive *B. garinii* strain G1, a shuttle vector was generated by using plasmid pKFSS1, a streptomycin-resistant derivative of pBSV2 [48]. The CRASP-4 encoding *erpC* gene plus its native promotor region was amplified from *B. burgdorferi* strain LW2 by PCR using primers containing the respective restriction sites and then sequenced (Table 1). The sequence of the *erpC* gene of *B. burgdorferi* strain LW2 is identical to that of *B. burgdorferi* type strain B31. Amplicons were hydrolyzed with HindIII and subsequently cloned into pKFSS1 at the corresponding restriction site, yielding shuttle vector pCRASP-4. The inserted sequence was subjected to nucleotide sequencing to verify that no mutations had been introduced during PCR and cloning procedures.

2.7. Transformation of Serum-Sensitive B. garinii. The noninfectious, serum-sensitive *B. garinii* strain G1 was grown in BSK medium and harvested at midexponential phase (5×10^7 to 1×10^8 cells/mL). Electrocompetent cells were prepared and transformed as described previously [39]. For selection of transformants, cells were diluted into 100 ml BSK medium containing 20 μg/mL streptomycin, then 200 μL aliquots were transferred into 96-well plates (Corning). After four to six weeks of incubation at 33°C, wells were evaluated for growth by color change of the medium and by dark-field microscopy for the presence of motile spirochetes. Several clones were expanded in 1 mL fresh BSK medium containing streptomycin (20 μg/mL) for 7 to 14 days. Transformed bacteria were then maintained in BSK medium containing 20 μg/mL streptomycin.

2.8. PCR Analysis of Transformed Borrelial Cells. Streptomycin-resistant clones of transformed *B. garinii* were characterized by PCR amplification of the introduced *erpC* gene and the recombinant plasmids streptomycin resistance gene (*aadA*) using specific primers (Table 1). The native *B. garinii flaB* gene was also amplified via PCR as a positive control. Ten microliter aliquots of bacterial cultures grown to midexponential phase were used for direct PCR. PCR was carried out for 25 cycles using the following parameters: denaturation at 94°C for 1 min, annealing at 50°C for 1 min, and extension at 72°C for 1 min. Reaction products were separated by agarose gel electrophoresis, and DNA was visualized by ethidium bromide staining and ultraviolet light.

2.9. SDS-PAGE, Western Blot, and Ligand Affinity Blot Analysis. Bacterial cell lysates were subjected to 10% Tris/Tricine-SDS-PAGE under reducing conditions and samples obtained by serum adsorption (last wash and eluate fractions) were separated by SDS-PAGE under nonreducing conditions as previously described [34].

For ligand affinity blot analysis, membranes were incubated for 1 h with normal human serum. After four washings with TBS containing 0,2% Tween20, membranes were incubated for 1 h with either a polyclonal goat CFH antiserum, polyclonal anti-CFHR1 antiserum that recognizes CFHR1, CFHR2, and CFHR5 and CFH or mAb JHD 7.10 which recognizes all three CFHRs but not CFH [38, 39, 49]. Following four washings with TBS containing 0,2% Tween 20, membranes were incubated with an appropriate peroxidase-conjugated secondary antibody for 1 h. Detection of bound proteins was performed using $3,3',5,5'$-tetramethylbenzidine (TMB) as a substrate.

For Western blot analysis, membranes were incubated for 1 h at room temperature with antisera recognizing CRASP-4/ErpC and CRASP-5/ErpA (αCRASP-4), CFH, CFHR1, or FlaB (L41 1C11). Following four wash steps with TBS containing 0,2% Tween20, membranes were probed with appropriate peroxidase-conjugated secondary antisera (Dako, Glostrup, Denmark) for 60 min at room temperature and bound antibodies were detected using TMB.

2.10. ELISA. Microtiter plates (Nunc-Immuno Module) were coated with CRASP-4 (5 μg/mL) over night at 4°C. Microtiter plates were washed with PBS containing 0.1% Tween 20 and treated for 1 h at RT with blocking buffer (AppliChem GmBH, Darmstadt, Germany). After washing, equimolar amounts (33 μM) of CFH, CFHR1, CFHR2, or CFHR5 were added and incubated for 1 h at RT. Thereafter, the wells were washed and bound CFH or CFHR proteins were detected with either goat CFH polyclonal antiserum or MAb JHD 7.10, which reacts with all three CFHRs [39, 49]. After washing, bound proteins were identified using appropriate secondary horseradish peroxidise-coupled antisera. Detection was performed with 1,2-phenylenediamine dihydrochloride as a substrate (OPD, DakoCytomation, Glostrup, Denmark) and absorbance was measured at 490 nm.

2.11. In Situ Protease Accessibility Experiments. Viable *Borreliae* were gently washed and resuspended in 500 μL PBS to obtain a density of $8 \times 10^5/\mu$L. Subsequently, proteinase K and trypsin (Sigma-Aldrich, Deisenhofen, Germany) were separately added to a final concentration of 25 and 100 μg/mL, respectively. Intact spirochetes without protease treatment served as a control. Following incubation for 2 h at room temperature, proteinase K and trypsin were inactivated by addition of phenylmethylsulfonyl fluoride (Sigma-Aldrich) (50 mg/mL in isopropanol). Cells were then washed gently twice with PBS-5 mM MgCl, resuspended in 20 μL of the same buffer, then lysed by sonication 5 times for 30 sec using a Branson B-12 sonifier (Heinemann, Schwäbisch Gmünd, Germany). Aliquots were separated using Tris/Tricine-SDS-PAGE as described above.

2.12. Serum Adsorption Assay. To assess binding of serum proteins to viable borrelial cells, a serum adsorption assay was employed as described previously [7, 50]. Briefly, borrelial cells (1×10^9 cells) grown to midexponential phase were washed and subsequently resuspended in 750 μL NHS supplemented with 34 mM EDTA (pH 8.0) to avoid complement activation. After 1 h incubation and four washes with PBS containing 0.01% Tween 20, proteins bound to the cells surface were eluted with 100 mM glycine-HCl (pH 2.0) for 15 min. Cells were removed by centrifugation at 14000 g for 10 min at 4°C, and the supernatant and the last wash were separated by SDS-PAGE under nonreducing conditions and analyzed by Western blotting as described above.

2.13. Serum Susceptibility Testing. Serum susceptibility of *B. garinii* isolate G1, G1/pKFSS1, and G1/pCRASP-4 was assessed by a growth inhibition assay as described previously [3, 42]. Briefly, aliquots (1.25×10^7 cells) of highly motile spirochetes were diluted into final volumes of 100 μL fresh BSK medium, which contains 240 μg/mL phenol red. As bacteria grow in BSK, the medium acidifies and the pH indicator dye turns from red to yellow. One hundred microliters of NHS or 100 μL heat-inactivated NHS was added to each aliquot of bacteria. Bacteria were then incubated in 96-well microtiter plates for 8 days at 33°C. For controls, aliquots of bacteria were also incubated with 100 μL BSK medium instead of NHS. Bacterial growth was monitored daily by measuring the ratio of culture medium absorbance at 562 versus 630 nm, using an ELISA reader (PowerWave HT; Bio-Tek Instruments, Winooski, VT). For calculation of the growth curves the Gen5 software (Bio-Tek Instruments, Winooski, VT) was used. Each experiment was conducted at least three times, and means ± SD were calculated.

2.14. Immunofluorescence Assay. Spirochetes grown to midexponential phase were harvested by centrifugation (5000 g, 30 min), washed, and resuspended in veronal buffered saline (VBS, supplemented with 1 mM Mg^{2+}, 0.15 mM Ca^{2+}, and 0.1% gelatin, pH 7.4).

For detection of deposited complement components on the bacterial surface, spirochetes (6×10^6) were incubated in 25% NHS and, as a control, in 25% heat-inactivated NHS for 30 min at 37°C with gentle agitation as previously described [3, 42].

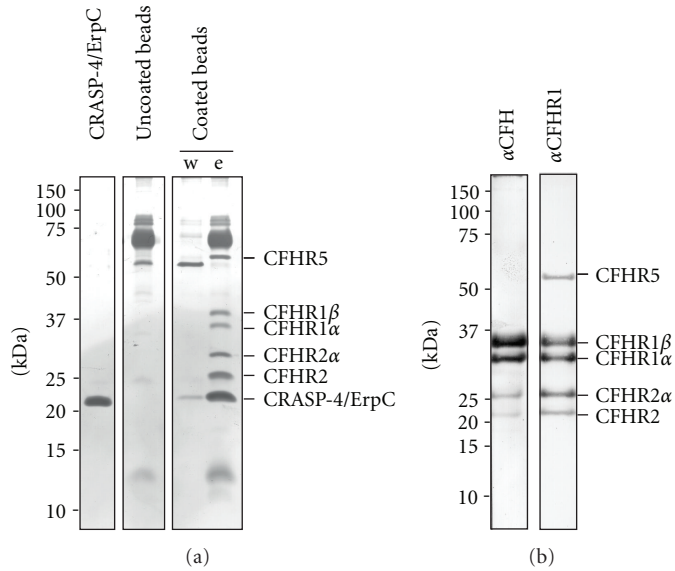

Figure 1: Identification of serum proteins that bind to recombinant CRASP-4. Recombinant, polyhistidine-tagged CRASP-4 was immobilized onto magnetic beads and incubated with NHS. Uncoated beads were also treated under the same conditions and used as a control to identify nonspecific binding of serum proteins. After extensive washing, bound proteins were eluted with 100mM glycine-HCl (pH 2.0) and the eluate fractions were separated by SDS-PAGE under nonreducing conditions. (a) Silver stain of a gel loaded with purified polyhistidine-tagged CRASP-4 (1 μg), eluate fraction of the uncoated beads, and the final wash and eluate fraction of CRASP-4-coated beads. (b) Western blot analysis of the eluate fraction of CRASP-4-coated beads using a polyclonal anti-CFH or a polyclonal anti-CFHR1 antiserum. Mobilities of molecular mass standards are indicated to the left.

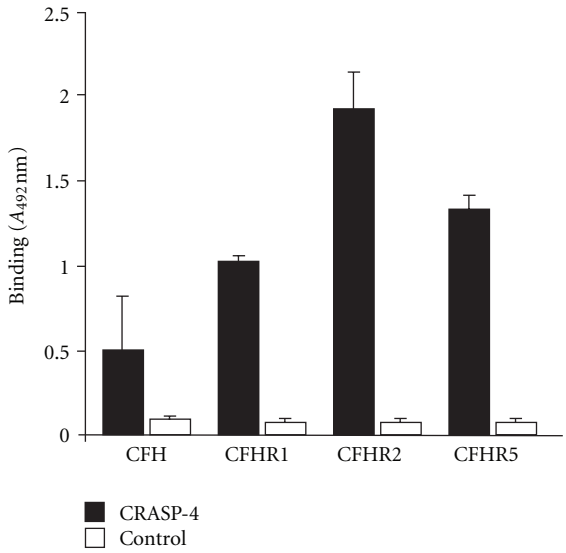

Figure 2: CRASP-4 binds distinct complement proteins. Binding of equimolar amounts of CFH, CFHR1, CFHR2, and CFHR5 (33 μM) to immobilized CRASP-4 (5 μg/mL) was analyzed by ELISA. Bound CFH or CFHR proteins were detected with either goat CFH polyclonal antiserum or mouse CFHR1 monoclonal antiserum (JHD 7.10), which reacts with all three CFHRs. Data represent the means and standard errors from three separate experiments.

In order to detect surface-exposed proteins, polyclonal rabbit anti-CRASP-4 antiserum (1:50 dilution) was added to the cells for 1h at 37°C with gentle agitation. After two washes with PBS containing 1% BSA, 10 μL aliquots of the cell suspensions were spotted on glass slides and allowed to air-dry overnight (= unfixed cells). Slides were then fixed in methanol for 10 min and air-dried for 1 h, followed by incubation with an adequate Alexa 488-conjugated antibody. Slides were then gently washed four times with PBS and mounted on ProLong Gold antifade reagent (Molecular Probes) containing DAPI before being sealed. Slides were visualized at a magnification of ×1,000 using an Olympus CX40 fluorescence microscope mounted with a DS-5Mc charge-coupled device camera (Nikon).

As a control, periplasmic FlaB was also investigated using unfixed as well as fixed spirochetes as described previously [39].

3. Results

3.1. CRASP-4 Interacts with Human Complement Regulators. To identify serum components that bind to CRASP-4, the purified his-tagged protein was immobilized on magnetic beads. Following incubation with NHS, beads were extensively washed and bound serum proteins along with CRASP-4 were eluted. The eluate fraction of the CRASP-4-coated beads and eluate fraction of uncoated beads as well as the final wash were separated by SDS-PAGE and analyzed by silver staining (Figure 1(a)). A bulk of proteins in the 60 to 80 kDa range was detected in the eluate fractions of CRASP-4-coated beads and also in the eluate fraction of uncoated beads. In the eluate fraction of uncoated beads and in the final wash fraction but not in the eluate fraction of CRASP-4-coated beads, a 55 kDa protein was found. In contrast,

proteins with apparent molecular masses of 60, 38, 35, 29, 25 and 22 kDa were detected only in the eluate fraction of CRASP-4-coated beads.

To identify the serum proteins bound to the recombinant CRASP-4 protein, Western blot analysis was performed using specific antisera. All serum proteins bound to CRASP-4 react with the polyclonal anti-CFHR1 antiserum that recognizes the two different glycosylated forms of CFHR1 (CFHR1α and CFHR1β) and CFHR2 (CFHR2, and CFHR2α) as well as CFHR5 (Figure 1(b)). By using a polyclonal anti-CFH antiserum, four signals corresponding to CFHR1α (35 kDa) and CFHR1β (32 kDa) and CFHR2 (25 kDa) and CFHR2α (22 kDa) could be detected. The 150 kDa CFH protein was not detected by either silver staining or Western blot, indicating that CRASP-4 did not bind CFH under these experimental conditions. The protein with an apparent mass of 22 kDa represents CRASP-4. Taken together, CRASP-4 binds to human CFH protein family members, including CFHR1, CFHR2, and CFHR5.

Next, binding of recombinant CRASP-4 to each of the three identified human serum proteins was analyzed by ELISA (Figure 2). CRASP-4 was immobilized onto a microtiter plate and binding of purified recombinant CFHR1, CFHR2, CFHR5 and serum-purified CFH was assayed. All three CFHR proteins, that is, CFHR1, CFHR2, and CFHR5 as well as CFH bound to the immobilized CRASP-4 protein, with the greatest apparent affinity being for CFHR2.

3.2. Generation of a CRASP-4-Expressing B. garinii Strain. Depending on the genetic composition, all serum-resistant *B. burgdorferi* isolates analyzed to date express at least two distinct CRASP molecules. In order to assess the contribution of an individual CRASP molecule in mediating complement resistance, the serum-sensitive *B. garinii* strain G1 (does not express any of these CRASP proteins during laboratory cultivation) was chosen for functional analyses of the CRASP-4 protein [34]. *B. garinii* G1 was transformed with the plasmid pCRASP-4, which harbors the entire CRASP-4 encoding *erpC* gene under the control of its native promotor, and with the empty shuttle vector pKFSS1. Transformants selected by the microdilution method were confirmed by PCR amplification of the CRASP-4 encoding *erpC* and the streptomycin resistance *aadA* gene (Figure 3(a)). Strain G1/pCRASP-4 yielded an amplicon corresponding to *erpC*, whereas the control strains G1 and G1/pKFSS1 did not. The *aadA* gene of the shuttle vectors was detected in the transformed cells, but not in the wild-type strain G1. Production of CRASP-4 in *B. garinii* G1 was verified through analysis of cell lysates from the CRASP-4 expressing cells and the nonexpressing control strains G1 and G1/pKFSS1 (Figure 3(b)).

3.3. Surface Exposure of CRASP-4 in B. garinii G1/pCRASP-4. CRASP-4 and other members of the Erp paralogous protein family are surface exposed proteins [44]. To confirm surface-exposure of these proteins in transformed *B. garinii*, intact spirochetes were treated with proteinase K and trypsin, followed by ligand affinity blotting of borrelial lysates (Figure 3(c)). Analyses of protease-treated cells revealed that CRASP-4 was highly susceptible to digestion by proteinase K but not trypsin, as previously described for the native protein [44]. Surface localization of CRASP-4 was also examined by immunofluorescence microscopy using live bacteria and polyclonal antibodies specific for CRASP-4 [44]. To avoid damage to the fragile borrelial outer membrane, intact bacteria were incubated with antibodies before fixation onto glass slides and sealed with mounting medium containing the DNA-binding dye DAPI. As shown in Figure 3(d), CRASP-4 positive cells showed a strong fluorescent staining, thus indicating that CRASP-4 was localized on the outer membrane. Integrity of the fragile borrelial outer membrane was confirmed by the lack of binding of antibodies directed against the periplasmic flagellar protein FlaB (Figure 3(d)). Control strains G1 or G1/pKFSS1 did not display fluorescence reactivity with the CRASP-4 antiserum.

3.4. Binding of Human Serum Proteins by B. garinii G1/pCRASP-4. Having demonstrated binding of CFHR1, CFHR2 and CFHR5 to recombinant CRASP-4, we next examined whether live G1/pCRASP-4 cells also bind the human complement regulators. To this end, serum-resistant *B. burgdorferi* LW2 (positive control), serum-sensitive *B. garinii* G1 (negative control), and transformants G1/pKFSS1 and G1/pCRASP-4 were incubated in NHS supplemented with EDTA (to prevent complement activation). After serum incubation, the final wash and elute fractions were separated by SDS-PAGE and subjected to Western blotting with a polyclonal antiserum that recognizes CFH and the CFH-related proteins CFHR1, CFHR1α, CFHR1β, CFHR2, CFHR2α, and CFHR5 (Figure 4). Serum-resistant *B. burgdorferi* LW2 bound CFH, CFHR1α, CFHR1β, CFHR2, and CFHR2α. In contrast, wild-type strain G1 and transformant G1/pKFSS1 did not bind CFH or any CFH-related proteins. Four prominent bands with apparent masses of 35, 32, 25 and 22 kDa were detected in the last wash and the eluate fraction of G1/pCRASP-4. Based on their mobilities, the 35 and 32 kDa proteins most likely correspond to the two glycosylated forms CFHR1α and CFHR1β. The 25 and 22 kDa bands were probably the nonglycosylated and the glycosylated forms of CFHR2. A barely visible band with an apparent molecular mass of 55 kDa could only be detected in the eluate fraction of G1/pCRASP-4, which was probably CFHR5. The bands with molecular masses of 40, 60 and >250 kDa seen in the eluate fractions of all strains represent unspecific binding of the antiserum. There was not any indication of binding the 150 kDa CFH protein. Taken together, CRASP-4 produced on the surface of live *Borreliae* strongly binds the human serum proteins CFHR1 and CFHR2, lesser amounts CFHR5 but no detectable CFH.

3.5. Serum Susceptibility of B. garinii Producing Surface-Localized CRASP-4. To define the roles of CFHRs and CRASP-4 in the complement resistance of *Borreliae*, a growth inhibition assay was used to examine the ability of transformant G1/pCRASP-4 to survive in the presence of complement active NHS (Figure 5). As expected, growth of *B. burgdorferi* LW2

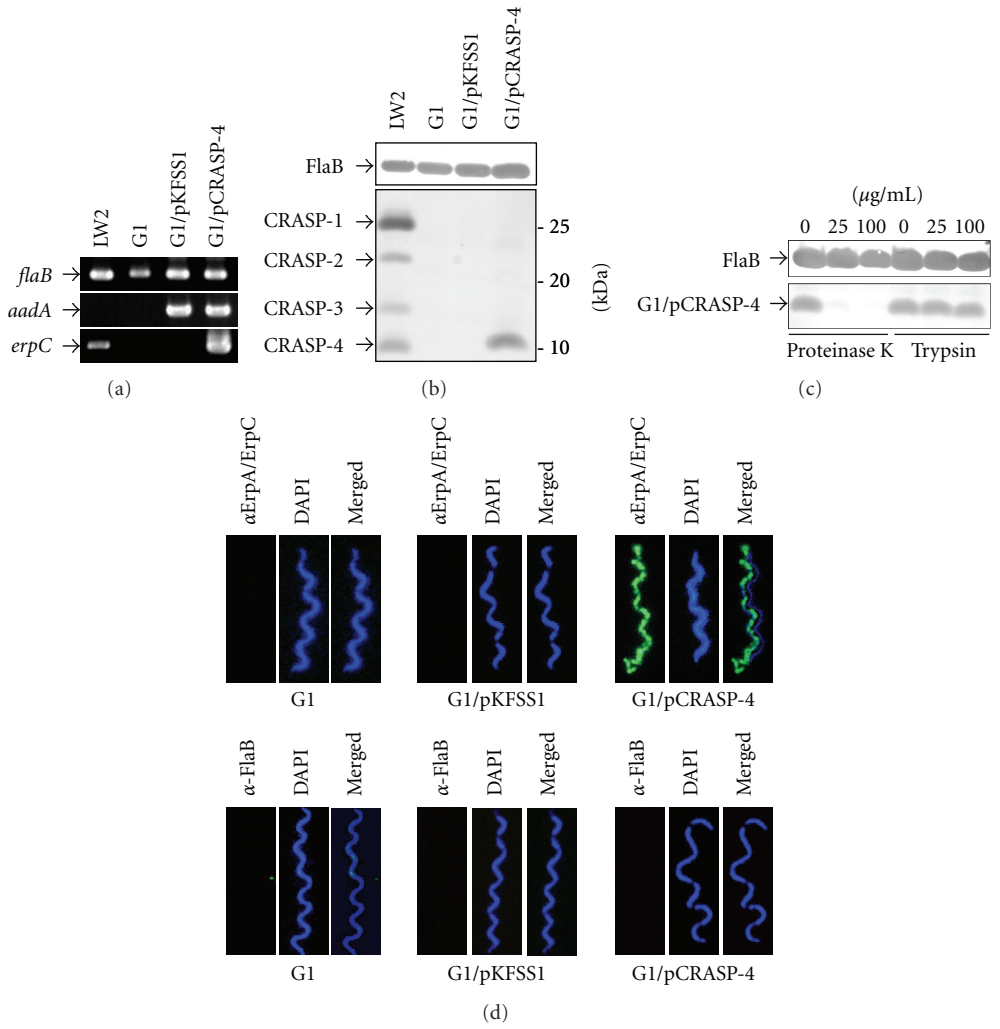

Figure 3: Characterization of *B. garinii* G1 producing CRASP-4. (a) *B. garinii* G1 and transformed strains G1/pKFSS1 and G1/pCRASP-4 were characterized by PCR amplification using *flaB*-, *aadA*-, and *erpC*-specific primers, as listed in Table 1. (b) Synthesis of CRASP-4 by transformed G1 was assessed using ligand affinity blotting. Whole cell lysates (15 μg each) of G1, G1/pKFSS1 and G1/pCRASP-4 were separated by SDS-PAGE, and transferred to nitrocellulose. After incubation with NHS, binding of CFH to CRASP-4 was identified using a polyclonal antiserum. A monoclonal antibody, L41 1C11, specific for the flagellin protein FlaB, was applied to show equal loading of borrelial lysates. (c) Surface localization of CRASP-4 in transformed G1 cells. Spirochetes were incubated with or without proteinase K or trypsin, respectively, then lysed by sonication, and total proteins were separated by SDS-PAGE. CRASP-4 was identified by ligand affinity analysis as described above. Flagellin (FlaB) was detected with MAb L41 1C11 (dilution 1/1000) by Western blotting. (d) Demonstration of surface expression of CRASP-4 by transformed *B. garinii* G1, by indirect immunofluoresecence microscopy of intact borrelial cells. Spirochetes were incubated with rabbit polyclonal anti-ErpA/ErpC antiserum before fixation. Periplasmic FlaB, used as control, was detected by mAb L41 1C11 using fixed and unfixed cells. For counterstaining, the DNA-binding dye DAPI was used to identify all bacteria. Slides were visualized at a magnification of $\times$1,000 using an Olympus CX40 fluorescence microscope mounted with a DS-5Mc charge-coupled device camera (Nikon).

included as control was unaffected, as indicated by a continuous decrease of the absorbance values (due to the colour change of the medium). In contrast, wild-type strain G1, G1/pKFSS1, and G1/pCRASP-4 survived in heat-inactivated NHS but not in native NHS. The failure of the CRASP-4 producing transformant to survive suggests that binding of CFHR1, CFHR2, and CFHR5 is not sufficient for mediating complement resistance.

Next we examined deposition of complement activations products C3, C6 and the membrane attack complex (MAC) on the surface of the transformant G1/pCRASP-4, *B. burgdorferi* LW2, and *B. garinii* G1. Following incubation in NHS, the majority of cells of G1/pCRASP-4 and wild-type strain G1 displayed strong fluorescence, suggesting that large amounts of C3, C6, and MAC were deposited on the borrelial cell surface (Figure 6). In addition, extensive bleb formation, cell fragmentation, and lack of DAPI staining indicate that spirochetes were lysed. In contrast, bacteria incubated with heat-inactivated NHS did not show evidence of complement deposition (data not shown).

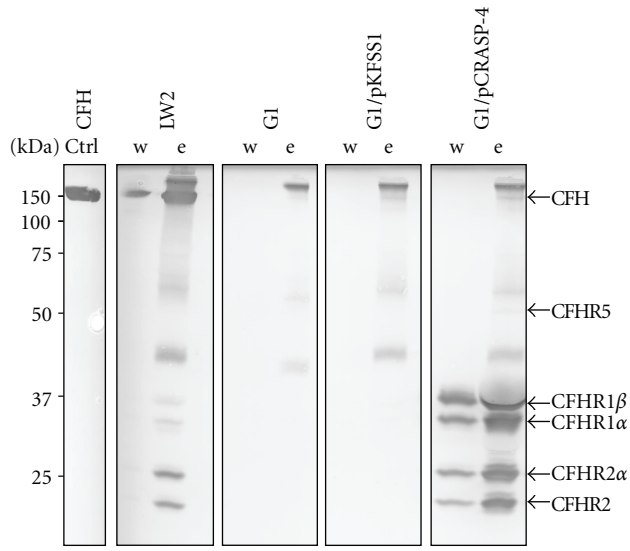

Figure 4: Binding of serum molecules by *B. garinii* transformants. *B. garinii* strains G1, G1/pKFSS1, and G1/pCRASP-4 and *B. burgdorferi* strain LW2 (used as control) were incubated in NHS plus EDTA to prevent complement activation and washed extensively, and bound proteins were eluted using 0.1 M glycine (pH 2.0). Both the last wash (w) and the eluate (e) fractions obtained from each strain were separated by SDS-PAGE and transferred to nitrocellulose. As an additional control purified CFH (1 μg) was also applied. Membranes were probed with a polyclonal anti-FHR1 antiserum which recognizes CFH, CFHR1, CFHR2, and CFHR5. Mobilities of molecular mass standards are shown to the left of the panels.

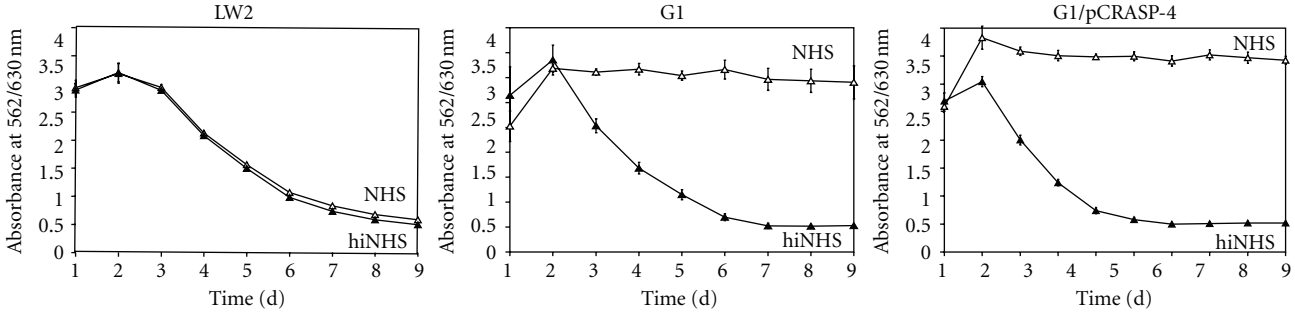

Figure 5: Serum susceptibility of transformed *B. garinii* G1. A growth inhibition assay was used to investigate susceptibility to human serum of *B. burgdorferi* strain LW2 and *B. garinii* strains G1 and G1/pCRASP-4. Spirochetes were incubated in either 50% NHS (open triangles) or 50% heat-inactivated NHS (filled triangles) over a cultivation period of 9 days at 33°C, respectively. Color changes were monitored by measurement of the absorbance at 562/630 nm. All experiments were performed three times during which each test was done at least in triplicate with very similar results. For clarity only data from a representative experiment are shown. Error bars represent ± SD.

Taken together, binding of CFHRs by CRASP-4 producing spirochetes does not sufficiently protect spirochetes from complement-mediated killing.

4. Discussion

To survive in different compartments and persistently infect their potential hosts, *Borreliae* have developed a variety of strategies that include overcoming destructive attacks by host complement. In particular, serum-resistant *B. burgdorferi*, *B. afzelii*, and *B. spielmanii* isolates bind the human fluid phase complement regulators CFH and FHL1 that allow spirochetes to finely regulate and inhibit complement activation on their cell surface [6, 7, 51]. In this study, we extend the characterization of molecular interaction of CFH/CFHR proteins and show that the infection-associated CRASP-4/ErpC protein of *B. burgdorferi* binds the host complement regulators CFHR1, CFHR2, and CFHR5, and to some extent CFH. However, CRASP-4 exposed to the outer surface of viable cells preferentially binds complement regulators CFHR1α, CFHR1β, CFHR2, and CFHR2α.

CFHR1 and CFHR5, and likely also CFHR2, exhibit complement regulatory activities. Thus, recruitment of these host proteins may help spirochetes to control complement activation. In agreement with our earlier observations of the interaction of CFH with native CRASP-3 and CRASP-5, the data presented herein showed that CFHR1, CFHR2, and CFHR5 alone or in concert are not sufficient to control complement activation at the borrelial surface. CFHR1 and CFHR2 are major constituents of serum lipoprotein particles that also contain apolipoprotein A-I, lipopolysaccharide-binding protein, phospholipids, and fibrinogen [52, 53]. Thus, it

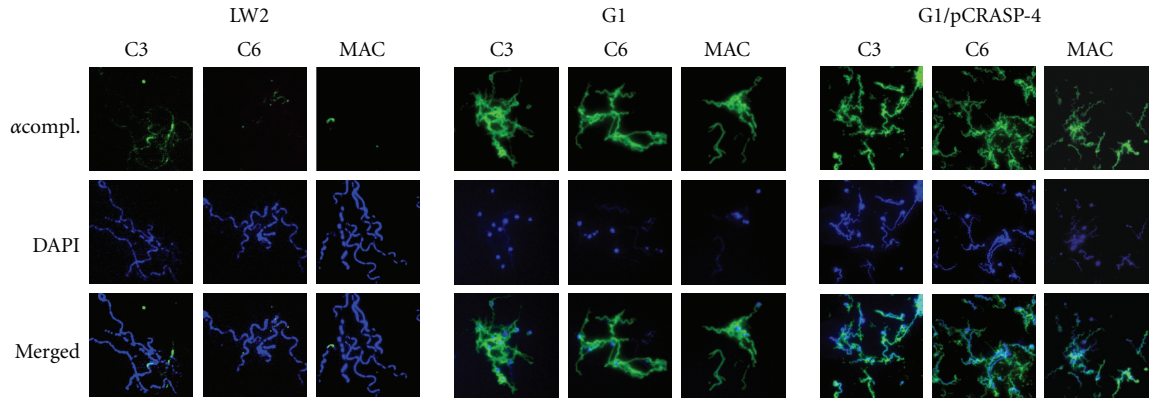

Figure 6: Deposition of complement components C3 and C6, and MAC on the surface of borrelial strains. Deposition of complement components on *B. burgdorferi* LW2 (control strain), *B. garinii* G1 and transformant G1/pCRASP-4 were detected by indirect immunofluorescence microscopy. Spirochetes were incubated with 25% NHS. Bound C3, C6, or MAC was detected using specific antibodies against each component plus appropriate Alexa-488-conjugated secondary antibodies. For visualization of intact spirochetes, the DNA-binding dye DAPI was used. Slides were visualized at a magnification of ×1,000 and the data were recorded via a DS-5Mc CCD camera (Nikon) mounted on an Olympus CX40 fluorescence microscope. Panels shown are representative of at least 20 microscope fields.

could be speculated that Lyme disease *Borreliae* capture lipoprotein particles through CFHR1 and CFHR2 to allow adherence to host epithelial cells and tissues, as has been described for CFH-coated *Streptococcus pneumoniae* [54].

Interaction with CFH has previously been reported for CRASP-4/ErpC and other closely related Erp proteins, for example, OspE paralogs from *B. burgdorferi*, *B. afzelii*, *B. spielmanii*, *B. garinii*, *B. lusitaniae*, *B. turdi*, *B. tanukii*, and *B. japonica* [3, 6, 11, 12, 33, 35, 37, 38, 43, 51, 55–58]. Here we demonstrate that recombinant CRASP-4 bound CFH in ELISA or ligand affinity blot experiments using borrelial cell lysates (Figures 2 and 3(b)). However, binding of CFH could not be detected if CRASP-4 was coated onto magnetic particles or was expressed on the surface of transformed borrelial cells (Figures 1(a) and 1(b) and Figure 4). Previous studies using surface plasmon resonance revealed that CRASP-4 in comparison to CRASP-3 and CRASP-5 displayed strong affinity for CFHR1 and the lowest binding affinity to CFH, suggesting a preferential binding to the smaller CFHR molecules [38]. CRASP-3 and CRASP-5, when heterologously produced in *B. garinii* G1 or in a high-passaged mutant strain B313 (a derivative of type strain *B. burgdorferi* B31 that carries only one copy of the CRASP-5-encoding *erpA* gene), similarly did not bind CFH [31, 39, P. Kraiczy unpublished data]. However, we cannot completely exclude conformational changes of surface-exposed CRASP-4 due to misfolding in *B. garinii*. Furthermore, heterologous production of the two CFH/FHL1-binding CRASP proteins CspA or CspZ in *B. garinii* G1 did not influence their functional activity to interact with CFH and FHL1, which might also argue for correct folding of borrelial proteins in this model organism [42, and P. Kraiczy unpublished data]. Conceivably, the stronger affinity of CRASP-4 to CFHR1 and CFHR2 may also favor preferential binding of these molecules to borrelial cells (Figure 4), even though CFH is present in a 10-fold higher concentration in human plasma than both CFHRs [38, 59]. As demonstrated earlier and in the present study, the individual CFH/CFHR-binding CRASP proteins (recombinant or native) possess different relative affinities for CFH, CFHR1, CFHR2, and CFHR5 [38, 39, 59]. When expressed on borrelial surfaces, none of those CRASPs bound CFH, but they did show prominent binding to CFHR1 and CFHR2. Binding of CFHR5 was more pronounced for CRASP-3 as compared to CRASP-4 and CRASP-5. Collectively, all three CRASPs displayed the strongest affinities for CFHR2.

Apparently as a consequence of the inability of CFH to bind to the microbial surface, bacteria accumulated destructive complement activation products, that is, C3 and MAC, on their surfaces and were killed (Figures 5 and 6). Displacement of CFH by CFHR1 or CFHR2, which exhibits sequence identities of 89 and 61% to the C-terminal SCRs 19 and 20 of CFH, respectively, or improper binding of CFH to CRASP-4 by other yet unknown factors may have led to that phenomenon. Once complement is activated, it appears that the inhibitory activity of CFHR1 on the C5 convertase and the capacity of CFHR5 (although bound in minuscule amounts on the bacterial surface) to inactivate C3b can not completely impede formation and insertion of the MAC, in particular when large amounts of C3b and downstream effector complement components are deposited on the bacterial membrane (Figure 6). This points to a crucial role of human CFH and FHL1 in complement resistance of *Borreliae*.

A CspA-deficient *B. burgdorferi* strain that carries two native copies of the *erpA* gene did not survive in human serum, indicating that CRASP-5 alone cannot sufficiently protect Lyme disease *Borreliae* from complement-mediated killing [40, 41]. However, heterologous production of CRASP-3 and CRASP-5 in the same CspA-deficient strain significantly increased spirochetal survival in the presence of 20% human serum, suggesting that both proteins exhibit a synergistic effect on complement resistance [43]. However, mutant strain B313, which lacks CRASP-1, -2, and -3, but produces native CRASP-4 and -5, did not bind CFH and was highly susceptible to complement-mediated killing by 50%

human serum (data not shown). Mutant strain B313 is a clonal mutant of B31 that lacks all that strain plasmids except cp32-1, cp32-2, cp32-3 cp32-4, cp26, and lp17 and therefore is unable to produce a variety of outer surface proteins, such as the major surface proteins OspA and OspB [60, 61]. The absence of a high number of outer surface proteins might influences the entire membrane composition and, thus, might effects the functional properties of these CFHR-binding CRASPs in the mutant strain B313. Conceivably, other proteins that are absent in B313 might serve as bystanders to promote optimal binding of the large CFH protein (which forms dimeric or oligomeric complexes in solution at physiological concentrations) to CRASP-3, CRASP-4, and CRASP-5.

Taken together, we identified complement proteins CFHR2 and CFHR5 as novel ligands for the infection-associated CRASP-4/ErpC protein of *B. burgdorferi*. CRASP-4 exposed to the borrelial surface preferentially binds CFHR1 and CFHR2 while binding of CFH and CFHR5 could only be detected under artificial experimental conditions. Although binding of CFHRs appears to be not necessary for complement resistance, the impact of these particular host proteins for immune evasion and pathogenesis of *Borreliae* warrants further investigations.

Acknowledgments

The authors thank J. Günnewig for skillful and expert technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft DFG, Project Kr3383/1-2. This work forms part of the doctoral thesis of C. Hammerschmidt.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 595427, 13 pages
doi:10.1155/2012/595427

Research Article

Evaluating Frequency, Diagnostic Quality, and Cost of Lyme Borreliosis Testing in Germany: A Retrospective Model Analysis

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Received 8 July 2011; Accepted 8 September 2011

Academic Editor: Piotr Lewczuk

Background. Data on the economic impact of Lyme borreliosis (LB) on European health care systems is scarce. This project focused on the epidemiology and costs for laboratory testing in LB patients in Germany. *Materials and Methods*. We performed a sentinel analysis of epidemiological and medicoeconomic data for 2007 and 2008. Data was provided by a German statutory health insurance (DAK) company covering approx. 6.04 million members. In addition, the quality of diagnostic testing for LB in Germany was studied. *Results*. In 2007 and 2008, the incident diagnosis LB was coded on average for 15,742 out of 6.04 million insured members (0.26%). 20,986 EIAs and 12,558 immunoblots were ordered annually for these patients. For all insured members in the outpatient sector, a total of 174,820 EIAs and 52,280 immunoblots were reimbursed annually to health care providers (cost: 2,600,850€). For Germany, the overall expected cost is estimated at 51,215,105€. However, proficiency testing data questioned test quality and standardization of diagnostic assays used. *Conclusion*. Findings from this study suggest ongoing issues related to care for LB and may help to improve future LB disease management.

1. Introduction

Lyme borreliosis (LB) is a vector-borne disease that is transmitted by ixodid ticks and is caused by the spirochetes of the *Borrelia (B.) burgdorferi* sensu lato (s.l.) complex. The 5 genospecies that are currently considered to be human pathogens are *B. burgdorferi* sensu stricto (s.s.), *B. afzelii*, *B. garinii*, *B. spielmanii*, and the proposed but not yet validated novel species *B. bavariensis* [1]. Over the last 20 years, LB has been recognized as a major public health problem in the United States and in Europe [2, 3]. Part of this status is related to variation in symptoms and the clinical picture of the disease [4]. In addition, further insecurity exists with the differential diagnostic considerations in LB patients, the natural trajectory of treated and untreated LB, and interpretation of diagnostic test results [2, 5, 6]. In Europe, the incidence of Lyme borreliosis is estimated to range from 0.6 to 155/100,000 [4, 7]. In Germany, the estimation of 60,000–100,000 incident cases per year is based on an older seroprevalence study in a single region [8]. Mandatory reporting of new LB cases was established in 2002, but only in the six new federal states of the northeastern part of

Germany. About 5,221 new cases of LB are reported for these states per year [9]. Little is known about the true incidence and distribution of LB in the other parts of Germany.

LB can manifest itself progressively as a multisystem disorder exhibiting a broad spectrum of clinical symptoms [4, 10]. The disease is usually diagnosed clinically based on a characteristic clinical picture, a history of tick bite, and the diagnosis then can be supported further by serological testing. However, both false negative and false positive serologic test results do occur, and together with a lack of standardization of current diagnostic methods can clearly impede a clear and concise diagnosis [11]. Moreover, current law in most European countries does not require profound clinical evaluation of such commercially available diagnostic test kits for LB prior to market registration. Most significant, however, is the high seroprevalence of anti-*B. burgdorferi* antibodies that correlates with manifest disease in only a minority of patients. Therefore, serology should only be used to confirm but not to primarily establish the diagnosis of LB. In addition, the antibody titers on followups do not reflect the success of antibiotic treatment [12]. These factors can lead to misdiagnosis and mismanagement of LB and patients after tick bites and/or unspecific symptoms. As reported in the US, these events may lead to inappropriate care for patients including adverse effects and unnecessary financial cost [2, 5].

The situation in Germany is less obvious. Currently, little data is available on health care utilization in Germany for patients with confirmed or suspected LB such as performance of diagnostic and therapeutic measures including those for unspecific chronic conditions which are attributed to LB by patients or their physicians. The aim of the German investigation on Lyme borreliosis: evaluation of therapeutic and diagnostic cost (GILEAD) study is a step-wise analytic approach to estimate the amount of diagnostic testing, assay quality, and cost related to manifest and suspected LB in Germany. In this analysis, we explore the relative frequency of diagnostic testing, the number of incident and prevalent diagnoses, and the cost of laboratory diagnostics by analyzing German health insurance claims' data. In addition, we performed a meta-analysis of results obtained during the regular German LB serology proficiency testing program to learn more about the accuracy and reliability of currently used serological tests in Germany. This was done to evaluate the hypothesis that, although guidelines for the diagnostic management of LB with well-defined diagnostic algorithms for suspected LB cases have been established in Germany [13–15], relatively high volumes of diagnostic efforts (i.e., serologic testing) related to LB are being performed repeatedly without necessarily adding any benefit to the management of such patients.

2. Materials and Methods

2.1. Analysis of Health Insurance Datasets. The basic dataset consists of health insurance data from a German statutory health insurance company (Deutsche Angestellten-Krankenkasse, DAK) which covers approx. 6.04 million individuals all over Germany. The population insured by DAK consists of more women than men (ratio 1.8 : 1). In a first step, relevant international classification of diseases (ICD 10-GM, 2004) diagnoses for Lyme borreliosis were defined as follows: ICD A69.2 for Lyme-specific erythema chronicum migrans, GO1* for LB-related meningitis, G63.0 for LB-related polyneuropathy, and M01.2 for LB-related Arthritis. Claims' data of the years 2007 and 2008 were derived from the underlying datasets (patient data, ambulatory treatment data, and medication data). In accordance with legal data protection requirements, all personal data were exclusively handled by DAK. Analyses were based on subject-specific data which did not allow the identification of individual persons. Informed consent is not required for these analyses in Germany. The quality of the data was checked for completeness, correct usage of inclusion criteria, and plausibility prior to analysis according to existing standards [16, 17]. Individuals insured at least since January 1, 2006, or January 1, 2007, respectively, in whom at least one laboratory diagnostic procedure performed for LB in either year 2007 or 2008, were included in our analyses. The diagnostic procedures according to the general laboratory health insurance claim code ("Einheitlicher Bewertungsmassstab", EBM [18]) included laboratory claim numbers 32586 (*B. burgdorferi* antibody/enzyme-linked immune assay, ELISA), 32662 (*B. burgdorferi* antibody/western blot), and/or 32743 (culture of *B. burgdorferi*). Individuals already having a coded diagnosis of Lyme borreliosis in 2006 were excluded from the analysis. We also extracted patient data (subject specifier, gender, year of birth, code for current residence, date of begin and end of insurance), ambulatory treatment data (subject specifier, quarter of the year, start and end of treatment period, diagnoses and diagnoses' assurance level, EBM codes, EBM date, area of specialty of physician), and medication data (subject specifier, date of prescription, agent, amount prescribed, cost).

2.2. Collecting Data from the German Lyme Disease Proficiency Testing Program. From March 2006 to November 2008, six LB serology proficiency testing surveys were conducted in Germany by the central reference laboratory for bacteriologic serodiagnostics of the Bacteriologic Infection Serology Study Group of Germany (BISSGG) now situated at the Institute of Laboratory Medicine, Northwest Medical Centre, Frankfurt/Main, in cooperation with the WHO Collaborating Centre for Quality Assurance and Standardization in Laboratory Medicine e.V. (INSTAND e.V.), Düsseldorf, and with the 9 reference laboratories of the BISSGG. The organisation and structure of the German proficiency testing program for bacteriologic infection serology is summarised elsewhere in more detail [11, 19, 20].

2.3. Selection of Serum Samples. Twelve serum samples were obtained from voluntary donors according to previously published strict criteria and after obtaining written informed consent [11, 19]. All subjects were clinically evaluated by experienced physicians. Six serum samples contained specific antibodies against *B. burgdorferi* as determined by various commercial test systems. All antibody-positive donors could recall a known history of a recent tick bite or active or past

LB, which also had been documented in the medical records of these patients by their physicians. Six samples tested negative for specific antibodies against *B. burgdorferi* and were used as negative controls. Current or very recent LB was excluded in these donors by careful physical examination, evaluation of patients' medical histories, and review of the medical records provided by the referring physicians. Two of the six negative samples contained anti-*T. pallidum* antibodies and were obtained from a donor with a past syphilis infection and a donor eight weeks after appropriate therapy. Table 1 provides a detailed description of the clinical data available for all twelve samples.

2.4. Study Conditions and Evaluation of Proficiency Testing Results. Assessment of reference test results was performed according to the current guidelines of the German Medical Association and the standard operating procedures of INSTAND for proficiency testing in infection serology as recently described in more detail [11, 19]. Qualitative and quantitative reference test results (Table 1) were determined for each pair of serum samples by 3 to 9 different specialised laboratories or university laboratories of the BISSGG with extensive expertise in the field of serodiagnostic testing for LB. Participation in the LB serology proficiency testing programs was not mandatory, but participating laboratories were required to register at INSTAND prior to their involvement. No preexisting criteria were established to exclude any laboratories from the survey. All participants were instructed to treat samples as routine samples and to perform their established serological test methods on the distributed samples blinded to additional clinical information to guarantee maximum objectivity. Qualitative and quantitative results had to be reported together with the methods used, the lot number and test manufacturer, and the laboratory machinery utilized [19]. Moreover, the laboratories reported interpretative statements as to whether the test constellation suggested a possible *Borrelia* infection and whether an early or late phase of infection was suspected. Reports were made in standardised form on defined evaluation sheets by use of a predefined code to permit statistical analysis after the surveys [19]. Only one test result per test method (indirect immune fluorescence assay: IFA, indirect hemagglutination assay: IHA, enzyme Immunoassay: EIA, chemoluminescence assay: CLIA, line blot, immunoblot, etc.) was reported to INSTAND by each participant. Participants were requested to return their reports to INSTAND for further computer-assisted evaluation of results within 10 days after receipt of samples [11, 19]. Qualitative results from participants were accepted as being *accurate* if their reported test results were congruent with the modal as determined by the reference laboratories (Table 1; Figure 1). Because the quantitative EIA results reported were so heterogeneous (Figure 2) owing to the different quantification methods of the test manufacturers, these results were not included in the evaluation listed below. Quantitative results of classical titre tests (IHA, IFA) were accepted as being *accurate* provided that results from participants were reported within a range of $\pm 2 \log_2$ unit dilutions around the median of the test results obtained by the reference laboratories. A qualifying certificate was forwarded to successfully participating laboratories for each parameter under the condition that their microbiological commentary, and qualitative and quantitative test results, for both samples using established assay systems met the above-listed criteria [11, 19].

2.5. Statistical Analysis. All included claims' data were analyzed both within the entire group of individuals and within the group of patients with a coded incident diagnosis of A69.2 in the year 2007 or 2008. To avoid misrepresentation, the population was analysed by age and gender and standardized with the general population of Germany (according to "Empfehlungen der Ersatzkassen und ihrer Verbände zur Umsetzung des §20 SGB V"). The numbers of all insured individuals were provided by gender, 5-year age groups, and 5-digit residence codes and used to compare to the group with a coded diagnosis of LB and/or a borreliosis test. Data analyses were descriptive and stratified by sex and standardized by age. Counts and incidence rates for LB-related diagnostic testing were calculated. All data including proficiency testing results were reported as absolute numbers, means, modals, and percentages including standard deviations (SDs) as indicated and helpful (Figure 1). In addition, mean pass rates (Table 2; Figure 1) were calculated from the specific pass rates of the individual surveys performed biannually.

3. Results

3.1. Results from the German Proficiency Testing Program. From March 2006 to October 2008, between 360 and 392 microbiological laboratories (mean: $N = 381$, SD = 11), including hospital laboratories, independent laboratories, physicians' office laboratories, and manufacturers of commercially available diagnostic LB assays took part in each of the six surveys that were held. Tests employed were those used routinely for the serodiagnosis of LB in the participating laboratories. Figure 1 gives an overview on the frequency of the various test systems used by the participants during the surveys. The laboratories most frequently performed a two-tier protocol, beginning with a sensitive EIA or CLIA screening (mean: $N = 312$ (81.9%), SD = 6.9) followed by immunoblot or line blot confirmation of the results (mean: $N = 282$ (74.0%), SD = 9), in compliance with the current recommendations of the CDC and most European scientific expert opinions [13, 21, 22]. On average, for direct immunoglobulin class-specific analysis of samples, IgG- and/or IgM-EIA were used by 259 (SD = 6) and 298 (SD = 9) of the participants, respectively, during the six surveys. An immunoblot confirmatory assay for IgG- and/or IgM-antibodies was performed by 239 (SD = 6) and 238 (SD: $N = 7$) of the laboratories, respectively. Some other traditional or more recently introduced serological test methods were employed much more rarely: IHA, mean: $N = 15$ (3.9%), IFA, mean: $N = 23.5$ (6.2%), polyvalent EIA, mean: $N = 33$ (8.7%), CLIA, mean: $N = 33$ (8.7%). Interestingly, there was a steady increase for new recombinant tests or hybrid assays (using native and/or recombinant protein preparations) and

Table 1: German LB proficiency testing program: characteristics of selected serum samples as determined by the reference laboratories of the BISSGG.

Sample	PHA	EIA (poly valent)	IFA-IgG	IFA-IgM	EIA-IgG	EIA-IgM	CLIA-IgG	CLIA IgM	Line blot	Immuno-blot IgG	Immuno-blot IgM	Clinical information (time of sampling after therapy)
31/2006	N (<80)	N	N (<40)	N (<20)	N	N	N	N	N	N	N	Syphilis stage I (4 yrs)
32/2006	P (1280)	P	P (320)	B/P (20)	P	B/P	P	B/P	P	P	B/P	Recent Lyme arthritis (1 yr)
61/2006	N (<80)	N	N (<40)	N (<20)	N	N	N	N	N	N	N	Healthy blood donor
62/2006	P (320)	P	B/P (80)	P (320)	B/P	P	P	P	P	B/P	P	Morbus Bannwarth (1.5 mo)
21/2007	P (160)	B/P	B/P (80)	N/B/P (≤40)	B/P	N/B/P	B/P	N/B/P	B/P	B/P	B/P	Seropositive but asymptomatic donor, several tick bites in recent medical history
22/2007	N (<80)	N	N (<40)	N (<20)	N	N	N	N	N	N	N	Healthy blood donor
61/2007	N (<80)	N	N (<40)	N (<20)	N	N	N	N	N	N	N	Syphilis stage II (8 weeks after treatment)
62/2007	P (1280)	P	P (320)	N (<20)	P	N	P	N	P	P	N/B	Seropositive but asymptomatic donor, several tick bites in recent medical history
31/2008	N (<80)	N	N (<40)	N (<20)	N	N	N	N	N	N	N	Healthy blood donor
32/2008	P (320)	P	P (160)	P (80)	P	P	P	P	P	P	P	Morbus Bannwarth, CSF pleocytosis, specific CSF antibody index (AI): 6.3
61/2008	P (1280)	P	P (160)	N/B (≤20)	P	N	P	N	P	P	N	Lymphocytoma (2 mo)
62/2008	N (<80)	N	N (<40)	N (<20)	N	N	N	N	N	N	N	Healthy blood donor

* Legend: P, positive, B, borderline, and N, negative. For some samples, combinations (i.e., N & B, B & P, or N/B/P) were accepted. Median titers as determined by the reference laboratories are given in parentheses.

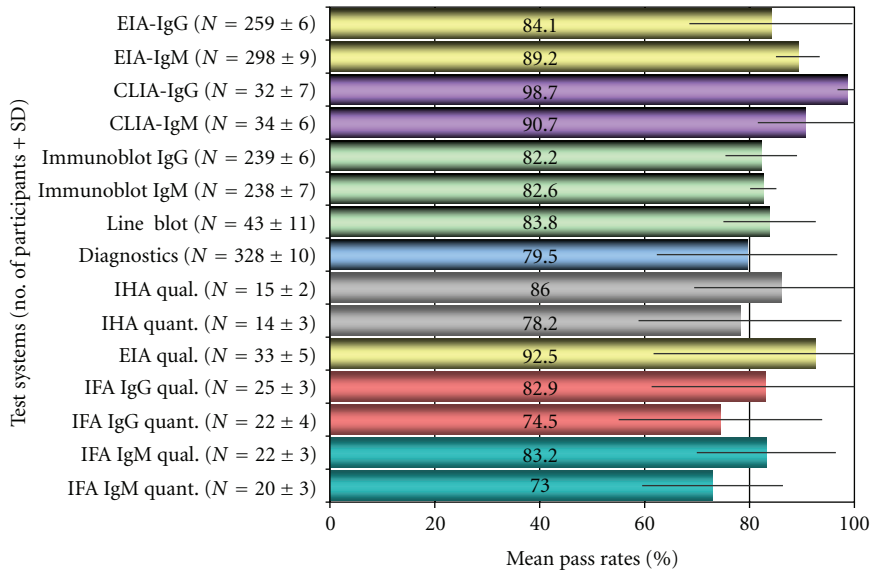

Figure 1: Average number of participants and mean pass rates (%) with standard deviations (bars) for different assay systems as observed between 2006 and 2008 in the German LB proficiency testing program.

Table 2: Mean pass rates of proficiency testing participants in regard to samples tested and assay system used.

Sample	IHA		Polyvalent EIA	IgG-EIA	IgM-EIA	IgG-IFA		IgM-IFA		IgG Blot	IgM blot	Line blot	IgG-CLIA	IgM-CLIA	Diagnostic Comment
Positive samples	Qual.	Quant.	Qual.	Qual.	Qual.	Qual.	Quant.	Qual.	Quant.	Qual.	Qual.	Qual.	Qual.	Qual.	Qual.
32/2006	100	88.9	95.5	97.0	90.7	93.5	93.5	64.3	61.5	97.1	95.1	97	95.7	11.5	98
62/2006	66.7	76.9	82	83.3	92.0	88.0	85.7	90.9	78.9	89.2	89	83.3	100	71.4	88.7
21/2007	93.3	82.4	90.9	96.1	95.7	79.3	80.8	100	100	93.9	89.4	94.1	96.8	100	51.7
62/2007	85.7	66.7	91.4	99.2	93.7	95.8	81.8	71.4	75	100	84.9	96.9	100	91.2	97.7
32/2008	78.6	78.6	96.3	97	91.4	95.7	81	80	75	88	88.5	96.2	100	100	71.0
61/2008	100	100	96.2	99.6	81.4	90.9	77.3	89.5	78.9	100	87.7	96.7	100	91.8	96.5
Mean	**87.3**	**82.3**	**92.1**	**95.4**	**90.8**	**90.5**	**83.4**	**82.7**	**78.2**	**94.7**	**89.1**	**94.0**	**98.8**	**77.7**	**83.9**
Negative samples	Qual.	Quant.	Qual.	Qual.	Qual.	Qual.	Quant.	Qual.	Quant.	Qual.	Qual.	Qual.	Qual.	Qual.	Qual.
31/2006	94.1	88.9	95.9	93.9	95.5	93.5	93.5	85.7	84.6	97.9	94.6	93.9	100	100	98.3
61/2006	100	100	99.6	100	93.4	92.0	90.5	95.5	94.7	96.1	95.6	100	100	96.4	99.4
22/2007	100	100	87.9	88.8	93.7	62.1	53.8	92.6	92	84.6	90.2	75	100	100	92.4
61/2007	69.2	71.4	11.8	53.5	94.1	41.7	40.9	95.2	95	77.7	96.9	87.9	100	91.2	87.4
31/2008	100	100	96.2	98.9	96.4	91.3	90.5	100	95	94.4	98.3	100	100	97.4	99.4
62/2008	100	100	96	96.8	97.1	86.4	81	100	94.4	93.8	98.2	91.7	100	93.9	98.1
Mean pass rates	**93.8**	**93.3**	**81.2**	**88.6**	**95.0**	**77.8**	**75.0**	**94.8**	**92.6**	**90.7**	**95.6**	**91.4**	**100**	**96.5**	**95.8**

new analytical test formats such as CLIA and line blots, from 5.8% and 8.5% in 2006 to 10.5% and 16.4% in 2008 (data not shown).

3.2. Accuracy of Test Results. Characteristics of the selected serum samples applied in the German LD proficiency testing program as determined by the nine reference laboratories are depicted in Table 1. The percentages of laboratories that reported correct results with their routinely applied assay systems on the twelve serum samples sent out for testing in the six surveys of the German LD proficiency testing program from 2006–2008 are summarised in Table 2 and Figure 1, as individual pass rates per method and sample or as mean pass rates over time. IgG tests (mean pass rate: 92.1%, range 41.7 to 100%) were only slightly more accurate than those for IgM tests (mean pass rate: 90.3%, range: 11.5 to 100%). Mean pass rates for immunoblot testing (mean pass rates: 92.6%, range: 75 to 100%) were less accurate than those for

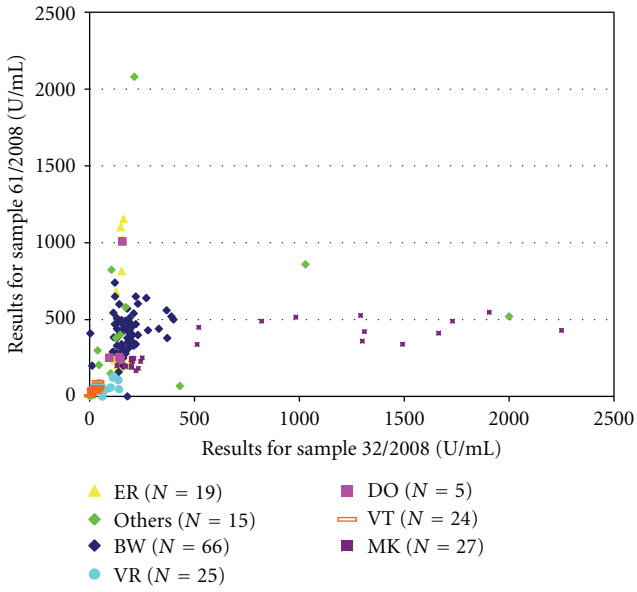

Figure 2: Youden's plot of different quantitative IgG-EIA results (U/mL) as obtained in two samples (no. 32 and 61) used for LB proficiency testing in 2008. For clinical information of samples, see Table 1. (ER, BW, DO, MK, VR, VT, others: anonymized abbreviations of different commercial EIA manufacturers).

EIA-testing (EIA mean pass rates: 92.5%, range 53.5–100%). Less frequently used tests such as polyvalent EIA, IFA, and IHA demonstrated mean pass rates for qualitative test results in the range of 11.8 to 99.6% (mean: 86.6%), 41.7 to 100% (mean pass rates: 86.5%), and 66.7 to 100% (mean pass rate: 90.6%). Newly introduced test systems such as CLIA and line blot showed a variable performance with pass rates from 11.5 to 100% (mean pass rate: 89.0%).

Serum samples from patients with long-lasting immune responses (Lyme arthritis, lymphocytoma, recent recurrent tick bites) showed that predominant IgG responses (32/2006, 62/2007, 61/2008) were reproducibly detected and correctly interpreted by most of participants. In contrast to this finding, samples from patients with shorter disease duration or lower titers of specific IgM and IgG antibodies, such as sera from neuroborreliosis cases (62/2006 and 32/2008), past infections (serum scar: sample 21/2007), and cross-reacting samples such as sera from syphilis patients (61/2007), posed more significant diagnostic problems. Here, especially IHA and IFA tended to fail in identifying these samples correctly (Tables 1 and 2).

Our observations with classical titer tests revealed that although the calculated median IFA and IHA titers of the reference laboratories and those of the participants in the majority of cases generally varied only for one to two $\log_2$ unit dilutions around the median; the ranges of titers in the group of participants revealed an enormous variability of test results (data not shown). Similarly, the quantitative results in EIAs demonstrated a very low level of interassay standardisation in all trials, resulting in a rather high heterogeneity of reported quantitative results (Figure 2). This finding is largely due to methodological differences of commercially manufactured assays and the variable methods of quantification (values of optical density (OD), indices, titers, U/mL) used. Similar to previous investigations [11], we decided not to include quantitative EIA results in the final evaluation of the proficiency testing surveys because of the obvious lack of assay standardisation.

Qualitative immunoblot test results were reported by the participants in all surveys that were performed (Tables 1 and 2). In addition, the laboratories reported the number and category of the specific IgG and IgM bands observed in their immunoblots for each of the serum samples (Figures 3(a) and 3(b)). The immunoblot results of the participating laboratories, however, showed that the individual results of the participants were not comparable in regard to the category and number of bands or the combination of bands (Figures 3(a) and 3(b)). Despite the high variability of serological test results, the microbiological interpretation of the different test constellations as reported by the laboratories was relatively homogeneous. Most participants (mean pass rate: 89.1%, range: 51.7 to 99.4%) correctly reported interpretative statements (Figure 1, Table 2) as to whether or not the assay results suggested a possible borrelial infection and whether an early or late phase of the specific antibody response was suspected.

3.3. False Positive and False Negative Test Results and Evaluation of Test Kit Quality. While using a variety of in-house tests and commercially manufactured LB test kits for the serodiagnosis of LB, participants reported a high number of false positive and false negative test results during the individual surveys. For IHA, false negative results were found in up to 33.3 of the reports and false positive results were reported in up to 30.8% of the participants. For the IgM-IFA, the rate of false negative results reached up to 35.7% and the rate of false positive results varied from 0 to 14.3% during our surveys. For the IgG-IFA, both false negative and false positive results were reported in up to 20.7 respective 58.3% of the participants. For polyvalent ELISA, the rate of false negative and false positive results ranged from 3.7 to 18% and from 0.4 to 88.2%. Class-specific ELISA testing also produced false positive results in up to 6.6% of the IgM- and in up to 46.5% of the IgG-ELISA reports. For immunoblot testing, false negative results were detected in 0–12% of the IgG- and in 4.9–15.1% of the IgM-assay reports. False positive blot results were reported in 2.1 to 22.3% of the IgG tests and in 1.7 to 9.8% of the IgM immunoblots. As depicted in Table 2 and Figures 1 and 4, the overall performance of assays was in part highly variable during the surveys and more or less depended on the assay type and manufacturer of the diagnostic test as shown for EIA in Figures 2, 4(a) and 4(b).

3.4. Outpatient Data Analysis of Patients with a Coded Diagnosis of LB. Throughout the years 2007 and 2008, an average of 6,042,531 individuals (male: female ratio 1 : 1.6, average age: 54.7 years, male subgroup: 53.2 years, female subgroup: 55.5 years) were insured by the German health insurance provider "*Deutsche Angestellten-Krankenkasse*" (DAK). In the years 2007 and 2008, a total of 22,282 and

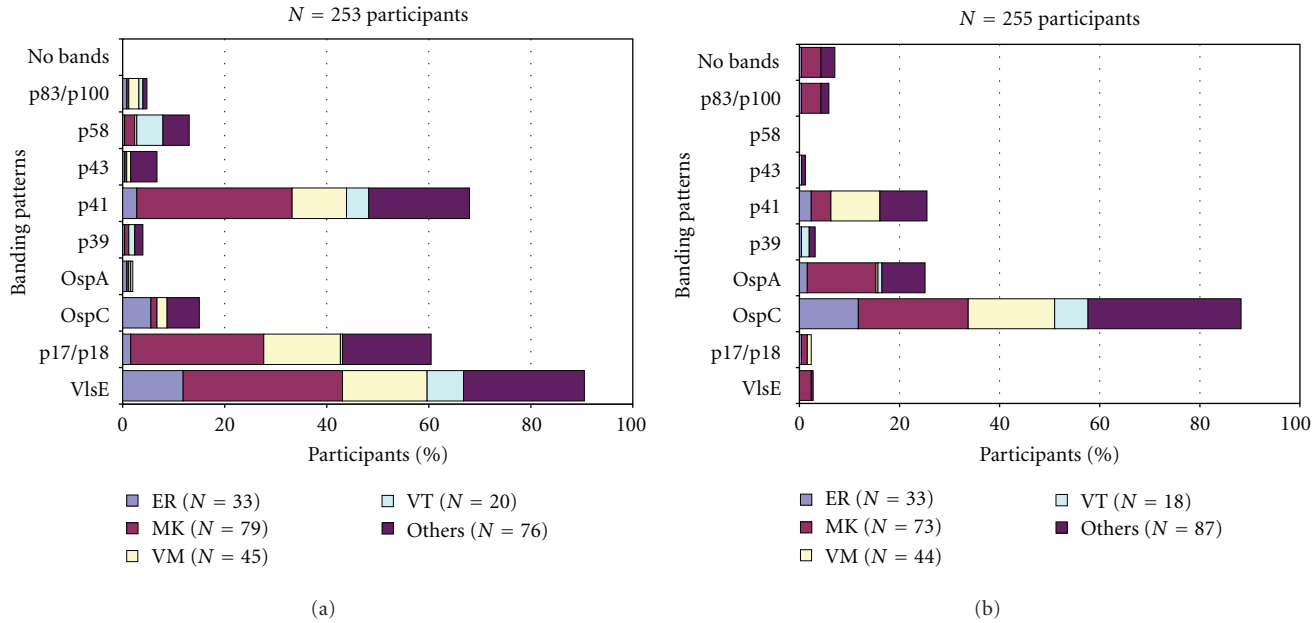

Figure 3: Recovery of LB-specific IgM- (a) and IgG- (b) immunoblot banding patterns (reported borrelial antigen bands: p83/100, p58, p43, p41, p39, OspA, OspC, p17/18, VlsE) as obtained from the participating laboratories for one sample (no. 32/2008) used for LB proficiency testing in 2008. Absolute frequency of participants reporting positive results for each antigen are depicted by bars. Relative frequency of positive reports for the different manufacturers are shown by colored boxes within the bars. (ER, MK, VM, VT, others: anonymized abbreviations of different commercial blot manufacturers). For clinical information of samples, see Table 1.

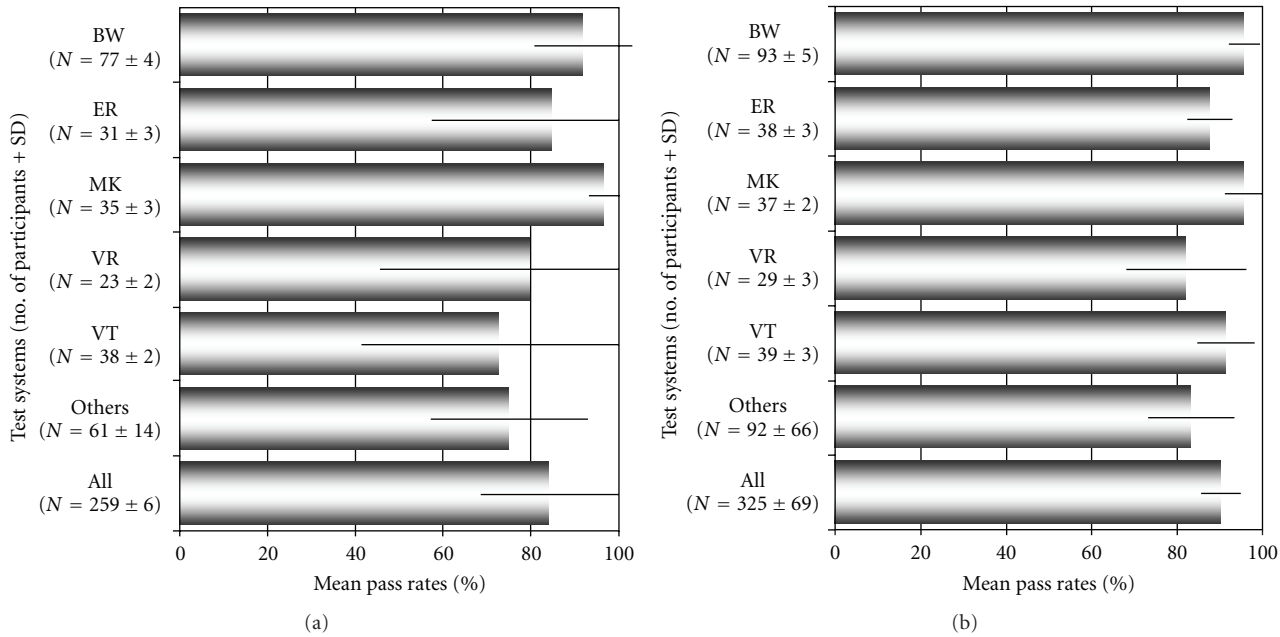

Figure 4: Average number of participants and average pass rates (%) with standard deviations (bars) for IgG- (a) and IgM- (b) EIAs of different manufacturers as observed between 2006 and 2008 in the German LB proficiency testing program (ER, BW, DO, MK, VR, VT, others: anonymized abbreviations of different commercial EIA manufacturers).

25,184 DAK patients were diagnosed with LB, respectively, which results in a prevalence of diagnoses of 365/100,000 (0.37%) and 442/100,000 (0.44%), respectively (average/year: 404/100,000). Sixty-six percent of diagnosed patients were female, and 34% were male. The average age of the cohort was 52.4 years (range: 0–98 years). However, a new diagnosis of LB was coded only in a fraction of these patients. For our current analysis, an incident diagnosis of LB was accepted only for the investigated individuals if a past diagnosis of LB could be excluded for both 2006

and in the preceding months of the analysed year 2007 and/or 2008. After exclusion of these (prevalent) diagnoses, 14,799 and 16,684 incident diagnoses remained for the years 2007 and 2008, respectively, leading to an average annual incidence for the diagnosis "LB" of 261/100,000 (diagnosis incidence of 242/100,000 and 279/100,000 individuals/year). Of these patients, 20,503 were female and 10,969 were male (m/f ratio: 1 : 1.9). Average age was 50.2 years (range: 1–98 years) for males and 53.6 years (range: 0–96 years) for females. Figure 5 shows the distribution of incident and prevalent diagnoses throughout the two years analysed for this study, plotted against the data resulting from the mandatory reporting of incident LB cases for 2007 and 2008 for the eastern German states where mandatory reporting of LB is in place [9]. As depicted in Figure 5, the annual distribution of ambulatory LB diagnosis fits well into the epidemiological pattern known for incident cases from the data reported for the eastern German states [9]. Per annum, 9,303 of the newly coded patients were tested for LB serology leading to an average of 20,986 EIA tests and 12,558 immunoblots per year. Using diagnostic claims code data for these procedures, the testing resulted in a total amount of 462,980€ in diagnostic cost annually. Moreover, 19,683 antibiotic treatment courses were administered in these patients resulting in average costs of 563,508€ for antibiotic treatments per year.

3.5. Analysis of Sickness Fund Data on Laboratory Diagnostics in All Insured Individuals. In 2007, a total of 164,634 EIAs were ordered in 94,699 individuals. 27,362 individuals were tested further using 46,627 confirmatory immunoblots. Using diagnostic claims code data, the overall cost for serological LB diagnostics resulted in 1,267,681.8€ (7.7€/EIA) for EIA and 1,119,048€ (24€/test) for confirmatory immunoblots. In 2008, a total of 185,007 EIAs and 57,934 immunoblots were performed in 112,150 and 35,002 individuals, respectively. Overall diagnostic cost in 2008 for serological diagnostics resulted in 1,424,554€ for EIAs and 1,390,416€ for western blotting. For the same time period, only 15 cultures of *B. burgdorferi* were claimed. In both years, the highest number of tests was performed in the 2nd and 3rd quarter of the year corresponding to the highest number of incident diagnoses (Figure 5). Assuming that our insurance sample (7.4% of the German population) is representative for the whole German population of approximately 82 million [23], in the years 2007/2008 an average of 213,913 incident cases could have been expected, but 2,362,439 EIAs and 706,493 western blots would have been performed, leading to a cost of 35,146,617€ for diagnostic testing. Adjusting these cost for an additional add on of 6,106,627.94€ for the relatively higher reimbursement of laboratory cost (EIA: 23.46€, blot: 53.62€) for the known 10.5% of individuals with private health insurance the calculated average annual cost would be even higher (41,253,240.24€). Moreover, 283,912 treatments would have been administered resulting in 9,961,865€ for antibiotic therapies. When extrapolating the findings from our cohort to the German population as a whole, this rather conservative calculation would translate into testing of

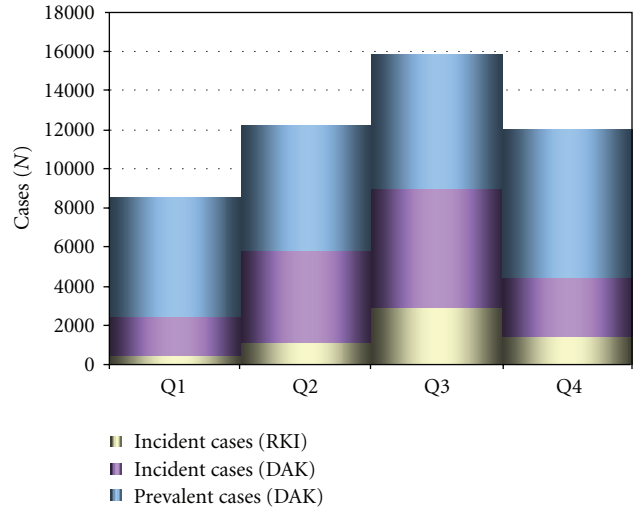

Figure 5: Average epidemiological annual distribution of coded incident and prevalent diagnoses of LB (incident DAK, prevalent DAK) as observed in the retrospective claims' data analysis depicted together with the average annual distribution of cases as reported by mandatory reporting (incident RKI) in the six new German states for 2007 to 2008. Q1–Q4: quarter of year.

1,397,628 individuals for suspected LB and total annual costs of 51,215,105.24€ for diagnostics and treatment. In contrast, focusing on incident LB cases, an average of 213,913 individuals would have been tested annually with 285,165 EIAs and 170,646 western blots leading to a cost of 6,291,290€ for diagnostic testing. Adjusting these cost for an additional add on of 1,002,617.17€ for the relatively higher reimbursement of laboratory cost (EIA: 23.46€, blot: 53.62€) for the known 10.5% of individuals with private health insurance would lead to average annual cost of 7,293,891.67€. Moreover, 237,000 treatments would have been administered resulting in another 7,614,973€ for antibiotic treatment. This calculation would translate to necessary annual costs of only 14,908,864.67€ for the diagnostics and treatment of incident LB patients in Germany, demonstrating a potentially significant gap between cost for indicated diagnostics and the high costs resulting from less selective healthcare services provided to the population as a whole.

3.6. Modelling the Influence of Diagnostic Test Quality and Cost on a Population-Based Scale. Applying available seroprevalence data from Germany [24–27] and our meta-analysed proficiency testing data to estimate the impact of the quality of diagnostic testing in Germany, we used mean pass rates for EIA and immunoblot as surrogate markers for average assay sensitivity and specificity. Given a projected number of 2,362,439 EIAs annually, we assumed these tests to be half IgG- and half IgM-specific tests. Assuming an average seroprevalence of 15% for Germany [24–27] and a mean IgG-EIA pass rate of 88.6% (SD = 16.1%) for negative and of 95.4% (SD = 5.5%) for positive samples (see above), IgG screening in Germany would lead to approximately 114,460 false positive and 8,150 false negative test results. Using mean

pass rates of 95.0% (SD = 4.4) for negative and 90.8% (SD = 1.9) for positive samples for the performance of IgM-EIA, IgM screening would have resulted in 50,202 false positive and 16,301 false negative test results annually. Putting into use mean pass rates for IgG and IgM immunoblot of 90.7% (SD = 7.2) (IgG immunoblot) and 95.6% (SD = 4.5) (IgM immunoblot) for negative and of 94.7% (SD = 4.6) (IgG immunoblot) and 89.1% (SD = 2.7) (IgM immunoblot) for positive samples, respectively, a given two-tier testing protocol would still result in 12,854 false positive and 26,495 false negative tests per annum on a population-based scale. In a different approach on a more test-specific basis, we also used our real world proficiency testing data (see above) to perform a fictive model calculation for a given two-tier testing protocol for specific IgG and IgM testing including computation of net sensitivities and net specificities. In the first model calculation, we used the mean proficiency testing pass rates for IgG- and IgM-ELISAs and immunoblots and obtained a net sensitivity of 90% for IgG and of 81% for IgM testing. The net specificity calculated for 98.9% for IgG and 99.8% for IgM.

In the second model calculation, we reduced the mean pass rates for IgG and IgM testing by the overall SDs as obtained throughout our surveys to adjust for slightly worse performing tests. This resulted in a loss of net sensitivity of 4.2% for IgM and of 9% for IgG and a loss of net specificity of 0.6% for IgM and of 3.4% for IgG. In this model, the reduction of net specificity and sensitivity led to an additional 192,716 immunoblot tests required and 4,625,183€ in additional cost. Moreover, the relatively small difference in net specificity added up to 6,191 additional false positive test results for IgM and 34,913 false positive test results for IgG. Finally, during our proficiency testing trials, we obtained a mean pass rate of 83.9% (SD = 17.2) in positive and 95.8% (SD = 4.5) in negative samples for the correct diagnostic interpretation of laboratory test results. Given these pass rates, the serological testing of 1,397,628 individuals annually for LB as projected above would lead to a misinterpretation rate of up to 12% ($N = 167,715$) even when it comes to the simple diagnostic question whether a positive or negative LB serology is present or whether an early or late phase of the antiborrelial immune response can be found.

4. Discussion

Similar to the situation in the United States with 20,000–30,000 LB cases reported annually [28], LB remains an important and very common indigenous infectious disease in Germany and Europe [8]. Although high morbidity can be expected from the disease due to the large number of cases and the potentially protracted course of the infection, little effort has been invested so far in investigations elucidating the epidemiological and financial impact of LB on the German health care system. This lack of health service data is striking when comparing LB with other common infectious diseases such as community-acquired pneumonia or nosocomial infections and is probably due to the readily available antimicrobial treatment options and the lack of mortality in LB. Likewise, such investigations are missing for most other parts of Europe making it difficult to assess the true dimension of the underlying medicoeconomical burden. Here, we used retrospective data analysis to examine (i) the epidemiology of LB, (ii) the quality of diagnostic testing, and (iii) the cost for diagnostic testing in Germany.

With official numbers absent for most parts of Germany, established mandatory reporting for 2007 and 2008 revealed about 5,624 annual incident cases (Figure 5) of LB (mainly erythema migrans) in the six new federal states (population: 16,507,263) in the north-eastern part of Germany [9]. Extrapolating these findings to the German population of 82 million, this would translate into approximately 27,958 incident cases for the entire country annually. This strongly suggests significant underreporting even when compared to the commonly cited numbers of 60,000–100,000 new LB cases per year as calculated from information available from previous seroprevalence investigations [8]. Thus, in a different approach, here, we used a retrospective outpatient data analysis performed between 2007 and 2008 on a cohort of 6,042,351 individuals insured by a German health care provider (DAK) to estimate the incidence and prevalence of LB in Germany by identifying the number of incident and prevalent diagnoses as a surrogate.

In the first step of our investigation, using these data, we were able to analyze a very large patient sample spread over the entire country and including all age groups. Importantly, possible bias due to recall, nonresponse, or the diagnostic process of attention is markedly reduced in such datasets compared to other study types [29]. Following strict definitions for the identification of "incident" and "prevalent" diagnoses, we identified 23,733 patients with a coded diagnosis of LB per year (prevalence: 404/100,000). An incident diagnosis of LB was coded in 14,799 and 16,684 individuals for the years 2007 and 2008, respectively, resulting in an incidence of 261/100,000 cases annually in the DAK cohort. Although the extrapolation of these numbers may lead to an overestimation due to clinical misdiagnosis and/or miscoding, our findings translate into 213,912 annual incident cases on a population-wide scale, which suggests more LB cases in Germany than projected previously in the available literature dealing with this topic.

Several recently performed interlaboratory studies have compared the diagnostic performance of serological tests for LB [5, 11, 30–35]. Such investigations, however, can provide only limited information on the overall performance and relative accuracy of diagnostic testing in general on a nationwide scale. Therefore, in a second step our study was aimed at supplying additional data on the quality of LB diagnostics at the national level over a well-defined period of time. In addition, we tried to identify limitations and overuse of current diagnostic approaches to LB over a well-defined period of time paralleling our retrospective patient data analysis as outlined above. According to most guidelines, LB serology should only be performed to support clinical diagnosis, not as a primary basis for making diagnostic and/or treatment decisions [4]. As shown in this investigation and previous studies, serologic testing can be flawed by problems with both sensitivity and specificity [11]. Not unexpectedly, different methodological approaches

in themselves can result, to some extent, in substantial differences with regard to test quality. Currently, a large variety of serological tests for the detection of LB are available in the European market, supplied by an increasing number of manufacturers. In the United States, a complex regulatory system for new in vitro diagnostics is in place which requires the manufacturer to compare its product substantially against an established device that has already been cleared by the FDA [36, 37]. In Europe, the institution of the new European IVD directive in 2000 did not legally insist in extensive, independent, and continuous *clinical* evaluation of commercially available serological test kits for LB before placing in vitro diagnostic tests on the market [38]. Instead, quality standards for the production quality and safety are enforced for in vitro diagnostic tests in their intended use [38, 39], and; consequently, test remakes are increasingly pushed onto the market. This trend is also supported by our observation showing a steady increase (~50%) of new test formats such as line blots and CLIA during the study period in the years 2006–2008. Currently, at least 55 different companies provide diagnostic tests for LB in Germany alone. Therefore, routine evaluations of microbiological laboratories by external quality control measures for LB serology appear to be attractive datasets to learn more about the relative frequency of certain test applications (i.e., EIA and immunoblot) in the diagnostic market, the amount of test standardisation, and the quality of performance in diagnostic infection serology [11, 19]. From 2006 to 2009, in Germany, between 360 and 392 microbiological laboratories took part in our proficiency testing surveys. Similar to findings of other investigations, most laboratories still relied on two-tier testing with EIA and immunoblot throughout the study period. Although qualitative testing by EIA and immunoblot showed mean pass rate ranges from 82.2 to 89.2% (Figure 1), quantitative EIA results and analysis of immunoblot banding patterns, however, demonstrated a very low degree of interassay standardisation (Figures 2 and 3). In addition, as already described in prior studies [11, 34, 35], a high number of both false negative and false positive test results became obvious from our surveys and was in part correlated with the diagnostic method, the manufacturer (Table 2, Figures 1 and 4), and the amount of specific antibodies present in different sera (Table 1). Our findings confirm the assumption that, in the routine laboratory, the quantity of detected antibody measured in titers or quantitative EIA results and, similarly, the number and category of specific immunoblot bands can vary greatly for the same sample. In addition, changes in qualitative and quantitative serologic test results may be misleading and can emerge simply by using different assay systems in different laboratories. As a consequence of the findings in this investigation and other recent studies, a more general implementation of diagnostic criteria for the interpretation of immunoblot results as suggested by expert recommendations [13] seems increasingly difficult in light of the relatively high assay variability (Figures 2 and 3). Most importantly, correlating the activity of LB and the success of subsequent therapies with quantitative serological testing as well as with qualitative changes in the test results as attempted by some physicians appears clearly unreliable. The extreme variability of test results reported by the participants in our surveys is concordant with the few available international studies on this topic [11, 35, 40–42]. To improve the value of LB serology in the routine microbiological laboratory, promotion of better interassay standardisation of the commercially available test kits is necessary [13, 35, 42] by implementing standards and procedures as suggested earlier by the Centers for Disease Control and Prevention (CDC) and the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD) conference on the serological diagnosis of LB [43]. Most importantly, more detailed and independent clinical evaluation of assays should be legally required before placing such products on the market [19].

So far, very few studies have examined the economic impact of quality and frequency of LB diagnostic testing on health care on the national level in the US and Europe. One study examined the medical and economical burden of LB in the United States. Using a decision analysis model and an estimated incidence of 4.7 LB cases per 100,000 persons led to direct and indirect costs including diagnostic testing of about 2.5 billion US Dollars over 5 years for the US [44]. For the state of Maryland, Zhang et al. calculated direct medical costs including diagnostics of 2,970 US Dollars plus indirect costs of 5,202 US Dollars per LB patient [45]. This would add up to approx. 200 million US Dollars per year for the United States. Another study estimated the cost of LB for the Scottish health care system. Although this study was limited to laboratory testing only, the authors estimated a total of GBP 47,000–615,000 for Scotland which seems to be a high financial impact for a country with a relatively low LB incidence [46]. All mentioned investigators used variable assumptions and economical models to assess the general cost of LB making it difficult to directly compare their findings to the results of our study. The results of these studies, therefore, cannot simply be transferred to the German health system due to differences in the epidemiology of the disease, of the methodological approaches, and in the health systems. Besides the cost for physician visits, consultation, and therapy, clearly, the cost for diagnostic testing, represents one of the major variables when calculating direct medical costs of LB on a population-wide scale. This is why, in the third step of our investigation, we tried to estimate the quantity and cost of diagnostic testing in Germany by modelling the combined information obtained from the DAK dataset and from the results of the regular German LB proficiency testing surveys run by INSTAND biannually to estimate the cost and medical quality of laboratory diagnostics on a nationwide level. By assessing the diagnostic frequency, quality, and cost of LB diagnostics in Germany, we estimated that 2,371,887 EIA tests and 709,331 western blots are performed annually. When modelling the influence of test quality for a given two-tier testing protocol including the calculation of net sensitivities and net specificities, it became obvious that using our real world proficiency testing data such tests would result in 12,854 false positive and 26,495 false negative test results annually on a population-wide scale. In this model, a small reduction in net specificity led

to an additional 192,716 immunoblot tests required and an amount of 4,625,183€ in extra cost. Moreover, the small reduction in net specificity added up to 6,191 additional false positive test results for IgM and 34,913 false positive test results for IgG. Finally, given the average pass rates for the correct diagnostic interpretation of laboratory test results obtained during our proficiency testing trials, the serological testing of 1,397,628 individuals annually as projected above would lead to a misinterpretation rate of up to 12%.

By further extrapolating the findings from our cohort of 6.04 million individuals insured by a statutory health insurance provider and adjusting our findings for 10,5% of privately insured patients with higher reimbursement, we project an overall expected cost of 51,215,105.24€ for LB serologic testing and treatment in Germany. These figures do not include patients' expenditure for nonrecommended tests (e.g., lymphocyte-transformation tests, LTT) which are not reimbursed but have to be paid out of the pocket. Similarly, in a study on LB management in primary care practices in Maryland USA, both diagnostic and therapeutic efforts were heavily overused [47]. In addition, Ramsey et al. showed that 80% of serology tests for Lyme borreliosis were regarded as inappropriate in a retrospective analysis in Wisconsin, USA [48]. Our findings on cost also come close to the study of Tugwell et al. who estimated 2.8 million tests in the US/year, leading to 100 million USD/year for serological testing [49]. The somewhat higher cost for testing in the US can be explained due to higher expenditure per test compared to the German situation. However, with our projected incidence of 214,000 cases per year, the overall frequency and cost of diagnostic testing and treatment clearly suggests a high amount of potentially inappropriate healthcare services in patients with a suspected or confirmed diagnosis of LB.

5. Conclusion

Our study is the first investigation of its kind in Germany and looks into the medical and economical burden of LB testing for the German healthcare system. Although suggesting a high amount of inappropriate diagnostic healthcare services, our analysis also shows limitations as it is focused on retrospective investigations of proficiency testing surveys and secondary claims data including external quality control datasets, coded diagnoses, and diagnostic and therapeutic services relevant for physician claims. Given our secondary data study design, other potential biases which cannot be accounted for including missing information on services provided outside the statutory health insurance and absence of information related to care provided which does not lead to (additional) claim codes (i.e., multiple visits within a quarter) [29, 50]. However, the findings coming from the GILEAD project are a first approximation of health care services provided related to LB. They will help to assess and better tailor the quality standards for diagnostic tests and the economics of current and future disease management and prevention programs for LB. Given the ongoing problems in Germany with the clinical management of LB, it seems important to closely monitor and evaluate health care utilization patterns including diagnostics and treatment for LB patients to both facilitate a better understanding of existing care and design intervention approaches to improve the clinical management for such patients.

Acknowledgments

This study was funded by a grant provided by INSTAND e.V., Düsseldorf, Germany. Part of the study was supported by a research grant of the German Federal Ministry of Education and Research (BMBF 01GY1137). I. Müller and M. H. Freitag contributed equally as first authors to the study.

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 294587, 10 pages
doi:10.1155/2012/294587

Research Article

Adaptive and Innate Immune Responsiveness to *Borrelia burgdorferi sensu lato* in Exposed Asymptomatic Children and Children with Previous Clinical Lyme Borreliosis

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Received 7 June 2011; Revised 25 August 2011; Accepted 29 August 2011

Academic Editor: Joanna Zajkowska

Why some individuals develop clinical manifestations in Lyme borreliosis (LB) while others remain asymptomatic is largely unknown. Therefore, we wanted to investigate adaptive and innate immune responsiveness to *Borrelia burgdorferi sensu lato* in exposed *Borrelia*-antibody-positive asymptomatic children ($n = 20$), children with previous clinical LB ($n = 24$), and controls ($n = 20$). Blood samples were analyzed for *Borrelia*-specific interferon (IFN)-γ, interleukin (IL)-4, and IL-17 secretion by ELISPOT and *Borrelia*-induced IL-1β, IL-6, IL-10, IL-12(p70), and tumor necrosis factor (TNF) secretion by Luminex. We found no significant differences in cytokine secretion between groups, but a tendency towards an increased spontaneous secretion of IL-6 was found among children with previous clinical LB. In conclusion, the adaptive or innate immune responsiveness to *Borrelia burgdorferi sensu lato* was similar in *Borrelia*-exposed asymptomatic children and children with previous clinical LB. Thus, the immunological mechanisms of importance for eradicating the spirochete effectively without developing clinical manifestations of LB remain unknown.

1. Introduction

Lyme Borreliosis (LB), caused by the spirochete *Borrelia* (*B.*) *burgdorferi*, is the most common tick-borne infection in both Europe and the USA [1, 2]. The infection may lead to a variety of symptoms by affecting different organs such as the skin, joints, heart muscle, or nervous system. The most common manifestation of LB is the migrating red skin lesion called erythema migrans (EM). LB in children follows a slightly different clinical course than in adults, and duration of symptoms is often shorter [3]. Children seem to have a better prognosis than adults and more seldom report persisting symptoms [4–6].

The cells of the innate immune system, constituting the first line of defense, recognize pathogen-associated molecular patterns (PAMPs) through pattern-recognition receptors (PRRs) like Toll-like receptors (TLRs) [7]. The spirochete *B. burgdorferi* contains a high proportion of lipoproteins that are mainly recognized by TLR2 [8]. Recognition of *B. burgdorferi* leads to the release of inflammatory mediators like interleukin (IL)-1β, IL-6, IL-10, and IL-12, and tumor necrosis factor (TNF) from monocytes, macrophages, neutrophils, and DCs [9–12]. These cytokines are important for recruitment of other components of the innate host immune response but also for signaling with the adaptive immune

system [12]. The adaptive immune system consists of T and B lymphocytes and comprises the second line of defense to eliminate the spirochete. Activated T helper (Th) cells differentiate into Th1, Th2, Th17 or T-regulatory cells [13]. Th1 cells are important for immunity against intracellular pathogens, whereas Th2 cells are involved in immune responses against extracellular parasites [14]. Typical Th1 and Th2 effector cells are macrophages and mast cells, respectively. Th1 and Th2 are defined by their signature cytokines IFN-γ and IL-4, which act antagonistically to counterbalance each other. Th17 cells, producing IL-17, are involved in the defense against fungi and some extracellular bacteria [15]. The immune response to *B. burgdorferi* involves both humoral and cell-mediated immune responses where both T-cell-independent and -dependent B cell responses are important during the adaptive immune response for killing the spirochetes [12].

Children and adults differ in the type of immune response they evoke when encountering *B. burgdorferi.*In adults, the immune response in LB is characterized by a strong Th1 responses with high numbers of *Borrelia*-specific IFN-γ-secreting cells and low levels of IL-4 [16, 17], whereas children seem to have a more balanced immune response with elevated secretion of both IFN-γ and IL-4 [18]. It has been hypothesized that the type of immune response evoked in the presence of the *Borrelia* spirochete may have significant effect on the clinical course and the outcome of the infection [19]. Persistent symptoms after LB were associated with a strong IFN-γ response but lacked the subsequent upregulation of IL-4 [19]. Thus, it appears that a later prominent Th2 immune response is necessary to downregulate the initial strong IFN-γ response in order to effectively terminate the infection and hinder unsatisfactory tissue damage. This could be one possible explanation why children usually experience a more benign course of the disease as they show both strong Th1 and Th2 immune responses [18].

The fact that some individuals may be exposed to *B. burgdorferi s.l.* without developing clinical symptoms is interesting from an immunological standpoint and could indicate a more effective immune response to the spirochete in these individuals. In adults, the term asymptomatic *Borrelia* infection is used for individuals who have been exposed to *B. burgdorferi s.l.* (i.e., with *Borrelia* IgG antibodies in serum) without known previous clinical LB [20]. These asymptomatic individuals have been found to have a higher secretion of the proinflammatory cytokines IL-12 and TNF than patients with clinical LB, suggesting an enhanced innate activity [21]. As for the adaptive immune responses, no differences have been found in *Borrelia*-specific IFN-γ and IL-4 secretion when comparing *Borrelia* exposed asymptomatic adults to patients with clinical LB [20]. To our knowledge, the innate and adaptive immune responses have not previously been studied in *Borrelia* exposed asymptomatic children.

The aim of this study was to investigate adaptive and innate immune responsiveness to *Borrelia* exposed asymptomatic children as compared to children with previous clinical LB to elucidate immunological mechanisms that might contribute to an effective eradication of the pathogen.

2. Materials and Methods

Children recruited to participate in this study were initially included in a larger prospective study, the ABIS (All Babies In Southeast Sweden) study, with the primary purpose of finding risk factors for immune-mediated diseases, mainly Type 1 diabetes (n = 17 055). These children were followed until at 5 years of age at primary health care centers. All participating families completed a validated questionnaire, and venous or capillary blood samples were collected in conjunction with the 5-year followup. Two thousand children were randomly selected and screened for *Borrelia* IgG antibodies in serum [22]. Information concerning gender, geographic location, known tick bites, previous LB, and antibiotic treatment for LB was collected from the questionnaire.

Of these 2000 children, a total number of 64 children, geographically spread, were chosen to represent three major groups: *Borrelia* exposed asymptomatic children (n = 20), children with previous clinical LB (n = 24), and controls (n = 20) (Table 1). *Borrelia* exposed asymptomatic children were characterized by having *Borrelia* IgG antibodies in serum, but they reported no previous symptoms or treatment for LB in the questionnaire. Children with previous clinical LB reported previous treatment for LB, and some of them had *Borrelia* antibodies in serum (4/24). The control group reported no symptoms or previous treatment for LB and had no *Borrelia* antibodies in serum (Table 1). The control group and children with previous clinical LB were matched for gender and geographic location with *Borrelia* exposed asymptomatic children (Table 1). All 64 children were included for analysis with the ELISPOT assay but some later had to be excluded (n = 16) due to low responses in postive controls (see *Data Handling*). These excluded children did not differ statistically concerning gender (female 7/16 versus 22/48) or geographic location (rural living 7/16 versus 19/48) compared to included children (n = 48). Samples from 41 children were used for analysis with Luminex due to insufficiencies in cell samples (see *Data Handling*). The excluded children (n = 23) did not differ statistically concerning gender (female 10/23 versus 19/41) or geografic location (rural living 10/23 versus 16/41) compared to included children (n = 41). Informed consent has been given by all participating families, and the study was approved by the Regional Ethical Committee at the Faculty of Health Sciences, Linköping, Sweden (Dnr 03-547). All laboratory work in this study (i.e., not the collection and cryopreservation of cells) has been carried out by one person (S. Hellberg, one of the authors).

2.1. ELISA Antibody Test for B. burgdorferi s.l. A commercial enzyme-linked immunoassay (ELISA) kit, based on the *Borrelia*-specific protein Flagellin, was used (IDEA *Borrelia burgdorferi* IgG kit, DakoCytomation, Glostrup, Denmark

Table 1: Subject characteristics at 5 years of age.

	Exposed asymptomatic ($n = 20$)	Previous clinical LB ($n = 24$)	Controls ($n = 20$)
Gender (f/m)	9/11	11/13	9/11
Rural living	7	11	8
Known tick bites	20	24	0
Previous clinical LB			
EM	0	22	0
NB, facial nerve palsy	0	5	0
NB, meningitis	0	1	0
Antibiotic treatment for LB	0	24	0
Borrelia IgG antibodies in serum*	20	4	0

Note. The data referred to in the table is given as numbers of children. Some of the children with previous clinical LB presented with several symptoms.
n: number; f: female; m: male; LB: Lyme borreliosis; EM: Erythema migrans; NB: Neuroborreliosis; IgG: immunoglobulin G
*Based on an ELISA (DAKO) kit for IgG antibodies for Borrelia-specific flagella antigen [24].

and Oxoid Limited, Hampshire, United Kingdom) [24], and cut-off for OD values was set according to the manufacturer's instructions.

2.2. Isolation, Cryopreservation, and Thawing of Peripheral Blood Mononuclear Cells (PBMCs). Blood samples were collected from the primary health centers, sent to the Division of Pediatrics, Linköping University, and prepared for isolation and cryopreservation as described in earlier studies [25]. When the time came for analyses, the samples were taken out of the nitrogen container and thawed in a 37°C water bath. Once thawed, the cell suspension was immediately transferred into a 15 mL polypropylene tube, and prewarmed (37°C) tissue culture media (TCM) containing 10% heat-inactivated fetal calf serum (FCS; Sigma Aldrich, Stockholm, Sweden) and Iscove's modification of Dulbecco's medium (GIBCO, Paisley, UK) supplemented with L-glutamine (Sigma Aldrich Sweden AB, Stockholm, Sweden) 292 mg L^{-1}, MEM (minimum essential media) 100 X nonessential amino acids 10 μg mL^{-1} (Invitrogen AB, Paisley, UK), penicillin 50 IU mL^{-1}, streptomycin 50 μg mL^{-1} (BioWhittaker Europe, Essen, Germany), and $NaHCO_3$ 3.024 g L^{-1} (Merck KGaA, Damstedt, Germany) were added dropwise along the wall of the tube to avoid osmotic shock in the cells. The cell suspension was centrifuged for 10 minutes at 400 ×g at room temperature and the supernatant discarded. The cells were washed twice in TCM at 400 ×g at RT for 10 min. The cells were counted using a Bürker chamber in a phase-contrast microscope (Carl Zeiss AB, Stockholm, Sweden). The cell membrane integrity, that is, viability of the cells, was assessed with trypan blue exclusion and ranged from 83% to 99% with a median of 95%. The concentration of cells was adjusted to 1×10^6 PBMC mL^{-1}.

2.3. Preparation of the Borrelia Outer Surface Protein-Enriched Fraction (OF) Antigen. The cells, both in ELISPOT assay and *in vitro* stimulation for Luminex assay, were stimulated by OF, consisting primarily of OspA and OspB from *B. garinii* strain Ip90. This antigen was chosen because it has previously been shown to be effective in differentiating between individuals with *Borrelia* infections and controls in ELISPOT assay for specific *Borrelia* IFN-γ and IL-4 secretion [17, 23]. The antigen was prepared as described in earlier reports [26, 27], and the optimal concentration of the *Borrelia* OF antigen was determined through testing of different antigen concentrations (54, 18, 6, and 2 μg mL^{-1}) on an adult patient sample with confirmed neuroborreliosis (NB) by ELISPOT analysis. The final optimal concentration of *Borrelia* OF antigen was therefore 6 μg/mL^{-1} in the ELISPOT assay. In the Luminex assay, a concentration of 12 μg/mL was used.

2.4. ELISPOT Analysis for IFN-γ, IL-4, and IL-17. To determine the T-cell response to *B. burgdorferi s.l.,* an ELISPOT assay was used to assess the number of *Borrelia*-specific IFN-γ-, IL-4- and IL-17-secreting cells. The ELISPOT analyses, originally described by Czerkinsky et al. [28], were performed according to the instructions provided by the manufacturer (Mabtech AB, Nacka, Sweden) and as described in detail in earlier studies [17, 23]. Tetanus toxoid (TT; Swedish Institute for Infectious Disease Control, Stockholm, Sweden) and phytohemagglutinin A (PHA; Sigma-Aldrich AB, Stockholm, Sweden) were used as positive controls, representing recall antigen and polyclonal stimulation, respectively, at a final concentration of 5 LF units mL^{-1} for TT and 20 μg mL^{-1} for PHA. A peptide pool consisting of 32 peptides derived from the human Cytomegalovirus, Epstein-Barr virus, and Influenza virus (CEF; Mabtech AB, Nacka, Sweden) was also included as an additional positive control for IFN-γ responses and used at a final concentration of 2 μg mL^{-1}. As for negative controls, wells with TCM were used without cells. All samples (except wells containing only PHA or cell medium alone) were assayed in triplicate although some samples could only be assayed in duplicate due to low cell count. The spots were counted manually by dissection microscope and by semiautomatic AID EliSpot Reader system/HR version 3.2.3 (AID autoimmune diagnostics GmbH, Strassberg, Germany) in a blinded manner on one single occasion by the same

person (S. Hellberg, one of the authors). Each spot counted represented one cytokine-producing cell.

2.5. Luminex Analysis for IL-1β, IL-6, IL-10, IL-12(p70), and TNF. Half a million PMBCs diluted in 0.5 mL TCM supplemented with 10% FCS (Sigma Aldrich, Stockholm, Sweden) were cultured together with OF from *B. garinii* and LPS (Sigma-Aldrich AB, Stockholm, Sweden) from *Salmonella typhimurium* at final concentrations of 12 μg mL^{-1}, 1 ng mL^{-1}, and 100 ng mL^{-1}, respectively, or without antigen, at 37°C, 5% CO_2, for 24 hours. After 24 hours, the cells were centrifuged (400 ×g at RT), and the supernatants were collected and frozen at −70°C until used for the Luminex analysis of cytokines in the samples. The levels of cytokines IL-1β, IL-6, IL-10, IL-12(p70), and TNF were measured in the PBMC supernatants by a Bio-Plex Pro Human Cytokine Panel Kit (Bio-Rad Laboratories, Calif, USA). All assays were carried out in accordance with the instructions provided by the manufacturer. The plates were then analyzed using Luminex 200 (Invitrogen, Merelbeke, Belgium). The analysis condition was set to a minimum of 100 beads per region. The raw data, median fluorescent intensity (MFI), was analyzed using xPONENT 3.1 (Luminex Corporation, Austin, Tex, USA). The quantifiable ranges for the standard curves were for IL-1β 1.91–1959.31 pg mL^{-1}, IL-6 1.54–25171 pg mL^{-1}, IL-10 1.48–6076 pg mL^{-1}, TNF 4.75–19438.75 pg mL^{-1}, and for IL-12(p70) 2.19–8988 pg mL^{-1}. Values below the lowest detection limit of the standard curve were assigned half the value of the detection limit, and values above highest detection limit were assigned double the value of the detection limit.

2.6. Data Handling. Regarding the ELISPOT assay, the median of the triplicates or duplicates was used for the analysis of cytokine-secreting cells. The method for determining *Borrelia*-specific secretion in this study has previously been implemented in other studies [18–20] and is based on both the unstimulated, spontaneous secretion and the antigen-stimulated secretion of the cells. The *Borrelia*-specific secretion was determined by subtracting the number of spots from the wells with cell suspension and medium (i.e., the spontaneous secretion) from the number of spots in the OF-antigen-stimulated wells (i.e., *Borrelia*-stimulated secretion) [20]. Both the *Borrelia*-specific secretion and the spontaneous secretion are interesting to show to give a full picture of immune responses, and both are therefore reported in Results, Discussion, and Figures.

All 64 samples were investigated by the ELISPOT assay. However, as there were low responses in some of the positive controls, defined criteria were used to assure that cells had the ability to respond. These criteria were mainly based on the PHA responses for IFN-γ. Samples with a high PHA response for IFN-γ (over 300 spots) were all included ($n = 27$). Samples with PHA response of 200–300 spots were included if they also had an apparent antigen-induced response for TT and CEF (for more than one cytokine) or a strong PHA response for both IL-4 and IL-17 ($n = 19$). Based on previous experience, samples with a PHA response for IFN-γ showing less than 200 spots were excluded ($n = 16$) with the exception of two samples showing strong antigen-induced responses for both TT and CEF (for more than two cytokines) and strong PHA responses for both IL-4 and IL-17 ($n = 2$). All these considerations were blinded from the belonging to groups and the *Borrelia*-stimulated secretion.

With regard to the Luminex assay, 41 samples were available and all were analyzed. The ability of the cells to respond to stimuli was assessed by the ratio between the samples stimulated with LPS and the spontaneous secretion. Samples were included if they had an LPS/spontaneous secretion ratio of five or more for any of the analyzed cytokines. Based on these criteria, all samples were valid and could be included for analysis ($n = 41$). The *Borrelia* induced secretion was obtained by subtracting spontaneous secretion from the samples stimulated with *Borrelia* OF antigen as previously implemented for the ELISPOT assay.

2.7. Statistical Analysis. Statistical Products and Service Solutions (SPSS), version 17.0 for Windows was used for the statistical analysis. The Kruskal-Wallis test was used as a pretest for comparison of the immunological parameters between the groups. Mann-Whitney U test was used as a post hoc test when the P value for Kruskal-Wallis test was $P < 0.08$. For Mann-Whitney, a level of $P < 0.05$ was considered statistically significant. Since the cytokines analyzed in this study were viewed as part of a pattern and not as separate events, no corrections for multiple comparisons were made.

3. Results

3.1. Number of IFN-γ-, IL-4- and IL-17- Secreting Cells Measured by ELISPOT. All groups displayed a predominance of *Borrelia*-specific IFN-γ-secreting cells as compared to IL-4- and IL-17-secreting cells. However, no significant differences were found between *Borrelia* exposed asymptomatic children and children with previous clinical LB regarding the number of *Borrelia*-specific IFN-γ-, IL-4-, and IL-17-secreting cells (Figure 1). Moreover, no significant difference could be found in comparison to the control group for any of the analyzed cytokines. Some of the individuals in all three groups had negative values for the *Borrelia*-specific secretion, that is, the spontaneous secretion was higher than the OF-stimulated secretion (Figure 1).

When analyzing the number of spontaneous secreting cells, indicating the nonstimulated background activity of cytokine secretion, no significant differences in IFN-γ, IL-4, or IL-17 was found between any of the groups (Figure 2). A ratio between the numbers of IL-4- and IFN-γ- spontaneously secreting cells, assessing the Th2/Th1 balance, did not show any significant differences across groups (data not shown). PHA-induced secretion, indicating the ability of the cells to respond to mitogenic stimulation, elicited a stronger response for IFN-γ than for IL-4 and IL-17, but no differences were found between groups (data not shown).

3.2. IL-1β, IL-6, IL-10, IL-12(p70), and TNF Secretion Measured by Luminex. The *Borrelia* induced secretion of IL-1β, IL-6, IL-10, and TNF were readily detectable with the

Table 2: The OF induced cytokine secretion in PBMCs by Luminex.

Cytokine	Exposed asymptomatic	Previous clinical LB	Controls
IL-1β	309 (183–846)	341 (132–1223)	340 (162–800)
IL-6	6805 (3528–12749)	7304 (3281–50303)	7335 (2578–12324)
IL-10	130 (38–442)	129 (42–572)	123 (37–684)
IL-12(p70)	1 (0-1)	1 (0-1)	1 (0-1)
TNF	676 (241–2311)	561 (209–1724)	628 (226–1373)

NOTE. The data referred to in the table is given as median values in pg/mL (range in parenthesis).
OF: outer surface protein enriched fraction; PBMCs: peripheral blood mononuclear cells; LB: Lyme Borreliosis; IL = interleukin; TNF: tumour necrosis factor.

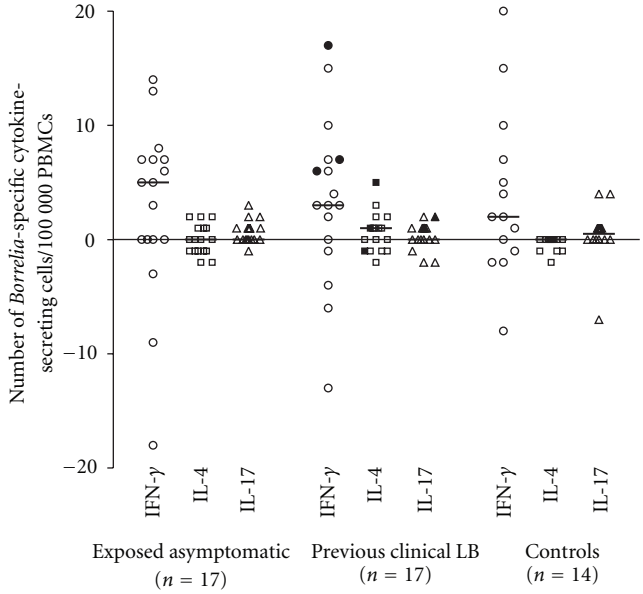

Figure 1: The number of *Borrelia*-specific IFN-γ-secreting cells (open circle), IL-4-secreting cells (open square) and IL-17-secreting cells (open triangle), per 100 000 PBMCs as measured by ELISPOT in different groups. The filled circles, squares, and triangles represent children in the previous clinical LB group with *Borrelia* seropositivity. The *Borrelia*-specific secretions are net values obtained after subtracting the number of spontaneous cytokine-secreting cells from the number of outer surface protein fraction (OF-) antigen-specific cytokine-secreting cells. The median values are noted as lines in the figure. No statistically significant differences were found between groups.

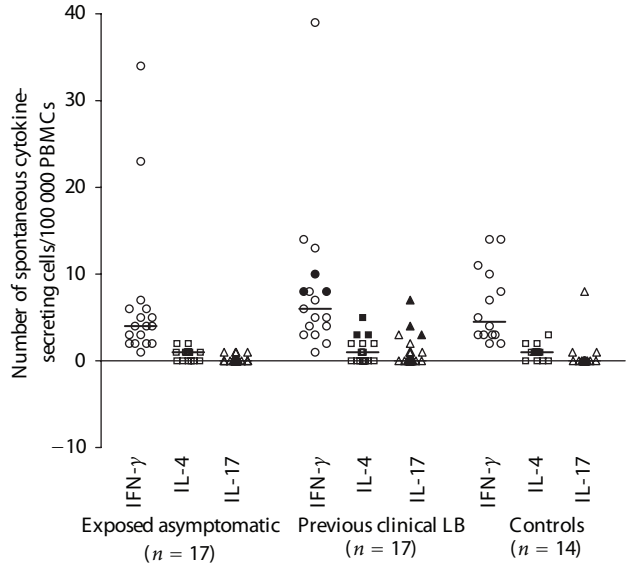

Figure 2: The number of spontaneously IFN-γ-secreting cells (open circle), IL-4-secreting cells (open square), and IL-17-secreting cells (open triangle) per 100 000 PBMCs as measured by ELISPOT in different groups. The filled circles, squares, and triangles represent the children in the previous clinical LB group with *Borrelia* seropositivity. The median values are noted as lines in the figure. No statistically significant differences were found between groups.

Luminex assay, whereas the *Borrelia* induced secretion of IL-12(p70) was undetectable in all samples (Table 2). Moreover, the IL-12(p70) levels were also below the detection limit also in 32 of the 40 samples stimulated with LPS and did not exceeded 8 pg/mL in the remaining 8 samples. Thus, levels of IL-12(p70) were not considered interpretable. Of the remaining cytokines, IL-6 was present in the overall highest concentration in all groups and IL-10 in the lowest concentration in all groups (Table 2).

As for the *Borrelia* induced secretion, no significant differences were found between *Borrelia* exposed asymptomatic children, children with previous clinical LB, and controls with regard to IL-1β, IL-6, IL-10, and TNF (Figure 3). The spontaneous secretion did not differ significantly between groups for any of the cytokines although there was a tendency ($P = 0.057$) towards an increased spontaneous secretion of IL-6 in children with previous clinical LB as compared to *Borrelia* exposed asymptomatic children (Figure 4). The ratio between LPS-stimulated secretion and the spontaneous secretion, indicating the ability of the cells to respond to a TLR4-agonistic stimulus, was (median values with range in parenthesis): 301(9–2051) for IL-1β; 314(8–4262) for IL-6; 134(17–450) for IL-10 and 66(7–652) for TNF (data not shown).

4. Discussion

In this study, we have investigated the *Borrelia*-specific (adaptive) and the *Borrelia* induced (innate) immune responses in *Borrelia* exposed asymptomatic children and children with

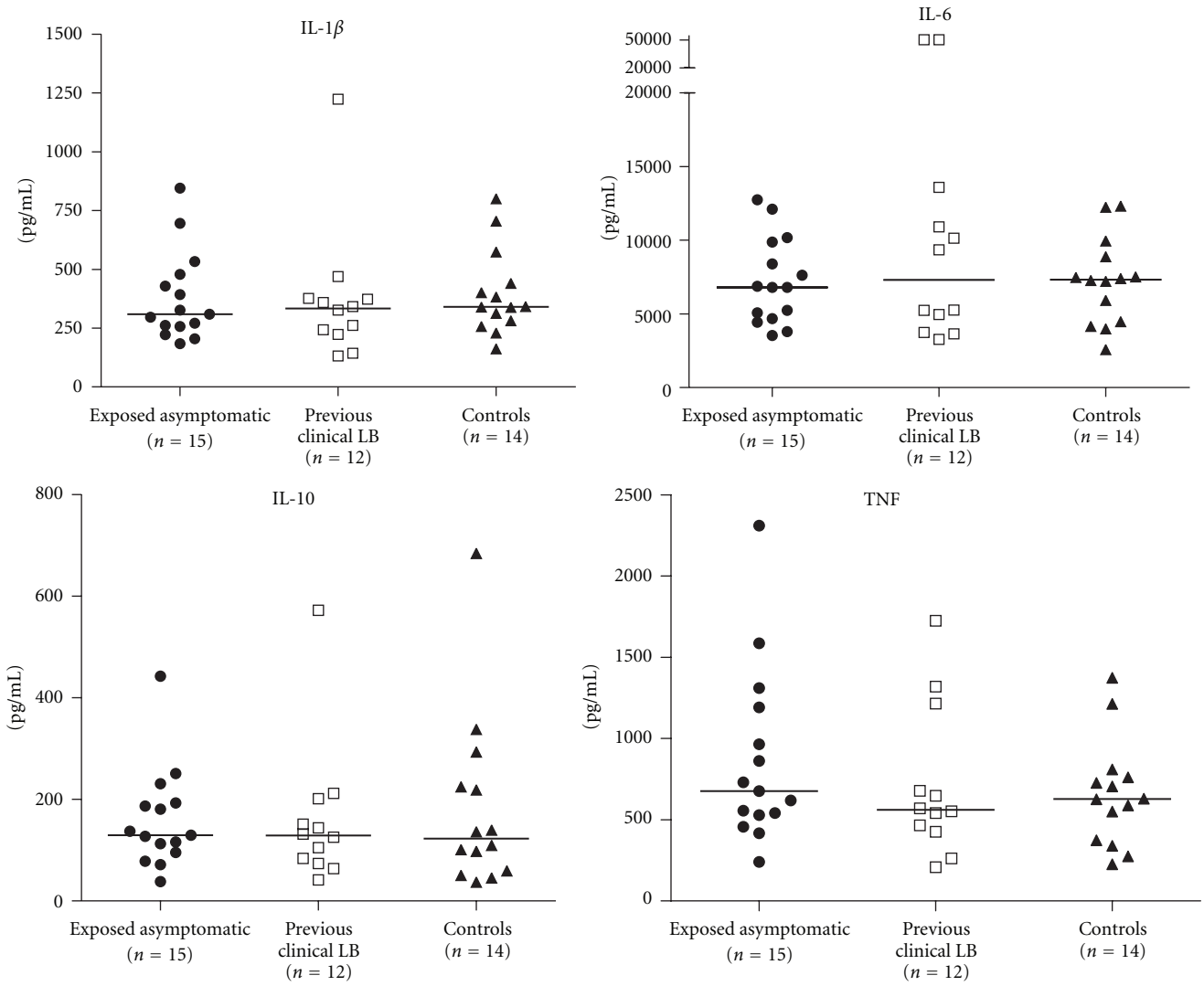

Figure 3: The *Borrelia*-induced secretion of IL-1β, IL-6, IL-10, and TNF in PBMC supernatants from *Borrelia* exposed asymptomatic children (filled circle), children with previous clinical LB (open square), and controls (filled triangle) as measured by Luminex. The *Borrelia* induced secretions are net values obtained after subtracting the level of spontaneous cytokine secretion from the level of outer surface protein-fraction (OF-) stimulated cytokine secretion. The median values are noted as lines in the figure. No statistically significant differences were found between groups.

previous clinical LB. Our aim was to better understand immunological mechanisms that could explain why some individuals develop clinical manifestations of LB and others do not. Interestingly, we found no differences in the number of *Borrelia*-specific IFN-γ-, IL-4-, and IL-17-secreting cells when comparing *Borrelia* exposed asymptomatic children, children with previous clinical LB, and controls. This lack of *Borrelia*-specific (adaptive) immune responsiveness is congruent with earlier studies on *Borrelia* exposed asymptomatic adults where no differences in the number of *Borrelia*-specific IFN-γ- and IL-4-secreting cells were found compared to patients with clinical LB [20]. Our negative findings are further supported by Jarefors et al. [29], who found no differences in IFN-γ secretion between asymptomatic adults and patients with previous clinical LB [29]. As for the innate immune responses, no significant differences in the *Borrelia* induced cytokine IL-1β, IL-6, IL-10, or TNF secretion between groups were found in our study, whereas in a previous study by Sjöwall et al. [21], an increased number of *Borrelia*-induced TNF-secreting DCs were found in asymptomatic adults as compared to patients with a history of NB [21].

We are well aware of the fact that the number of patients in each group is rather low; but with well-characterized patient groups, a proper design of the study and nonparametric statistic calculations, the results should be reliable. Furthermore, the fact that the number of *Borrelia*-specific IFN-γ- and IL-4-secreting cells was generally lower as compared to earlier studies [17, 18, 20] led us to consider that the responsiveness of the cells to stimuli might have been reduced due to the quality of the cells after freezing (previous studies were performed on freshly isolated cells).

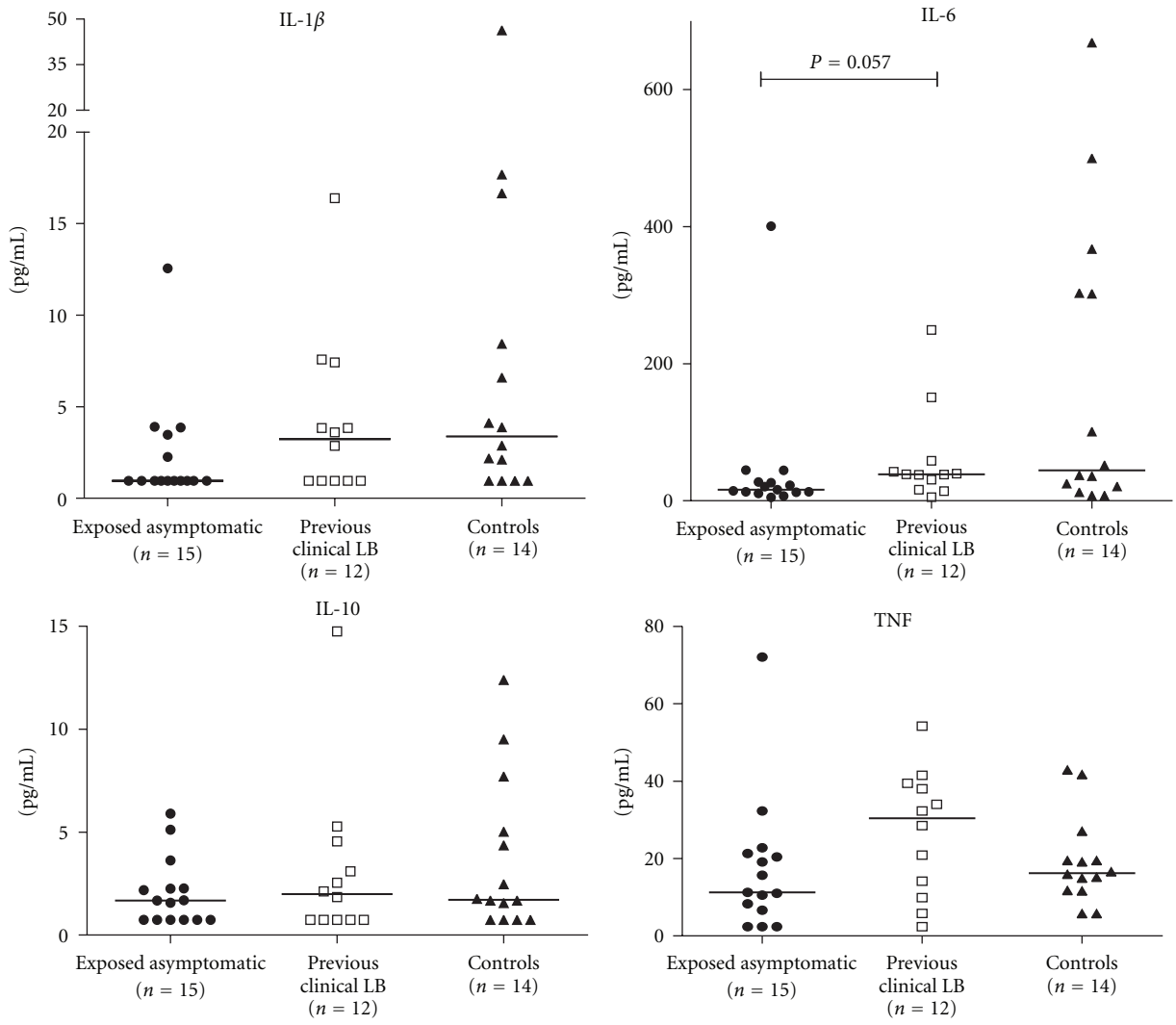

Figure 4: The spontaneous secretion of IL-1β, IL-6, IL-10, and TNF in PBMC supernatants from *Borrelia* exposed asymptomatic children (filled circle), children with previous clinical LB (open square), and controls (filled triangle) as measured by Luminex. The median values are noted as lines in the figure. There was a tendency to higher IL-6 in children with previous clinical LB compared to *Borrelia* exposed asymptomatic children, otherwise no statistically significant differences were found between groups.

However, the response to PHA was similar to that obtained from another study on LB in adults [17], and additionally the response to LPS was substantially higher than the spontaneous secretion which, considered together, confirms the ability of the cells to respond to stimuli. The use of cryopreserved PBMCs in this study was necessary due to the design of the study, as it would have been impossible to test cellular immune responses in freshly isolated PBMCs from thousands of unselected children. Earlier reports on cryopreservation effects on cytokine secretion by ELISPOT showed a general decrease in the IL-4 secretion in cryopreserved cells as compared to fresh cells both in spontaneous and allergen-induced secretion, whereas IFN-γ secretion was less affected [30]. Thus, to some extent, the cryopreservation could explain the low levels of IL-4-secreting cells found in this study as compared to earlier studies with freshly prepared PBMCs from children (1–17 years old) [18]. It is also important to note that blood samples from children were taken postinfection and not during the actual infection, in accordance with the design of the study.

The young age of the children has also to be taken into consideration. The ability to respond with IFN-γ is impaired in neonates [31] and develops during childhood [32, 33]. The children in our study were all 5 years of age, and thus one would expect full capacity to respond with IFN-γ to stimuli [34, 35]. A similar capacity was found when comparing the PHA-induced IFN-γ secretion in our present study to the PHA-induced IFN-γ secretion in adults [17].

Whether or not children with previous clinical LB are "truly" *Borrelia* exposed, patients may be a matter for discussion. Data in our present study is based on self-reported information that may have weaknesses. However, most patients did report a previous EM, which is a clear clinical diagnosis, and although self-reported, the diagnosis

was stated to be set by a physician and treated with antibiotics. Very few of the children with previous clinical LB were *Borrelia* seropositive ($n = 4$, Table 1), but this was expected since EM is a clinical diagnosis set by a physician, the sensitivity of the test in EM is low and antibody levels may be reduced after antibiotic treatment [36]. Previous antibiotic treatment in this group could theoretically also have influenced immune responses. Moreover, one must remember that our study mainly evaluates children with previous EM (only 5 children with facial palsy and 1 with meningitis), thus conclusions on immune responses in disseminated LB could not be drawn from our data.

Furthermore, whether or not *Borrelia* exposed asymptomatic children are "truly" *Borrelia* exposed or falsely seropositive may also be a matter for discussion. We have not carried out any confirmatory test since the specificity of the test is high [37], and in earlier studies, a *Borrelia*-specific T-cell response in PBMCs was noted in *Borrelia* exposed asymptomatic adults, certifying a true exposure [38]. Thus, we believe that false seropositive specimens should not be a problem in our material.

We found a tendency towards higher levels of spontaneous secretion of IL-6 (by Luminex) in children with previous clinical LB (Figure 4). IL-6 is a pleiotropic cytokine that mainly mediates proinflammatory effects, and it induces secretion of IL-17 from naïve T cells [39] and may therefore, together with IL-17, be involved in pathogenesis of LB. Recently, it was suggested that IL-17 might contribute to the pathogenesis in Lyme arthritis and in NB [40, 41]. Thus, one could speculate that IL-6 together with IL-17 may be involved in inflammatory mechanisms contributing to clinical manifestations of LB. However, in the present study, we found only a tendency of elevated spontaneous secretion of IL-6 but no *Borrelia* induced IL-6 or IL-17 secretion. This could possibly be explained by the fact that we have analyzed PBMCs after inflammation and not specimen from immune privileged sites during active inflammation. Thus, the role of IL-6 together with IL-17 is certainly interesting regarding the pathogenesis in LB but needs further investigation.

Finally, why some individuals develop a clinical disease upon encountering *B. burgdorferi s.l.* and some do not remains unclear. Whether or not specific properties of the host's immune system are of importance is still not understood, and, admittedly, there might be other important aspects. For example, the *Borrelia* genospecies infecting the human might play a substantial role in the different clinical outcomes observed in LB. It is well established that the different genospecies of *B. burgdorferi s.l.* can cause different clinical manifestations, and it is also well known that different genospecies have different abilities in escaping the complement system, thereby avoiding elimination [42]. These aspects, as well as the individual genetic predisposition might be crucial factors in understanding mechanisms in the spirochete-host interaction, and future studies are warranted in these fields.

In conclusion, our results show no differences in adaptive or innate immune responsiveness to *B. burgdorferi s.l.* when comparing *Borrelia* exposed asymptomatic children and children with previous clinical LB. Thus, immunological mechanisms of importance for eradicating the spirochete effectively without developing clinical manifestations of LB remain unknown.

List of Abbreviations

ABIS:	"All Babies In Southeast Sweden"
B. burgdorferi s.l.:	*Borrelia burgdorferisensu lato*
CEF:	Peptide pool consisting of 32 peptides derived from human Cytomegalovirus, Epstein-Barr Virus, and Influenza virus
CSF:	Cerebrospinal fluid
DC:	Dendritic cell
ELISA:	Enzyme-linked immunosorbent assay
ELISPOT:	Enzyme-linked immunospot assay
EM:	Erythema migrans
f:	Female
FCS:	Fetal calf serum
IFN-γ:	Interferon gamma
Ig:	Immunoglobulin
IL:	Interleukin
LB:	Lyme borreliosis
LPS:	Lipopolysaccharides
m:	Male
NB:	Neuroborreliosis
OF:	Outer surface protein-enriched fraction
OD:	Optical density
PAMPs:	Pathogen-associated molecular patterns
PBMCs:	Peripheral blood mononuclear cells
PHA:	Phytohaemagglutinin A
PRRs:	Pattern recognition receptors
SPSS:	Statistical Products and Service Solutions
TCM:	Tissue culture medium
Th1:	T helper lymphocyte type 1
Th2:	T helper lymphocyte type 2
Th17:	T helper lymphocyte type 17
TLR:	Toll-like receptor
TNF:	Tumor necrosis factor
TT:	Tetanus toxoid.

Conflict of Interest

No conflict of interests is stated by the authors.

Acknowledgment

The authors are most grateful to all participating children/parents. They also wish to thank the staff at the Child Health Services in the region as well as the excellent research nurses and the technicians connected to the ABIS study, in particular Ingela Johansson and Gosia Smolinska. Also special thanks are due to Mari-Anne Åkeson and Petra Cassel for excellent expertise on ELISPOT and Luminex analysis, respectively. In addition, they are most grateful to the staff at the Department of Molecular Biology, Umeå University for supplying the *Borrelia* OF antigen. The study was supported by grants from the Research Council in Southeast Sweden

(FORSS), the County Council in Östergötland, the Swedish Child Diabetes Foundation, the Juvenile Diabetes Research Foundations, the Holmia Foundation and the Center of Clinical Research in Dalarna (CKF).

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Hindawi Publishing Corporation
Clinical and Developmental Immunology
Volume 2012, Article ID 138069, 8 pages
doi:10.1155/2012/138069

Research Article

ELISA-Based Measurement of Antibody Responses and PCR-Based Detection Profiles Can Distinguish between Active Infection and Early Clearance of *Borrelia burgdorferi*

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Received 8 July 2011; Accepted 19 August 2011

Academic Editor: Joanna Zajkowska

Borrelia burgdorferi is a spirochetal bacterium that causes Lyme disease. These studies address whether current research methods using either ELISA to detect seroconversion to *B. burgdorferi* antigens or PCR quantification of bacterial DNA within tissues can accurately distinguish between a productive infection versus a *B. burgdorferi* exposure that is rapidly cleared by the innate responses. Mice receiving even minimal doses of live *B. burgdorferi* produced significantly more *B. burgdorferi*-specific IgM and IgG than groups receiving large inocula of heat-killed bacteria. Additionally, sera from mice injected with varied doses of killed *B. burgdorferi* recognized unique borrelial antigens compared to mice infected with live *B. burgdorferi*. Intradermal injection of killed *B. burgdorferi* resulted in rapid DNA clearance from skin, whereas DNA was consistently detected in skin inoculated with viable *B. burgdorferi*. These data indicate that both ELISA-based serological analyses and PCR-based methods of assessing *B. burgdorferi* infection clearly distinguish between an established infection with live bacteria and exposure to large numbers of bacteria that are promptly cleared by the innate responses.

1. Introduction

Borrelia burgdorferi (Bb) is a spirochetal bacterium that causes Lyme disease [1]. Introducing this pathogen into the skin of susceptible hosts, either via the bite of an infected tick or by injection of culture-grown bacteria, leads to their subsequent dissemination to several tissues, including heart, joint, and neural tissues [2]. These spirochetes are notable in their ability to persist for months to years within host tissues, with intermittent reemergence promoting the acute localized inflammatory lesions that characterize Lyme disease. While these persistent bacteria elicit strong innate and adaptive immune responses, their fastidious growth requirements have hindered *in vitro* analyses to determine which elements of host immunity are most important for controlling these infections [3–7].

Most studies to assess immune responses against *B. burgdorferi* are performed using a well-described murine model of Lyme disease. Mice are a natural reservoir for *B. burgdorferi*, and persistent bacteria within certain inbred strains are associated with similar tissues and produce inflammatory pathology consistent with that exhibited in human patients, though the severity of disease can vary widely between different inbred mouse strains [8–10]. Infection studies using inbred strains have allowed identification of specific immune mediators that affect host clearance, such as Toll-like receptor 2 (TLR2) [11, 12], MyD88 [13, 14], CD14 [15, 16], IL-10 [17, 18], the chemokine KC [19], and the production of antibodies against critical *B. burgdorferi* antigens [20–24].

Studies elucidating the basis of *B. burgdorferi* clearance have relied heavily on two parameters, namely, seroconversion to bacterial antigens and detection of bacterial DNA in host tissues. Production of high antibody titers against certain *B. burgdorferi* antigens, which have been further characterized using western blot analyses, can protect animals from both tick-mediated and syringe challenge with *B. burgdorferi* [9, 22, 25]. The specific effects of antibodies and other immune mediators on *B. burgdorferi* clearance have traditionally been measured qualitatively by culturing murine

tissues in sterile BSK medium and determining whether resident spirochetes can grow from these cultures [26]. More recently, real-time PCR techniques have been developed that can accurately quantify even minute *B. burgdorferi* levels in murine target tissues [17, 27, 28], and similar methods have been used to compare the upregulation of targeted murine and bacterial gene products within infected tissues [18, 29, 30]. The refinement of these techniques have greatly improved the usefulness of the murine model of Lyme disease, particularly in identifying immune mediators that are effective in controlling these unique pathogens.

While both ELISA techniques, to measure antibody levels, and PCR analyses, to determine *B. burgdorferi* levels, are widely used to assess the development of Lyme disease in infected animals, questions have been raised regarding how accurately these techniques assess the infection status. *B. burgdorferi* are known to be highly immunogenic, largely due to the wide range of lipoproteins that are produced in response to different environmental cues [6, 31, 32]. These lipoproteins all possess a triacyl modification on their amino terminus [33] that not only activates many different host immune cells through interaction with TLR2 [11, 34–36] but also provides potent adjuvant activity that significantly enhances antibody responses to these lipoproteins [37, 38]. This raises the possibility that mice receiving a significant inoculum may produce substantial *B. burgdorferi*-specific antibodies that do not truly reflect a response to an active infection, but alternatively reflect an antibody response to an initial inoculum that was quickly cleared; in these cases, differences between active and subclinical infection would only be apparent by subsequent western blot analyses. A second issue is that *B. burgdorferi* can persist in many different tissues, but the precise extracellular or intracellular microenvironment in which they persist, as well as the immunoprivileged status of that niche, is still being defined [39–42]. It is plausible that bacterial products from killed bacteria, such as DNA, might escape timely or complete clearance from those tissues, and, thus, subsequent assessment could falsely indicate that viable *B. burgdorferi* were persisting in those tissues. To address these issues, we have injected mice with various doses of live and heat-killed bacteria to determine whether significant and characteristic differences in both antibody production, as assessed by ELISA analyses, and detection of *B. burgdorferi* DNA, by PCR, can accurately reflect whether the mice were actively infected or were only exposed to a threshold level of bacterial antigens.

2. Materials and Methods

2.1. Infection of Mice with Borrelia burgdorferi. C57BL/6NCr (B6) mice were obtained from the National Cancer Institute: Frederick Animal Production Program (Frederick, MD). Mice were housed in the Department of Lab Animal Resources at the University of Toledo Health Sciences Campus according to the National Institutes of Health guidelines for the care and use of laboratory animals. All protocols were reviewed and approved by the Institutional Animal Care and Usage Committee.

The clonal N40 isolate [43] of *B. burgdorferi* was generously provided by Steve Barthold (University of California, Davis) as a passage two culture after isolation from the urinary bladder of a Rag-$1^{-/-}$ mouse. For all infections, a passage 4 culture was grown in BSK-II medium supplemented with 6% rabbit serum (Sigma Chemical, St. Louis, Mo, USA) for 3–5 days at 33°C and directly enumerated using a Petroff-Hauser's chamber and dark field microscopy. B6 naïve mice were infected with the indicated numbers of viable or heat-killed *B. burgdorferi* in a 20 μL volume by intradermal injection into a shaven back. These bacteria remain intact after heat killing (55°C for 1 hour) based on visual inspection and counting by dark field microscopy but are subsequently unable to grow in BSK medium (data not shown).

2.2. Immunoglobulin (Ig) Quantification. Serum was obtained at the indicated times by either retroorbital bleeding or exsanguination, and Ig content was assessed using previously described ELISA techniques [17]. Briefly, microtiter plates were coated with either sonicated *B. burgdorferi* or goat antibodies to mouse IgG, IgM, and IgA (Southern Biotech, Birmingham, Ala, USA). Multiple serum dilutions were added to plates for 90 min at 37°C, and bound murine Ig was detected by addition of isotype-specific HRP-conjugated antibodies (Southern Biotech). Ig content was quantified by comparison to standard curves constructed by using purified Ig of the appropriate isotype (Southern Biotech).

2.3. Western Blot Analysis. One hundred twenty μg of sonicated cN40 isolate were electrophoresed in a 4–12% Bis-Tris gel (Invitrogen) containing a single large well, transferred to Immobilon-P membrane (Millipore, Bedford, Mass, USA), and immunoblotted using a Surf Blot apparatus (Idea Scientific Company, Minneapolis, Minn, USA). Immune sera used to blot the membrane were obtained from B6 mice at day 28 after inoculation with viable or heat-killed *B. burgdorferi*. Antibody-antigen complexes were detected by addition of HRP-conjugated antibodies specific for total murine Ig (Southern Biotech) and visualized by chemiluminescence. Multiple film exposure times were acquired for each blot to ensure that all protein bands were recorded irrespective of concentration variances between samples.

2.4. DNA Preparation. Murine skin tissues encompassing (6 mm diameter) the Bb injection site were harvested from experimental animals sacrificed at the indicated times after injection, and DNA was prepared from individual tissues as previously described [10]. Briefly, tissue specimens were incubated in 0.1% collagenase A (Roche Diagnostics, Indianapolis, Ind, USA) at 37°C overnight, followed by the addition of an equal volume of 0.2 mg/mL proteinase K (Invitrogen, Carlsbad, Calif, USA) and incubation overnight at 55°C. DNA was recovered by multiple phenol-chloroform extractions and ethanol precipitation and includes digestion of contaminating RNA in 1 mg/mL DNase-free RNase (Sigma), with the final sample resuspended in 500 μL of TE buffer. The DNA content was quantified by absorbance at 260 nm, and working samples were diluted to 50 μg/mL for quantitative real-time PCR analyses.

2.5. Quantification of B. burgdorferi in Mouse Tissues. The number of spirochetes resident in the different murine target tissues were determined via PCR analyses using a LightCycler (Roche Diagnostics) rapid fluorescence temperature cycler based on our previously described protocols [17, 28]. Briefly, amplification was performed on 100 ng of template DNA in a 10 μL final volume containing 50 mM Tris (pH 8.3), 3 mM $MgCl_2$, 4.5 μg of bovine serum albumin, 200 μM of each deoxynucleoside triphosphate, a 1 : 10,000 dilution of SYBR Green I (Molecular Probes, Eugene, Ore, USA), 1 μM of each primer (Integrated DNA Technologies, Coralville, Iowa, USA), and 0.5 U of Platinum *Taq* DNA Polymerase (Invitrogen). Copy numbers for the mouse *nidogen* and *B. burgdorferi rec*A genes present in each sample were calculated by extrapolation to standard curves using LightCycler software (Roche Diagnostics). The reported data represents *rec*A values that were corrected by normalization based on the *nidogen* (*nid*) gene copy number. The oligonucleotide primers used to detect mouse *nidogen* were nido.F (5′-CCA GCC ACA GAA TAC CAT CC-3′) and nido.R (5′-GGA CAT ACT CTG CTG CCA TC-3′). The oligonucleotide primers used to detect *B. burgdorferi rec*A were nTM17.F (5′-GTC GAT CTA TTG TAT TAG ATG AGG CTC TCG-3′) and nTM17.R (5′-GCC AAA GTT CTG CAA CAT TAA CAC CTA AAG-3′).

2.6. Statistical Analyses. The statistical significance of the quantitative differences between the different sample groups was determined by application of Student's two-tailed *t*-test; *P* values that were ≤0.05 were considered significant.

3. Results

3.1. Quantification of Bb-Specific Ig Levels in Serum. We initially wanted to determine whether distinct quantitative differences are detectable in antibody levels produced during an active Bb infection versus bacterial exposures that are quickly resolved. To address this, groups of B6 mice were injected with different doses of live or heat-killed bacteria, and the Bb-specific Ig content of individual sera collected either 2 or 4 weeks after infection was compared by ELISA analysis. Sera from control mice that were injected only with BSK II medium contained no Bb-specific IgG and minimal levels of Bb-specific IgM (Figure 1), which reflects the presence of natural Bb-specific IgM in naïve mice, as previously reported [44, 45]. Mice injected with live bacteria showed higher Bb-specific IgM levels at 2 weeks (Figure 1(a)) than at 4 weeks (data not shown), and while low levels of Bb-specific IgG were seen at 2 weeks after infection (data not shown), the levels were much higher at 4 weeks post-infection (Figures 1(a) and 1(b)). Injecting a single dose of heat-killed Bb into B6 mice, with or without CFA, did not elicit significantly enhanced IgM levels compared to mice receiving BSK II medium alone (Figure 1(a)), even at a dose of 5×10^7 killed Bb. However, injection of as few as 250 live bacteria increased the Bb-specific IgM levels by over 200-fold compared to mice receiving the highest dose of killed bacteria ($P \leq 1.6 \times 10^{-5}$) by 2 weeks post-infection. A

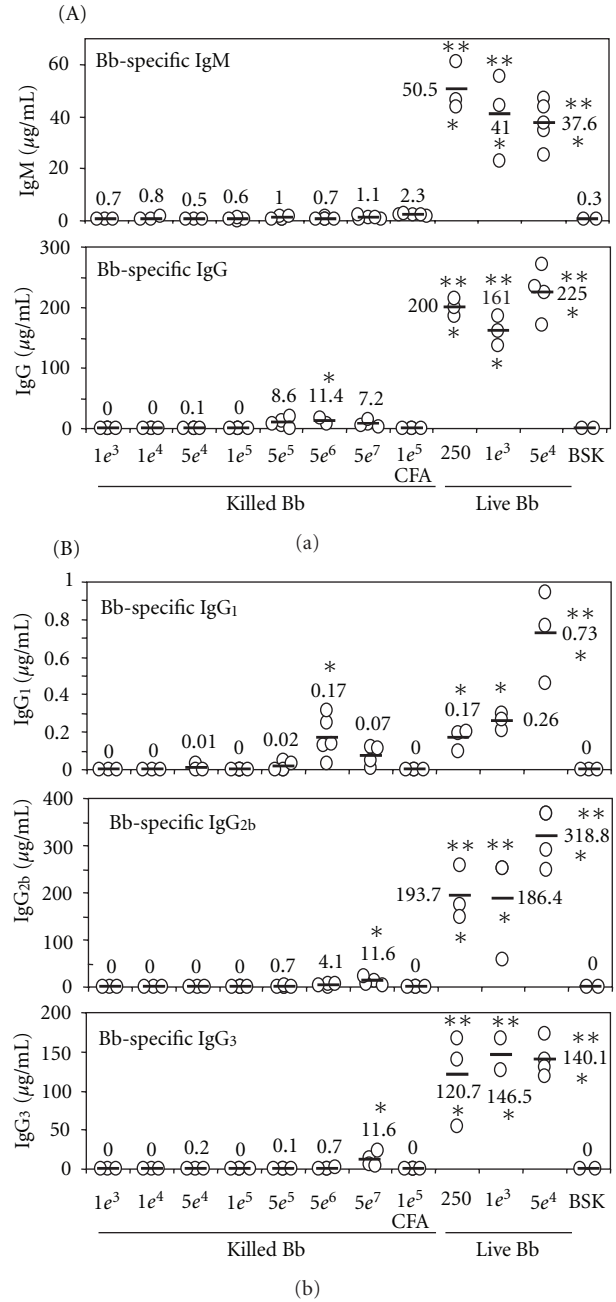

Figure 1: Comparison of Bb-specific antibody levels produced during active infection versus exposure to high numbers of killed bacteria. (a) Groups of B6 mice were injected with a single dose of live or heat-killed Bb, and serum was collected at either 2 or 4 weeks after infection; in some cases the killed Bb inoculum included an equal volume of complete Freund's adjuvant (CFA). The doses injected ranged from 1000 ("$1e^3$") to 5×10^7 ("$5e^7$") killed bacteria, and 250 to 5×10^4 ("$5e^4$") live Bb. Individual sera were assessed for Bb-specific IgM content at 2 weeks after infection and for IgG content at 4 weeks after infection by ELISA analyses. Each circle represents the serum value for an individual animal, and the number beside the bar indicates the average value for that group. *indicates values that are significantly different from control mice (Un); **indicates values that are significantly different from mice injected with killed bacteria. (b) The sera assessed for IgG content in (a) were also assessed for the levels of the indicated individual IgG isotypes using similar ELISA techniques.

somewhat similar trend was seen in IgG levels at 4 weeks post-infection, where a dose of 5×10^6 killed Bb was required to significantly increase Bb-specific IgG levels compared to naïve mice ($P = 0.011$). Again, injection with as few as 250 live bacteria increased the Bb-specific IgG levels by over 20-fold compared to mice receiving the highest dose of killed bacteria ($P \leq 2.3 \times 10^{-6}$) by 4 weeks post-infection. These data indicate that significant quantitative differences in Bb-specific antibodies are readily detected in sera from animals that sustain an active infection compared to those exposed to relatively high numbers of killed Bb that are quickly cleared.

To determine whether these differences in IgG production extended to all IgG isotypes, similar ELISA analyses were performed and developed using isotype-specific detection antibodies (Figure 1(b)). The production of IgG_{2b} and IgG_3 isotypes showed a similar trend as reported for Bb-specific IgG, in that significant levels of Bb-specific antibodies were only observed at the very highest dose of killed bacteria, while administration of as few as 250 live Bb resulted in ≥10-fold increase in Bb-specific antibodies ($P = 0.001$ and 0.01, resp.). In contrast, IgG_1 production appeared much more responsive to killed bacteria, with significant increases in Bb-specific IgG_1 elicited in response to 5×10^6 killed bacteria, and these levels were similar to those produced in response to all live bacteria inocula, except for the highest dose. However, the overall quantities of IgG_1 antibodies were ≥200-fold less than the other isotypes assessed, and thus represent a minor component of the total IgG response.

3.2. Comparison of Bb Antigens Recognized by Antisera to Live and Killed Bb. Bb can rapidly modulate a large number of surface-exposed and other lipoproteins during their natural infection cycle from arthropod to vertebrate host [31, 32, 46–48]. Therefore, it is likely that the antigens that are immunologically recognized during an active infection differ from those in a killed Bb exposure that is rapidly cleared, and could be used to further confirm the significant differences in antibody production observed in the ELISA analyses. To address this, sera were collected from mice injected with various doses of live and killed Bb at 4 weeks post-infection, and used for western blot analyses to compare the range of Bb antigens that are recognized by each serum (Figure 2). Mice injected only with BSK II medium (lanes 2-3) possessed no detectable Bb-reactive antibodies, while a mAb specific for Bb OspA (lane 1) appeared to detect both the monomeric and dimeric forms of this lipoprotein. As expected based on the ELISA analyses (Figure 1), an approximately 20-fold higher concentration of serum from animals injected with killed bacteria was needed to effectively visualize Bb antigens compared to sera generated against live bacteria (Figure 2). Sera from mice that received either a single (lanes 7–9) or boosted dose (lane 13) of killed Bb demonstrated a protein recognition pattern that was distinct from mice receiving either a low (lanes 10–12) or high (lanes 14–16) dose of live Bb. These studies indicate that significant quantitative and qualitative differences are apparent in the antibodies produced between mice that undergo an active infection with

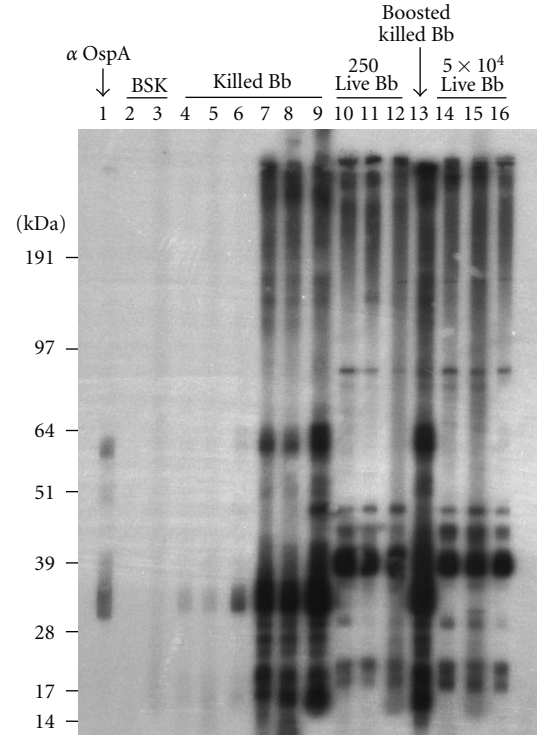

Figure 2: Antibodies produced against live and killed spirochetes recognize unique Bb antigens. Sera were collected from individual mice 4 weeks after injection with a single dose of either BSK II medium (lanes 2-3), 5×10^7 killed Bb (lanes 4–9), 250 live Bb (lanes 10–12), or 5×10^4 live Bb (lanes 14–16); one group initially received 10^5 killed Bb + CFA, with a boost 3 weeks later with 10^5 killed Bb + IFA, and sera collected 3 weeks later (lane 13). All sera were diluted either 1 : 25 (lanes 2-3 and 7–9) or 1 : 500 before using to immunoblot membranes containing electrophoresed Bb. A mAb specific for Bb OspA, H5332 [49], was included as a marker for OspA reactivity (lane 1).

Bb and those which rapidly clear a relative large bacterial inoculum.

3.3. Persistence of Bb DNA in Skin Tissues. PCR-based assays are commonly used to quantify differences in Bb levels within host tissues for a number of model systems; however, it is not clear how long the DNA from killed bacteria might persist in those tissues and provide inaccurate estimation of the viable Bb numbers. To address this, parallel groups of mice were injected intradermally with either live or killed Bb, and skin tissues were harvested and assessed at different times after injection for the presence of bacterial DNA by real-time PCR using our described protocols [17, 28]. Skin tissues harvested immediately after injection of equal numbers of live or killed Bb showed the presence of similar numbers of Bb genomes by PCR analyses (Figure 3), and, by 8 h after injection, these numbers have substantially decreased to similar low levels in animals receiving both live and killed bacteria. After 8 h after injection, almost no Bb genomes were detected in skin tissues of animals receiving killed bacteria (Figure 3), and more extensive studies showed that no Bb genomes were detected in skin, ear, ankle, or heart tissues of mice receiving killed

bacteria at 2 and 4 weeks after injection (data not shown). Alternatively, mice receiving live bacteria showed a gradual increase in skin Bb levels after 8 h following infection, with the highest bacterial numbers detected at 96 h after infection (Figure 3), and reduced but consistent levels noted at 2 and 4 weeks after injection (data not shown). These findings suggest that killed Bb are rapidly cleared from skin tissues and that their genome content can no longer be detected by PCR within hours of being killed.

4. Discussion

The spirochetal pathogen *B. burgdorferi* (Bb) is an obligate parasite that cycles efficiently between vertebrate and arthropod hosts and persists for extended periods within various host tissues. Antigenic variation has been described to play a putative role in immune evasion, but other mechanisms by which these bacteria evade clearance from immunocompetent hosts are not well defined. The fastidious growth requirements of these spirochetes make it difficult to design stringent *in vitro* analyses that accurately reflect host conditions. Therefore, infection studies using inbred mouse lines are the gold standard for addressing host-Bb interactions. Both ELISA and western blot analyses have been useful in measuring the critical antibody responses to Bb infection, including the identification of immunoreactive bacterial products that can confer protective immunity and in determining infection rates by seroconversion. Similarly, the recent development of quantitative real-time PCR techniques has allowed researchers to distinguish important differences in immune clearance between different murine model strains and, thus, identify mechanisms that are critical for clearance of these persistent bacteria. The sensitivity and specificity of these assays are well documented, but questions have arisen as to their abilities to differentiate between active infections versus those produced by residual and/or stimulatory bacterial products that persist subsequent to bacterial killing. Our study attempts to clarify the limitations of these techniques in assessing the murine infection model of Lyme disease.

B. burgdorferi is notable in that ≥8% of putative protein-coding genes contain a signal peptide "lipobox" region, suggesting that these gene products are recognized by lipid modification enzymes that produce triacylated lipoproteins [6, 7, 33]. These lipoproteins can be secreted across the cytoplasmic membrane to the outer membrane [50], where they are not only recognized by the adaptive immune responses but can also interact with TLR2 on a number of different immune cell types to induce inflammatory responses [11]. As a result of these interactions, Bb lipoproteins are highly immunogenic and possess endogenous adjuvant activities that make them attractive vaccine candidates [38, 51, 52], such as the OspA-based LYMErix vaccine [53]. Infection with as few as 20 organisms can lead to the production of high levels of Bb-specific antibodies by 2 to 4 weeks after infection, which corresponds with a subsequent decrease of bacterial numbers in host tissues, suggesting the importance of these antibodies in controlling bacterial numbers [22, 54, 55].

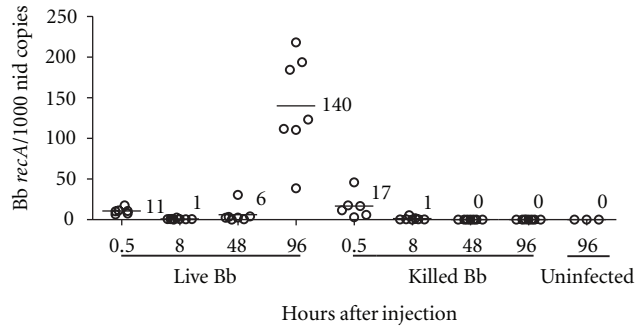

Figure 3: DNA from killed Bb is rapidly cleared from infection sites and is undetectable by PCR. Groups of mice were injected intradermally with 10^4 of either live or heat-killed Bb. Mice were sacrificed at the indicated times after injection, and a 6 mm skin sample encompassing the injection site was excised for DNA preparation. The relative Bb levels were assessed by real-time PCR using *recA* primers and normalized relative to the murine *nid* content. Each circle represents Bb *recA* levels relative to 1000 *nid* copies for an individual animal, and the number beside the bar indicates the average value for that group. These data reflect the combined results of two separate experiments.

While increases in Bb-specific antibody levels are often used as an indicator of infection, it is possible that this correlation is misleading, since even a Bb inoculum that is rapidly cleared by the innate immune responses would result in the presentation of a substantial number of immunogenic lipoproteins to T and B cells, which could potentially elicit antibody levels that approach those produced by infected animals. However, we found that mice injected intradermally with a single dose of either live or heat-killed Bb produced sera with distinct differences. A dose of at least 5×10^6 killed bacteria was required to elicit detectable Bb-specific antibodies, but even 5×10^7 killed bacteria elicited 20- to 400-fold less Bb-specific antibody levels than any tested doses of live bacteria. These large differences were noted in all Ig isotypes tested except for IgG_1 production. Overall, these data suggest that significant differences are apparent between the antibody levels produced in response to a productive Bb infection versus exposure to killed bacteria and that properly controlled ELISA analyses can reliably distinguish the two.

During their natural infection cycle, Bb must quickly adapt to a wide range of arthropod and vertebrate environments in order to survive. Many of these changes are associated with rapid expression changes involving multiple surface lipoproteins [56]. For example, OspA is highly expressed on the surface of Bb within the tick midgut or when cultured in BSK medium at ambient temperatures [47]. However, the process of tick feeding or increases in temperature cause the rapid downregulation of OspA and subsequent upregulation of numerous lipoproteins associated with mammalian infection, such as OspC [46, 57], the Erp family proteins [30], and different modifications of the *vlsE*-expressed lipoprotein [58]. Thus, Bb introduced into a murine host will differentially express a large number of different lipoproteins during the course of infection and should subsequently present a much broader range of antigens to host immune cells than Bb that are rapidly killed.

Our studies determined that the Bb proteins recognized by sera from mice injected with killed Bb were distinct from the proteins recognized by sera from mice injected with live Bb. Based on size and the OspA control, the response to killed Bb appeared largely directed against protein bands consistent with OspA monomers (~31 kDa) and dimers (~62 kDa), while the response to live Bb appeared directed to proteins with band sizes consistent with p39 (~39 kDa), flagellin (~41 kDa), and p93 (~93 kDa); these different reactivities to proteins recognized on live Bb have been previously noted by different investigators [8, 46, 59–61]. Because these changes in antigen expression have been shown to correspond with active infection, our western blot data confirms that the significant differences in ELISA values can accurately distinguish between animals that experienced a progressive Bb infection versus an exposure to bacteria that could not adapt to escape host clearance.

Subsequent to inoculation in a murine host, Bb are known to disseminate from the skin and persist in a wide range of tissues (including the skin). While the precise environment that these bacteria prefer to persist within is not well defined, they are believed to largely exist extracellularly. Bb can associate with collagen bundles [62, 63], decorin-associated tissues [41, 64], or relatively avascular spaces throughout their host [65], all of which might provide some protection from immune recognition/clearance. Alternatively, Bb might be able to persist within some immune cells subsequent to phagocytosis, with both live and killed bacteria appearing to remain intact for extended periods of time [66]. These possibilities suggest that even Bb that are killed within host tissues might persist for extended times and, thus, could allow their cellular contents to be detected by assays designed to detect viable persistent bacteria, such as PCR of infected tissues. We addressed this by injecting mice with live and killed Bb and following the Bb content in the skin over time by PCR analysis. Levels of PCR-detectable bacteria declined to similar low levels by 8 h after injection in both groups, likely due to both the degradation of DNA content from killed Bb, as well as the dissemination of live Bb away from the harvested skin injection site. However, the Bb DNA content in tissues receiving live bacteria increased to reach peak values by 96 h and subsequently remained low but significant. This pattern is consistent with previous reports of Bb persistence within skin tissues [8, 12]. Our current studies suggest that killed Bb and their cellular content are efficiently cleared in skin tissues and should not be detectable by PCR methodologies within hours of being killed. In summary, both serological and PCR-based methods of assessing Bb infection clearly distinguish between an established infection with live bacteria and exposure to even large numbers of bacteria that are cleared early by the innate responses.

Acknowledgments

This work was supported by the Scientist Development Grant 0335148N from the American Heart Association (R. M. Wooten) and, Public Health Service Grant R01-AI073452 from the National Institute of Allergy and Infectious Diseases (R. M. Wooten). The authors wish to thank Robert Blumenthal and Isabel Novella for helpful discussions in writing this paper.

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