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HUMAN LYME NEUROBORRELIOsis

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HUMAN LYME
NEUROBORRELIOsis

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Absence of evidence is not evidence of absence.

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From the time that Dr. Allen C. Steere, a rheumatologist at Yale University, first discovered Lyme disease when a strange cluster of cases occurred in families in the area of Lyme, Connecticut, during the summer of 1976, medical scientists have fervently pursued research on this so-called mystery illness. So-called because initially Lyme mimicked a myriad of other diseases and finding its cause was certainly not an easy task. It wasn’t until 1982, in fact, that the etiologic agent, Borrelia burgdorferi, was finally identified by Dr. Willi Burgdofer in the bodies of tiny “deer ticks” of the genus Ixodes, namely scapularis (dammini) and pacificus in the United States. There remains to this day, almost 40 years later, a great deal of complexity and difficulties on many fronts of Lyme disease especially with regard to diagnosis and treatment. Dr. David S. Younger’s new book entitled, “Human Lyme Neuroborreliosis” sheds a great deal of light on the most significant issues of Lyme Disease, and, as a neurologist, he concentrates his efforts on Lyme neuroborreliosis (LNB). As Dr. Younger succinctly put it, “Lyme disease has moved into the spotlight because of the annual toll of neurological disease most prominently, early childhood facial palsy, subacute adult meningitis, cranial neuritis, and radiculoneuritis, and late-stage peripheral neuropathy and encephalopathy, some of which have the potential for improvement with appropriate antimicrobial therapy”.

An extensive historical overview is provided in the books’ first chapter wherein Dr. Younger describes historical aspects of Lyme in both American and European patients, caused by Borrelia burgdorferi, sensu stricto and sensu lato, respectively.

In chapter two, of Dr. Younger’s seven-chapter text, he presents the epidemiology of Lyme disease. About 30,000 cases are reported annually in
the US, but the CDC estimates that there are actually about 300,000 cases. As such, “it is the most commonly reported vector-borne illness in the United States”. Younger also provides case definitions for surveillance purposes, which are important for epidemiologic purposes, but he astutely warns, such “rigorous case definition in clinical practice would exclude many patients with less compelling, incomplete, or atypical presentations from empiric treatment”.

The Blood-Brain Barrier (BBB) is the topic of the third chapter, which provides an extensive overview and review of the historical background of BBB starting with Dr. Paul Ehrlich’s 1885 experimental observations in mice, the neuronal development of BBB, its ultrastructure and function, and its implications for Lyme neuroborreliosis. Dr. Younger cautions that while breakdown or disruption of the BBB that accompanies a variety of inflammatory, autoimmune, neoplastic, infectious and neurodegenerative CNS disorders, is also common to Lyme neuroborreliosis, but “has yet to be critically analyzed.”

Non-human Primate animal models are discussed in Chapter four. Some of the great pioneering animal studies in the US and Great Britain were discussed which actually shed some light on several important aspects of human Lyme borreliosis and neuroborreliosis. It turns out that the rhesus monkey provides a good model for human B. burgdorferi infection because most of the salient phases and features of the disease are present in that animal. Several interesting findings were gleaned from these reported experiments. One such finding was that CNS and PNS tissues “were identified as major reservoirs of spirochetal infection and they demonstrated that a strong, well developed anti-B. Burgdorferi humoral immune response did not clear spirochetes from the animals during the months of infection, especially in the nervous system.” There was other interesting, as well as, helpful findings presented, and it is clear that the rhesus monkey has proven to be a useful animal model for B. burgdorferi infection.

The neurological clinical aspects of Lyme disease encompassing three major systems, the CNS which include the brain and spinal cord, the PNS which includes exiting cranial and spinal nerves coalescing to form distal peripheral nerves, and lastly the autonomic nervous system comprising a complex network of central and peripheral fibers that begin in the hypothalamus, and end in organs and the tissues it innervates, is discussed in Chapter 5. Dr. Younger emphasizes that Lyme disease can affect one or all of the aforementioned systems resulting in characteristic clinical features of neuroborreliosis. He differentiates clinical manifestations of this infection in
other parts of the world as well as discusses the different species of ticks involved. In Europe I. ricinus is the common tick while I. persulcatus predominates in Asia. In North America the only bacterium involved is B. burgdorferi sensu stricto, while in Europe and Asia B. afzelii and B. garinii can cause the disease. Dr. Younger also cautions that “early treatment might lead to falsely negative serological test results as may also occur in immune-compromised individuals and immune-competent patients with true infection that are tested before a Lyme IgM Western blot immune response is mounted.” Younger discusses the etiopathogenesis of nervous system damage in LNB relating that “it is not well understood but likely related to aspects of the infectious process and post-infectious autoimmune host factors”.

Chapter six entitled “Serological Diagnosis” is most important because testing for Lyme disease is at the heart of many controversies. The most commonly used test is the two-tier ELISA and immunoblot or Western blot assays. This testing has a varied efficacy (sensitivity and specificity) dependent upon the manufacturer of the test as well as the laboratory performing it. Also Lyme ELISA can result in false positives due to cross reactivity with antigenically similar organisms such as spirochetes like Syphilis, and treponemal periodontal disease. Younger cites one review of commercially available ELISAs, sensitivities ranged from 29% to 75% but no more than 68% if specificity was not compromised. In a similar review of western blotting, sensitivities range from 46% to 50% for commercial kits and no more than 80% for reference tests. In a well-defined patient study eight different ELISA systems were based on whole cell antigens supplemented with V1sE and assays used exclusively recombinant proteins. A subset of samples was tested in five immunoblots. The number of IgM and/or IgG-positive ELISA results in the group of patients suspected of Borrelia infection ranged from 34 to 59%. Dr. Younger reported that “the concept of the two-tiered testing, which employs a screening ELISA that, if positive is confirmed by a western blot assay, was developed for epidemiological surveillance of suspected cases of Lyme borreliosis, and is not intended to be used for diagnosis”. He also discusses PCR testing of synovial fluid, biopsies of EM, and ACA rashes achieved fairly good results but had a poor sensitivity often shows false negative results with blood and CSF specimens. However new molecular methods and techniques are on the horizon. Further he discusses the recent culture technique for Borrelia using a modified BSK-H media for both short (6 days) and long term (8 and/or 16 weeks) which resulted in positive culture result in 47% of sera in 6 days, 83% at 8 weeks, and 94% at 16 weeks. As of this writing that method has not yet been confirmed. Certainly in clinical

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microbiology finding the etiologic agent of a disease has been the gold standard. Hence a perfected culture technique might prove to be invaluable asset for the diagnosis of Lyme disease. There are newer versions of this type culture method already on the horizon. Younger concludes this chapter by discussing C6 ELISA test and then significance of CXCL13 Chemokine as a biomarker for LNB. In reality, except for culture or PCR, there is no practical means of detecting the presence of the organism, as serologic studies only test for antibodies of Borrelia. Complicating all of this testing is the reality that Ixodes ticks can carry other tick-borne agents of disease like Babesia, Bartonella and Ehrlichia which have been know to co-infect patients.

The last chapter in the text discusses treatment of LNB. Younger reports that while the most effective antibiotics used for treatment include doxycycline, amoxicillin, cefuroxime and ceftriaxone, for localized early, or late stage systemic and nervous system infections, they are all relatively inexpensive drugs. However he cautions, “early treatment and prevention are the mainstays of treatment in those with early Lyme disease to forestall neurological involvement”. Younger lists suggested therapies based on patients’ neurologic symptoms, as well as the suggested first, second, and third line treatment regimens for both adults and children. He concludes the chapter by saying that “LNB can be treated with oral and parenteral antibiotics in uncomplicated patients, and combined with immune modulatory therapy for complicated cases”.

The content of this text will prove to be a valuable resource for both neurology specialists, as well as internists, general practitioners, and for that matter any other medical practitioners interested in Lyme disease. This text concisely addresses the most salient issues involving Lyme neuroborreliosis in a succinct manner, which adds to its appeal.

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Preface

I was trained to critically analyze diseases in medical school and later during internal medicine and neurology residencies and postdoctoral fellowships. In writing articles, I found it most rewarding to start by establishing the historical background of the illness I was researching and then to read all of the original articles I cited to be sure that I could formulate my own impressions. In my clinical practice I was fortunate to be able to see a variety of patients with Lyme disease who had neurological complications. Over the past several years I have pursued Masters Degrees in Public Health and Epidemiology to better understand disease processes.

Lyme disease is a disorder of immense proportion and importance. I devoted the past six months to a concerted effort to produce a volume on Lyme neuroborreliosis that would have scholarly appeal to investigators in biology, medicine, pharmaceutical and investigative research. It has been organized in the same manner as I conceptualized my understanding of the illness beginning with an overview of the epidemiology and a comprehensive history of Lyme neuroborreliosis. The non-human primate animal model of the diseases is reviewed as well as a comprehensive chapter on the blood-brain barrier with implications for Lyme neuroborreliosis. The volume continues with a detailed account of the clinical presentation and aspects of neurological laboratory diagnosis, followed by an up-to-date review of serological assessment. The final chapter delves into the background of antimicrobial treatment and asserts recommended regimens for affected children and adults.

I am thankful to Nova Biomedical for allowing me to author this volume. I wish to extend my sincere appreciation to two stellar New York University colleagues, Steven Galetta MD, Philip K. Moskowitz MD Professor and Chair of Neurology, and Cheryl Healton DPH, Director of the Global Institute of

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Public Health, for allowing me to work alongside them and other inspired colleagues. I was assisted by New York University Research Associate Kyra Doumlele in the preparation of the manuscript and figures. Finally, I would not have written this book without the support of my spouse Holly, sons Seth and Adam, and our dog Delilah, who all stood patiently while I assiduously worked to finish this book by the start of tick season.

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Historical Overview

Abstract

First described by Garin and Bujadoux in 1922 as meningoradiculitis associated with Lyme disease in European cases, nervous system manifestations have been recognized in endemic regions of the United States since at least 1972 when investigators commenced initial retrospective analysis of children and adults in contiguous Connecticut communities with recurrent episodes of arthritis sometimes preceded by an expanding an erythema migrans rash. Contemporaneously identified as a triad of meningitis, cranial neuritis and radiculoneuritis, the spirochete etiology of Lyme disease was later revealed to be due to the bite of *Ixodes dammini* ticks infected by the bacterial organism *Borrelia burgdorferi*. The nervous system manifestations of Lyme disease have since been well studied revealing a far wider reaching disorder affecting the central and peripheral nervous system often with unpredictable early and late manifestations. This chapter reviews the historical aspects of Lyme disease and Lyme neuroborreliosis.

Introduction

The historical aspects of nervous system Lyme disease have been previously reviewed [1]. The disease was originally named for Lyme and Old Lyme Connecticut, wherein a tight clustering of recurrent attacks of childhood and adult asymmetric oligoarticular arthralgia occurred beginning in 1972. Recognition of the nervous system manifestations later followed later
revealing the full spectrum of peripheral and central nervous system involvement due to the tick-borne spirochete *Borrelia burgdorferi*, hereafter referred to as *B. burgdorferi*, sensu stricto in North America [differentiated from *B. burgdorferi*, sensu lato for European Lyme disease].

**European Patients**

In 1922 Garin and Bujadoux published the first report of meningoRADICULITIS associated with Lyme disease. Originally published in the Journal de Medicine de Lyon [2], the original translated case [3] described a 58-year-old man admitted to a French hospital ward with pain, paralysis and wasting of the right arm and left sciatic and low back pain. Three months earlier he had been bitten by a tick on the left buttock which was the site of later sciatic pain followed by pain radiating from the right brachial plexus to the right cubital fossa. An inflammatory ring of skin redness was noted on the left buttock with a bite mark at the ring’s center which later increased, becoming large, red and uniform without vesicles and occupying the entire buttock. Examination of the nervous system showed marked paralysis of the right deltoid without objective sensory loss and normal tendon reflexes. Cerebrospinal fluid (CSF) showed 75 lymphocytes with an elevated albumin level and normal glucose. He slowly improved following treatment with four injections of the arsenical neoarsphenamine, used to treat syphilis.

Two decades later in 1944, Bannwarth [4] described 13 patients with aseptic meningitis accompanied by radicular pain and radicular sensory and motor disturbances, sometimes associated with cranial nerve palsies predominantly involving the facial nerve. Although considered a rheumatic disorder, in 1983, Sköldenberg and colleagues [5] described 21 patients with persistent or progressive chronic meningitis preceded by localized erythema migrans (EM) rash during the summer or fall with a preceding tick bite and later development of fatigue, malaise, weight-loss and fever followed by facial nerve paralysis, motor and sensory peripheral radiculoneuropathy, and myelitis. Noting antibodies against *Ixodes (I.) dammini* spirochetes in the CSF of three patients, and a favorable response to a 2-week course of intravenous penicillin, Sköldenberg and colleagues [5] postulated a European spirochete vector, similar to the vector of Lyme disease isolated one year earlier by Burgdorferi and colleagues [6].

In 1987 Vallat and colleagues [7] described 10 adults from rural areas around Limoges, France who were bitten by *I. ricinus* tick in the summer and
Historical Overview

fall later developing a cutaneous rash along the leg close to the bite followed by asymmetrical radicular pain, leg weakness, cranial nerve involvement and areflexia. Objective sensory loss was limited to affected nerve roots. CSF showed lymphocytic meningitis with elevation of the protein content. Positive anti-\textit{B. burgdorferi} antibodies were detected in 3 patients including one with convalescent titers that showed a reduction in the antibody titer along the course of the disease. Electrodiagnostic studies and sural nerve biopsy confirmed an inflammatory peripheral neuropathy without vasculitis. Such findings agreed with the histopathological findings of a postmortem confirmed case [8] that included perivascular meningeal, spinal and nerve root inflammation by lymphocytes and plasma cells. The patients described by Vallat and colleagues [7] too were reminiscent of those first encountered by Garin and Bujadoux [2] and Bannwarth [4] with the added characteristic feature of the conspicuous absence of arthritic signs [9], although a few such cases with joint involvement had been reported [10].

\textbf{United States Patients}

Lyme arthritis, a new form of inflammatory arthritis was occurring in eastern Connecticut at least since 1972 with a peak incidence of new cases in the summer and early fall [11, 12]. In 1977 Steere and colleagues [11] studied 51 residents including 39 children and 12 adults in three contiguous Connecticut communities that constituted a tight geographic clustering of cases. Epidemiologic analysis of the clustering suggested transmission of a causative agent by an arthropod vector to humans in whom 25\% described an expanding annular EM rash before onset of the arthritis. Cultures of the synovium and synovial fluid did not suggest infection with agents known to cause other forms of arthritis. Malaise, fatigue, chills, fever, headache and stiff neck were the commonest symptoms associated with onset of the skin lesion, even preceding it by a few days. Some patients had backache, myalgia, nausea and vomiting, and sore throat that lasted for a few days but in some were recurrent or more persistent. Skin biopsy specimens taken at the center and borders of the initial lesions in 8 of 12 patients showed edema of the papillary dermis and heavy infiltration by mononuclear inflammatory cells around blood vessels and all layer of the dermis in addition to keratin thickening, intracellular and extracellular edema, and hemosiderin epidermal deposits. Those who developed arthritis appeared to have significantly elevated erythrocyte sedimentation rates (ESR), lower third and fourth components of
complement (C3, C4), higher serum IgM levels, and serum cryoprecipitates at the time of the skin lesions compared to those who remained well. In retrospect, in addition to arthritis, 24 (48%) of patients developed neurological symptoms or signs typically after the skin rash and before development of arthritis. Two patients developed unilateral facial palsy, one of whom also demonstrated asymmetrical sensory radiculopathy with belt-like tightness and pain along the T10 dermatomes. Two developed clinical and CSF evidence of lymphocytic meningitis. Twelve others demonstrated fever, headache and stiff neck during the first few days of the illness associated with the skin lesions, and 8 had similar complaints 2 to 11 weeks before arthritis in the absence of skin lesions equally suggestive of aseptic meningitis, only one of whom had a lumbar puncture that showed acellular CSF. Eight patients had transient regional or generalized hyperesthesia to touch or temperature.

Two years later, Reik and colleagues [13] summarized the neurological abnormalities of Lyme disease among 18 patients with neurological involvement diagnosed by the presence of EM in 14, and arthritis in 4, 43% of whom recalled being bitten by ticks at the site of the exanthema 3 to 20 days before onset, including one patient who claimed to being bitten at the site of EM 2 years previously. Their illness generally began with EM along with the first signs of neurological involvement while the skin lesion was still present in 8 patients and 1 to 6 weeks after it faded in 6 patients. In the 4 patients diagnosed with Lyme arthritis without EM, neurological involvement preceded arthritis by 5 to 7 months in 3 patients, but arthritis preceded neurological involvement by 2 months in the remainder. Widespread neurological involvement was common including meningitis, encephalitis, chorea, cerebellar ataxia, cranial neuritis, motor and sensory radiculoneuritis, mononeuritis multiplex, and myelitis. However, headache and stiff neck were most common, with involvement limited to a single site in only 3 patients including encephalitis in 1 patient, and 2 patients with facial palsy alone. Nine patients experienced a single episode lasting 2 weeks to 3 months, while the other 9 had multiple episodes separated by asymptomatic periods lasting 2 days to 3 months with a usual pattern of fluctuating meningoencephalitis and superimposed cranial and peripheral neuropathy or radiculopathy.

In 1982 Burgdorfer and colleagues [6] isolated a spirochete from *Ixodes dammini* that bound immunoglobulins of patients convalescing from Lyme disease and recorded the development of lesions resembling EM in New Zealand White rabbits 10 to 12 weeks after being fed upon by the ticks. Electron microscopy of midgut diverticula revealed spirochetes closely associated with the microvillar brush border of the gut epithelium (Figure 1).
Irregularly coiled spirochetes ranging in size from 10 to 30 \( \mu \)m in length of 0.18 \( \mu \)m to 0.25 \( \mu \)m in diameter with ends tapered and four to eight filaments inserted subterminally at each end were observed. The \textit{I. dammini} spirochetes were isolated by inoculating 0.1 ml of a suspension prepared from midgut tissues of four infected ticks into modified Kelly’s medium and after 5 days of incubation at 35 degrees, the culture tubes contained spirochetes that could be regularly subcultured and maintained at that temperature. Sections of skin biopsy specimens from the rabbits that emerged after placement of the feeding ticks were stained with hematoxylin and eosin (H&E) showing thickened hyperkeratotic epidermis, with dermal dense mononuclear cell infiltration of the superficial layer of skin. However attempts to isolate spirochetes from suspensions of biopsied skin lesions in Kelly’s medium were negative. When tested by an indirect immunofluorescence (IF) method \[14\], antibodies to the spirochetes were present in the serum of all rabbits on which the ticks had fed 30 to 60 days earlier at titers of \( \geq 1:1280 \). The antigenic basis of the relation to Lyme disease of the \textit{I. dammini} spirochete was established by a positive reaction ranging from 1:80 to 1:1280 among serum samples of nine patients with clinically diagnosed Lyme disease by means of indirect IF compared to non-reactivity among 4 other serum samples from individuals from New York and 10 from Montana with no history of the disease in whom titer ranged no higher than 1:20. Their results established not only the susceptibility of the domestic rabbit to the \textit{I. dammini} spirochete but the possible value of indirect IF test as a diagnostic tool for Lyme disease. Furthermore, their results indicated the need for investigations to look into the epidemiology and ecology of Lyme disease and related disorders such as EM of Europe notwithstanding the nature of the relation between the spirochete and its \textit{I.dammini} vector.

One year later in the same volume of \textit{The New England Journal of Medicine}, Steere and coworkers \[15\] and Benach and colleagues \[16\] described the spirochetal etiology of Lyme disease. Steere and coworkers \[15\] reported recovery of the newly recognized spirochete from the blood, EM lesions or CSF of 3 of 56 patients with Lyme disease and from 21 of 110 nymphs or adult \textit{I. dammini} ticks in Connecticut. In patients, specific IgM antibody titers reached a peak between the third and sixth week after onset of the disease whereas specific IgG antibody titers rose more slowly generally becoming highest months later when arthritis was present. Among 40 patients with EM alone, 90% had an IgM titer \( \geq 1:128 \) between EM and convalescence, whereas among 95 patients with later manifestations including involvement of
the nervous system, 94% had elevated titers of IgG of ≥1:128 in comparison to normal IgG titers among 80 control subjects.

Benach and colleagues [16] isolated spirochetes from the blood of 2 of 36 patients in Long Island and Westchester County, New York with signs and symptoms suggestive of Lyme disease that were morphologically similar and serologically identical to organisms known to infect I. dammini ticks, endemic to the area and epidemiologically implicated as vectors of Lyme disease. Disease onset in Patient 1, a 21-year-old man and summer resident of Fire Island commenced with EM rash emerged two days after a tick bite of the lower back followed four days later by fever, myalgia and generalized pain. The disease in Patient 2, a 70-year-old also summer resident of Fire Island commenced with an EM rash of the inner thigh without known tick bite, followed by flu like symptoms, myalgia of the back and neck, and swelling of the left knee. The mode of antibiotic therapy which was unspecified in patient 1, and noted to be parenteral penicillin in Patient 2, was associated with sustained improvement. In both patients there was a rise in specific anti-

Figure 1. Electron micrograph of I. dammini spirochetes (SP) associated with microvillar brush border (MV) of the tick’s midgut (X55, 440). Insert shows cross-section of spirochetes (X122, 100).
spirochetal antibodies in paired specimens of serum concluding that the I. dammini spirochete had an etiologic role in Lyme disease.

Two years later, Pachner and Steere [17] studied 38 patients with neurological manifestations of Lyme disease. In 34 of the 38 patients, the first stage of the illness began in the summer with EM in whom 38% recalled a tick bite at the site of the lesion within 1 to 2 weeks of onset. EM was often accompanied by malaise, fatigue, headache, stiff neck, fever, myalgia, arthralgia, dysesthesia, sore throat, and abdominal pain. In 3 patients, meningitis was the first manifestation of the disease, and 1 remaining patient experienced only prolonged prodromal symptoms before onset of neurological abnormalities. The second stage of neurological manifestations commenced about a month after onset of EM after a latent period with a characteristic triad of meningitis, cranial neuritis, and radiculoneuritis in respective frequencies of 89%, 50% and 32%. However different clinical patterns of neurological involvement were encountered. One pattern was lymphocytic meningitis recognized by severe headache, stiff neck, and nausea and vomiting after EM. A second pattern was EM and associated severe headache followed weeks later by cranial neuritis and two months later by radiculoneuritis also in the setting of lymphocytic pleocytosis on lumbar CSF analysis. A third pattern was EM followed two weeks later severe headache, cranial neuritis and relapsing mononeuritis multiplex or radiculoneuritis in association with later lymphocytic CSF pleocytosis. A fourth pattern was lymphocytic meningitis followed 2 months later by cranial neuritis, bilateral radiculoneuritis and mononeuritis multiplex, also in the setting of lymphocytic pleocytosis on CSF analysis. Although the duration of neurological symptoms varied according to the timing and mode of treatment, in 16 patients treated with high-dose parenteral penicillin, symptoms of meningitis resolved within 10 days of therapy. The clinical picture of Lyme neuroborreliosis (LNB) presented by Pachner and Steere [17] was similar to the European cases [2, 5, 7] differing however in the absence of arthritis.

In 1987 Halperin and colleagues [18] summarized the peripheral nervous system (PNS) manifestations of LNB. Of 36 patients with proven late Lyme disease seen in the Lyme Disease Clinic at University Hospital, State University of New York, Stony Brook between 1985 and 1986 with compatible clinical histories consistent with the diagnosis of and immunologic evidence of reactivity to B. burgdorferi, 14 (36%) had prominent limb paresthesia, and all but one had electrodiagnostic evidence of peripheral neuropathy. Although 10 patients had been treated with parenteral penicillin therapy [19], twelve other patients were studied before and after antibiotic
treatment all with significant clinical improvement. Sural nerve biopsies in 2 patients showed changes of segmental demyelination without changes of axonal degeneration, segmental demyelination, focally positive epineurial IgM, C3 and properdin deposition, and negative B. burgdorferi IF. Their observations lead them to conclude that many patients with Lyme disease had significant PNS abnormalities that were resolvable with appropriate antibiotic treatment. In some instances neuropathy continued to evolve despite treatment with parenteral penicillin therapy [19] whether due to insensitivity of the spirochete or inadequate penetration of the antibiotics into the nervous system. It was striking that several patients responded to more prolonged or higher dose penicillin regimens, as well as to treatment with other agents known to cross the blood-brain barrier (BBB) such as ceftriaxone and chloramphenicol. Quite separate from central nervous system (CNS) manifestations of Lyme disease, PNS involvement appeared to be a consequence of the direct effect of infection with spirochetes and not a purely immune-mediated phenomenon.

In 1989, Halperin and coworkers [20] evaluated 85 patients with serological evidence of B. burgdorferi infection noting encephalopathy in 41, neuropathy in 27, meningitis in 2, multiple sclerosis (MS)-like illness in 6, and psychiatric disorders in 3. Twelve of 18 patients with encephalopathy, meningitis or focal CNS disease had evidence of intrathecal synthesis of anti-B. burgdorferi antibodies compared to none with either MS-like or psychiatric disorders or 2 of 24 with neuropathy. The authors concluded that intrathecal concentration of specific antibody was a useful marker of CNS B. burgdorferi and that Lyme disease itself was the cause of encephalopathy. The authors used two different methods to compare CSF and serum antibody concentrations. First, enzyme linked immunosorbant assays (ELISA) were performed on serum diluted 1:500 and on CSF diluted 1:1 and optical density (OD) were measured. Concentration of IgG in serum and CSF were measured by laser nephelometry and a CSF Lyme antibody index was calculated according to a described methodology [21]. Another method measured relative IgG concentrations in serum and CSF, diluting the serum 1:500 and the CSF so that the final IgG concentrations were identical to that in the diluted serum and performing ELISA simultaneously on the same plate. The CSF Lyme antibody index was then calculated by determining the ratio of the OD of the CSF to that of the serum. Indices greater than 1.0 were interpreted as evidence of intrathecal synthesis of specific antibody as previously described for European LNB [22]. Of 17 who underwent magnetic resonance imaging (MRI), 10 were normal and 7 demonstrated multiple small white matter lesions that were hyperintense on proton-weighted and T2-weighted images.
but isointense on T1-weighted images. Four such patients with abnormal MRI lesions were found to have CSF Lyme indices exceeding 1.0 and of scans repeated following antibiotic treatment in 6 of the patients, 3 showed signal hyperintensity resolution. The authors concluded that there were a broad range of possible disorders apart from meningitis, cranial neuropathy and painful radiculitis, including a mild chronic encephalopathy especially in those with long-standing Lyme disease. Altogether, 10 of 13 patients with encephalopathy and one-half of those with focal CNS disease had evidence of intrathecal synthesis of specific anti-*B. burgdorferi* antibody in contrast to 2 of 24 patients with peripheral neuropathy. The high concentration of specific antibody in the CSF of patients with encephalopathy suggested that CSF anti-*B. burgdorferi* antibody concentration was a useful indicator of CNS involvement, especially in those with chronic LNB associated with subtle difficulty of concentration and memory presumably due to a reversible low-grade inflammatory process.

Later that year in 1989 Steere [23] summarized the causation, vector and animal hosts, clinical manifestations, pathogenesis, and treatment of human Lyme disease. Three stages of infection due to *B. burgdorferi* were recognized, each with different clinical manifestations. Stage 1 followed injection by the tick with spread of *B. burgdorferi* locally in the skin in 60 to 80% of patients resulting in EM that faded in 3 to 4 weeks but could occur often accompanied by fever, minor constitutional symptoms or regional adenopathy. At this time the patient’s mononuclear cells responded minimally to spirochete antigens and even specific antibody might be lacking. Stage 2 of early infection followed days or weeks after inoculation with bloodstream or lymphatic spread to many organ sites. More common in the United States (U.S.) than in Europe, widespread dissemination resulted in recovery of spirochete from tissue specimens of meninges, brain, myocardium, retina, muscle, bone, synovium, spleen, and liver [24]. In the rat model of the disease, the spirochete could be cultured from all organs five days after inoculation but positivity gradually disappeared from most sites [25] such seemed likely to occur in patients as well. After hematogenous spread, those with stage 2 nervous system involvement in the U.S. developed frank neurological involvement [13, 17] that typically included meningitis and superimposed cranial neuritis or peripheral neuropathy more commonly and occasionally encephalomyelitis, compared to European LNB cases that most often commenced with radicular pain followed by CSF pleocytosis with less frequent meningeal or encephalitic signs [26, 27]. By this time mononuclear cells demonstrated heightened responsiveness to specific *B. burgdorferi* antigens
and mitogens, with elaboration of specific IgM antibody responses often directed at the 41-flagella antigen of the spirochete. Such as frequently associated with polyclonal activation of B-cells with elevated total levels of IgM antibody and presence of cryoprecipitate and circulating immune complexes. This was followed by the elaboration of specific IgG antibody and an array of spirochetal polypeptides notably to 31, 34, and 66 KD outer surface proteins and the 55/58 KD antigen. Histologically affected tissues demonstrated infiltration of lymphocytes with plasma cells with some degree of vascular damage or hypercellular occlusions. Late infection characterizing stage 3 disease was typified by episodes of arthritis lasting months or rather than weeks and even more chronic involvement. Late syndromes in the CNS and PNS included reports of progressive encephalomyelitis in European LNB cases [28] and subacute encephalitis, dementia and demyelinating diseases although typically without intrathecal production of anti-B. burgdorferi antibody [29-31]. More commonly, the U.S. patients manifested subtle CNS and PNS syndromes late in the illness including intermittent distal paresthesia or radicular pain for more than a year after onset of the disease [18] or subtle symptoms of CNS involvement such as memory loss, somnolence or behavior changes after the more classic signs of LNB disappeared. With inconsistent evidence of intrathecal production of the specific anti-B. burgdorferi antibody, it was difficult to tell whether symptoms were related to active CNS infection. Steere [23] note that stage 2 neurological involvement resolved upon parenteral antimicrobial therapy in all patients with objective neurological abnormalities except those with facial palsy and no abnormalities of the CSF. Ceftriaxone became the most readily used antibiotic agent because it crossed the BBB more readily than intravenous penicillin and required once-a-day administration. Treatment of stage 3 joint or neurological abnormalities was more problematic however ceftriaxone remained the drug of choice in a randomized comparison of ceftriaxone and penicillin that demonstrated lack of response in 5 of 10 patients who received intravenous penicillin compared to only 1 of 13 who received ceftriaxone [32].

One year later, Logigian and colleagues [33] defined chronic PNS and CNS abnormalities of Lyme disease in a cohort of 35 patients with an overall median duration of symptoms of 12 months (range 3 to 168 months), respectively beginning 16 months (range 1 to 156 months) and 26 months (range 1 to 168 months) after EM. Ten patients with memory difficulty, depression or headache were excluded with normal neurologic tests and negative or indeterminate antibody responses to B. burgdorferi or underactivity of mononuclear cells to B. burgdorferi antigens; as were 5 others
with dementia, demyelinating disease or headache that were instead deemed to have Alzheimer disease, MS or brain tumor in spite of positive anti-\textit{B. burgdorferi} antibody responses in 4 patients. The remaining 27 patients that comprised the study group had: 1) Neurological abnormalities caused by infection with \textit{B. burgdorferi} for at least 3 months that could not be attributed to another cause; 2) Available neurological evaluations including lumbar puncture, detailed electrodiagnostic testing, and MRI data; and 3) Current evidence of humoral or cellular immunity to \textit{B. burgdorferi} as shown by elevated serum IgG or IgM antibody titer, five or more IgG antibody bands to spirochetal polypeptides [34] or a stimulation index of 10 or more in response to \textit{Borrelia} antigens [35]. Altogether 19 (70%) of patients had polyneuropathy and all but 2 had encephalopathy, while 24 (89%) had mild encephalopathy with prominent memory difficulty in 22 (81%), depression in 10 (37%), and fatigue in 8 (30%). Brain MRI in 3 patients with encephalopathy and polyneuropathy showed small areas of T2-signal intensity as did another with encephalopathy alone. One patient had leukoencephalitis with asymmetric spastic diplegia, periventricular white-matter lesions, and intrathecal production of antibody to \textit{B. burgdorferi}. Treatment with a 2 week course of intravenous ceftriaxone led to sustained improvement in 17 (63%), temporary benefit in 6 (22%), and no improvement in 4 (15%) patients. The authors concluded that months to years after initial infection with \textit{B. burgdorferi}, patients with Lyme disease could manifest chronic encephalopathy, polyneuropathy, or less commonly leukoencephalitis and that these chronic neurologic abnormalities usually improved with intravenous antibiotic therapy.

One year later, Krupp and colleagues [36] evaluated neurobehavioral functioning following treatment in a cohort of 15 patients, that included oral antibiotic therapy in 9 patients who received 3 weeks of oral amoxicillin or doxycycline, and 6 patients who received ceftriaxone therapy with a mean interval between treatment and neuropsychological testing of 6.7 months (range, 3 to 12 months). All patients underwent neurological and neuropsychological evaluation that included a bedside mental status evaluation, MRI, total anti-\textit{B. burgdorferi} antibody activity employing OD measures [35], with values greater than 3 standard deviations above the mean for controls considered positive. The concentrations of anti-\textit{B. burgdorferi} IgG were measured in the serum and CSF and adjusted by dilution until the final CSF and serum IgG concentrations were identical. ELISA was performed on CSF and serum simultaneously on the same plate and CSF for anti-\textit{B. burgdorferi} was considered positive if the CSF OD exceeded the serum negative cutoff value equivalent to a CSF antibody index of $\geq 1.0$. Intrathecal
synthesis of anti-\textit{B. burgdorferi} antibody was considered positive when the ratio of CSF to serum OD was $\geq 1.0$ \cite{20}. Neuropsychological evaluation consisted of measures to assess global intellectual ability, verbal fluency, conceptual reasoning, visual spatial ability, and memory functioning. In addition, subjects were screened for depressive symptoms and fatigue by appropriate scales. The results of neuropsychological testing were compared to a cohort of 10 healthy controls matched for age and years of education.

Altogether, neuropsychological evaluation revealed evidence of cognitive impairment in 9 (60\%) patients that was rated as mild in 1, moderate in 6, and severe in 2 patients. In 6 other patients, the neuropsychological evaluation was normal. CSF in 11 patients showed positive anti-\textit{B. burgdorferi} titers that exceeded the negative serum cutoff values in 5 patients, only one of whom was considered positive for intrathecal synthesis; and in 6 whose CSF antibody titer was less than the negative serum cutoff value. MRI of the brain performed in 8 patients showed normal results in 6, while 2 patients demonstrated one or more small areas of increased signal intensity on $T_2$-weighted images in subcortical white matter. The study and control groups differed significantly in verbal memory functioning with the former showing substantially poorer performance. Among the 9 patients who received oral antibiotic therapy, 3 demonstrated normal cognitive functioning and 6 showed impaired cognitive function. Of the 6 patients treated with intravenous antibiotic therapy, cognitive therapy was equally normal and impaired in 3 patients respectively. There was no relation between neuropsychological function and either MRI or CSF findings, however depression and fatigue were both correlated with memory performance, with depression inversely related to memory deficits. The authors conceded that patient selection might have been biased and generalizability of the results was limited. In the absence of a correlation between encephalopathy and intrathecal production of anti-\textit{B. burgdorferi} antibody, the authors \cite{36} suggested that encephalopathy might have been caused by the immunological consequences of systemic infection including release of pathogenic cytokines or other toxic-metabolic factors. Since some of the treated patients had entirely normal neuropsychological results but high scores on a depressive symptom scale, the encephalopathy in such patients might have been confounded by concomitant depression.

The next year, Luft and coworkers \cite{37} conducted a prospective study of CNS involvement in acute disseminated \textit{B. burgdorferi} infection by measurement of \textit{Borrelia}-specific deoxyribonucleic acid (DNA) using the polymerase chain-reaction (PCR) assay with comparison to standard serological tests. Among 12 patients with acute disseminated Lyme borreliosis
and less than two weeks of active disease and 16 control sera, 4 of 6 study cases with EM and 4 of 6 with cranial neuritis without EM, demonstrated evidence of CSF B. burgdorferi-specific DNA, compared to none of 16 control samples. Only 4 of 8 study cases found to have spirochetal DNA in their CSF had complaints referable to the CNS, whereas no other abnormalities were noted in 3 of the 8 PCR-positive CSF samples. The authors concluded that B. burgdorferi invaded the CNS early in the course of infection and therefore careful consideration of antibiotics should be undertaken to achieve adequate levels in the CSF to treat those with disseminated infection.

**Conclusion**

Since its recognition at the turn of the twentieth century in Europe and its identification several decades ago in the U.S., investigators and clinicians have come to appreciate the depth and complexity of the clinical infectious and associated immunological response elicited by B. burgdorferi, the causative organism of Lyme disease. The recognition and successful treatment strategies of nervous system Lyme disease occurred through systematic public health-like analysis of waves of affected patients initiated by Allan Steere and his colleagues at Yale University beginning in 1977. Since its definition as a unique human model for an infectious cause of arthritis, Lyme disease has moved into the spotlight because of the annual toll of neurological disease most prominently, early childhood facial palsy, subacute adult meningitis, cranial neuritis, and radiculoneuritis, and late-stage peripheral neuropathy and encephalopathy, some of which have the potential for improvement with appropriate antimicrobial therapy. Further understanding of LNB will be obtained as investigations continue into the spirochete microbiome, and human host genome and immune defense mechanisms with the development for latent autoimmunity.

**References**


Epidemiology

Abstract

Lyme disease is the most commonly reported vector-borne illness in the United States and the fifth most common disease in the National Notifiable Diseases Surveillance System. There are clinical and laboratory criteria for the ascertainment of confirmed, probable, and suspected cases of Lyme disease however these criteria are neither applicable nor intended for individual patient diagnosis or selection of antibiotic regimens. Cases of Lyme disease collected by state and local health departments are shared with the Centers for Disease Control and Prevention and are tabulated in final numbers in the Morbidity and Mortality Weekly Report. These data are also summarized in the annual Morbidity and Mortality Weekly Report Summary of Notifiable Diseases and available in datasets for epidemiologic analysis and public health responses. While the number of incident cases overall has not steadily increased in states and counties, there has been an overall increase in the geographic distribution.

Introduction

Lyme disease is caused by the bacterium *B. burgdorferi* and is transmitted to humans through the bite of infected blacklegged ticks. Typical symptoms include fever, headache, fatigue, and a characteristic skin rash called erythema migrans EM. Undiagnosed and therefore untreated, infection disseminates to musculoskeletal, cardiovascular and nervous system. Lyme disease is
clinically diagnosed based on symptoms, physical findings and the probability of exposure to infected ticks in endemic geographic areas. Laboratory testing is helpful when performed utilizing evidenced and validated methods. Systemic and nervous system Lyme disease or Lyme neuroborreliosis LNB is highly treatable with oral and parenteral antibiotics depending upon the severity of infection.

Lyme disease is the most commonly reported vector-borne illness in the United States U.S. and the fifth most common disease in the National Notifiable Diseases Surveillance System (NNDSS). According to the Centers for Disease Control and Prevention (CDC) [1], there were 22,014 confirmed and 8,817 probable incident cases of Lyme disease reported in the U.S. during 2012. However one important development was an increase in the geographic distribution. In 2012, a total of 356 counties had a reported incidence of ≥10 confirmed cases per 100,000 persons, as compared to 324 counties in 2008 (Figure 1 A-C, Table 1).

![Reported Cases of Lyme Disease -- United States, 2001](A)

Figure 1 A-C. (Continued).
Figure 1 A-C. Confirmed cases of Lyme disease displayed on maps of the United States corresponding to 2001 (A), 2008 (B) and 2013 (C) illustrating the increase in geographic distribution. Exact case counts for each year as well as those displayed in the maps are listed in Table 1.
Table 1

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<tr>
<td>U.S. Total</td>
<td>17,029</td>
<td>23,763</td>
<td>21,273</td>
<td>19,804</td>
<td>23,305</td>
<td>19,931</td>
<td>27,444</td>
<td>28,921</td>
<td>29,959</td>
<td>22,551</td>
<td>24,364</td>
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In 2013, 95% of confirmed incident cases were reported from fourteen northeast and mid-western states including Connecticut, Delaware, Maine, Maryland, Massachusetts, Minnesota, New Hampshire, New Jersey, New York, Pennsylvania, Rhode Island, Vermont, Virginia and Wisconsin. Surveillance methods used for case ascertainment include the application of rigorous clinical and laboratory criteria to verify the diagnosis for reporting purposes. However the methodology and specific criteria employed in case ascertainment for epidemiological and public health activities were never intended to be applied to routine clinical diagnosis or in the selection of antibiotic regimens.

Case Ascertainment and Surveillance

It is important to understand the process of Lyme disease case ascertainment. Cases of Lyme disease have been collected and verified by state and local health departments in accordance with a legal mandate and surveillance practices in the U.S. since 1991. After removal of personal identifiers, selected information on cases is shared with CDC. Public surveillance data available on the CDC website (www.cdc.gov) is subject to several limitations. There can be misclassification associated with under-reporting in highly endemic areas and over-reporting in non-endemic areas. There may be logistical reasons for the ability of a given state or local health department’s ability to capture and classify cases based upon budgetary and personnel conditions. Moreover, surveillance data are captured by county of

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residence not that of exposure. There may be differences in the closure of annual surveillance datasets between the states. Following implementation of the surveillance program in 1991, case definitions for Lyme disease were modified in 1996 and again in 2008, and although minor, these modifications may impact on the interpretation of trends. Final case counts of confirmed cases are published at year end after all states and territories have verified their data and tabulated in final numbers in the Morbidity and Mortality Weekly Report (MMWR) in early August of the following year and summarized in the annual MMWR Summary of Notifiable Diseases (www.cdc.gov/mmwr/mmwr_nd/). The CDC developed a public use dataset that provides the number of confirmed cases by county for the years 1992 to 2011, four 5-year intervals enabling investigators to access direct data and download that information into compatible research-driven computer software for epidemiological analysis.

To improve public health, CDC has been conducting three complementary projects. The first project is to achieve an estimate of the number of people diagnosed with Lyme disease based on medical claims information from a large insurance database. The second study is to estimate the number of people who test positive for Lyme disease based on data obtained from a survey of clinical laboratories. A third study aims to estimate the number of people who report that they have been diagnosed with Lyme disease in the previous year. Preliminary results from the three different evaluation methods suggest that the number of people diagnosed with Lyme disease each year in the United States is around 300,000 persons. However, these new estimates would not necessarily bear upon our understanding of the geographic distribution of Lyme disease.

It should be emphasized that the methodology and specific criteria employed in case ascertainment for epidemiological and public health activities are not intended to be meaningful or even applicable to routine clinical diagnosis or in the selection of antibiotic regimens. So applied, a sizable population would be excluded from consideration of the diagnosis, including those with less compelling, incomplete or atypical presentations who might otherwise benefit from empiric antibiotic therapy.

For surveillance purposes, the clinical description of Lyme disease is a systemic tick-borne disease with protean manifestations including dermatologic, rheumatologic, neurologic, and cardiac manifestations. The most common clinical marker for the disease is the EM rash, the initial skin lesion so noted in up to three-quarters of confirmed cases. Late Lyme manifestations include musculoskeletal (joint swelling, mono- and
oligoarthritis), nervous system (lymphocytic meningitis, cranial neuritis, radiculoneuropathy, and encephalomyelitis), and cardiovascular (high-grade heart block and atrioventricular conduction defects). Arthralgia, myalgia, and fibromyalgia; headache, fatigue, paresthesia, stiff neck; and palpitation, bradycardia, bundle branch heart block and myocarditis, which may be highly suggestive of an index case, consistent with Lyme disease-related musculoskeletal, neurologic, and cardiac disease, are not specific criteria for case designation.

The specific laboratory criteria for case ascertainment according to the CDC [2-5] includes a positive $B. burgdorferi$ culture OR one of the following: 1) A positive result of two-tier testing interpreted using established criteria where a positive IgM titer is used for symptom onset ≤30 days and a positive IgG titer for any point during the infectious illness; 2) Single-tier IgG immunoblot or Western blot seropositivity; and CSF positivity for $B. burgdorferi$ by enzyme-linked immunoassay (EIA) or IF assay notably when the titer is higher in CSF than in serum. The terminology of Lyme “exposure” often employed in clinical notes, is defined as having been in wooded, brushy, or grassy areas, all potential tick habitats, in a county where Lyme disease is endemic. The term endemic refers to a county in which at least two confirmed cases have been acquired or a country with a population of known tick vectors infected with $B. burgdorferi$. A confirmed case of Lyme disease for surveillance meets the criteria of EM rash with known exposure OR EM with laboratory evidence of infection without known exposure OR one with at least a late clinical manifestation with laboratory evidence of infection. Suspected or probable cases respectively are those respectively with EM and/or laboratory evidence of infection. A history of tick bite in not required for case ascertainment.

**Conclusion**

Case definitions for surveillance purposes are an invaluable resource in the epidemiological understanding of Lyme disease and LNB. Although indirectly applicable to the clinical and laboratory diagnosis of individual patients, rigorous case definition in clinical practice would exclude many patients with less compelling, incomplete or atypical presentations from empiric treatment. Data set analysis of surveillance is useful in establishing trends and an explanation for the epidemiologic aspects of Lyme disease and the nervous system manifestations.

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References


Chapter 3

The Blood-brain Barrier

Abstract

There has been extraordinary research in the blood-brain barrier (BBB) over the past decade. Once considered a static anatomical barrier to the traffic of molecules in and out of the central nervous system when fully developed in adults, and otherwise irrelevant to neuroscience and disease, the BBB is now known to be fully functional in development, and vital in normal cerebrovascular angiogenesis. The cellular components and other molecular constituents of the BBB contained in a neurovascular unit protect the central nervous system from injury and disease by limiting the passage of toxins, pathogens, and inflammatory effectors of the immune system. Blood-brain barrier breakdown has been recognized as an important factor in a variety of primary and secondary neurological disorders. However BBB disturbances, while common to Lyme neuroborreliosis, have yet to be critically analyzed. This chapter reviews the history, neurodevelopment, ultrastructure, function, and clinicopathologic correlation of the BBB with a particular relevance to Lyme neuroborreliosis.

Introduction

The past decade has witnessed an expansion of knowledge in the properties possessed by the BBB in health and disease summarized in several excellent recent reviews [1-5]. In essence, the neurovascular unit of the BBB is comprised of capillary vascular and neural cells, extracellular matrix...
components, and a variety of immune cells that mediate local immunity. The schematized and electron microscopic appearance of cerebral capillaries in the BBB shown in Figures 1 and 2, demonstrate layers of pericytes adherent to the abluminal or parenchymal surface of endothelial cells, together surrounded by a layer of basal lamina comprised of extracellular matrix protein molecules. The end feet of neighboring astrocyte processes ensheath the blood vessels. Monolayers of adjacent endothelial cells that form tight junctions (TJ) strands connect adjacent endothelial cells by adhesions of transmembrane (occludin, claudin, and junctional associated molecules [JAM]) across the intercellular space while cytoplasmic scaffolding and regulatory proteins such as zona occludens type 1 and 2 [ZO-1, ZO-2]) provide linkage to the actin cytoskeleton and initiate several signaling mechanisms via protein-protein interactions. Endothelia BBB cells are also linked by adherens junctions composed of vascular endothelial (VE)-cadherin, which mediates cell-cell adhesion interactions, linking adherens junctions to the actin cytoskeleton via catenins [2, 3]. Perivascular macrophages that reside between astrocyte endfeet and the vessel wall, mast cells associated with specific regions of the central nervous system (CNS), resident microglia that act as antigen presenting cells (APC), circulating leukocytes that can penetrate the intact BBB via interactions with cellular adhesion molecules (CAM) to mediate bidirectional crosstalk between immune cells and endothelium for normal surveillance, constitute the extended neurovascular unit [2].

Figure 1. A. Cross-section schematic representation of a capillary in the human blood-brain barrier over an endothelial tight junction. B. The insert shows the molecular composition of tight and adherens junctions. See text for details. Reproduced from reference 1, with permission of the publisher.
Breakdown or disruption of the BBB that accompanies a variety of inflammatory and autoimmune, neoplastic, infectious, and neurodegenerative CNS disorders, notably stroke, multiple sclerosis, brain trauma, human immunodeficiency virus (HIV), infection, and Alzheimer disease. These disorders are associated with the abnormal entry of plasma components, immune molecules and cellular elements that leads to further neural dysfunction and varying degrees of irreversible neural degeneration. Although there is limited understanding of the role of the BBB in the etiopathogenesis of LNB, future progress could lead to improved understanding with the prospect of improved outcome in early and late manifestations of the disease, taking advantage of the selective expression of membrane bound proteins expressed by brain endothelia cells or circulating leukocytes to target new drugs, as well as improving the effectiveness of conventional oral and parenteral antibiotics. This chapter reviews the history, neurodevelopment, ultrastructure, function, and clinicopathologic correlation and relevance to Lyme neuroborreliosis.

**Historical Background of the BBB**

In 1885, Ehrlich [6] provided the first suggestion of the presence of a barrier when a parenteral injection of vital dye into the bloodstream of mice
penetrated practically every systemic organ except the brain turning them dark purplish-blue, leaving the brain and spinal cord pale white-yellow. Ehrlich himself thought that this difference was due to a low binding affinity. The existence of a barrier at the level of the cerebral vessels was postulated by Bield and Kraus [7] and later by Goldman [8] and Lewandowsky [9] at the turn of the twentieth century, who jointly interpreted their experience in favor of a true BBB, later termed Blut-Hirn-Schranke. In 1967, Reese and Karnovsky [10] demonstrated a structural barrier to an intravenous injection of horseradish peroxidase (HRP) demonstrating exogenous peroxidase to the lumina of blood vessels and in some micropinocytotic vesicles within endothelial cells, but none beyond the vascular endothelium. The relatively scarce number of vesicles was a morphological feature of a functioning BBB. Their findings localized at a fine structural level a barrier composed of the plasma membrane and the cell body of endothelial cells and TJ between adjacent cells of the cerebral cortex. In 1969, electron microscopic studies by Brightman and Reese [11] in the mouse conclusively demonstrated endothelial and epithelia TJ that occluded the interspaces between blood and parenchyma or cerebral ventricles, constituting the ultrastructural basis for the blood-brain and blood-cerebrospinal fluid barriers. Feder [12] noted active exclusion of the small electron-dense tracer microperoxidase by intact TJ after parenteral injection supplementing the findings of Reese and Karnovsky [15]. Nagy and colleagues [13] examined fracture faces of cerebral endothelium in normal and hyperosmolar mannitol-treated rat brains to elucidate the organization of TJ in various segments of the cerebral vascular bed and the structural basis of BBB opening in hyperosmotic conditions. Their findings provided no direct evidence for the structural basis of BBB opening in hyperosmolar mannitol-treated rat brains noting extended TJ regions in capillaries and postcapillary venues. Shivers and coworkers [14] studied isolated rat brain capillaries employing freeze-fracture images of interendothelial ZO revealing complex arrays of intramembrane ridges and grooves characteristic of TJ. The ZO of these capillary endothelial cells were considered very tight.

**Development of the BBB**

In contrast to the neuronal development, the vascular system undergoes blood vessel formation through the two distinct processes, vasculogenesis and angiogenesis. The former commences with endothelial differentiation from angioblasts to vascular plexuses, while the latter is associated with sprouting

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of new vessels from existing ones. Both vasculogenesis and angiogenesis are influenced by vascular endothelial growth factor (VEGF), a major attractive molecule for extending blood vessels especially endothelial tip cells [15], as well as by other molecules, blood flow and contact with surrounding tissues. Found at the growing end of extending vessels, the shaft of extending endothelial tip cells is composed of an endothelial cell chain made of stalk cells similar to the axon growth cone and its associated axonal shaft. In vivo imaging employing green fluorescent protein (GFP) depicts the advancing endothelial tip cell navigating the environment and sprouting from existing vasculature [16].

Several experimental observations have suggested the importance of the neurodevelopment of the BBB [5]. First, there are shared molecular and cellular mechanisms in both neurogenesis and angiogenesis [17, 18]. Various axon guidance pathways from members of the four major families of axon guidance ligand-receptor pairs including Slit/Robo, semaphoring/plexin/neuropilin, Netrin/Unc5/DCC, and Ephrin/Eph, that mediate complex cellular navigational programs within axons as both chemoattractants and repellents, also direct angiogenic tip cells toward their final destinations. Neuropilin-1 is necessary for endothelial tip cell guidance in the developing central nervous system [19]. Moreover, axonal terminal arborization parallels vessel sprouting. Similar to hypoxic tissue that secretes VEGF via hypoxia-inducible factor (HIF), a transcription factor that promotes cell survival through the downstream activation of numerous genes including VEGF [20], axonal terminals devoid of synaptic input secrete nerve growth factor (NGF), the expression of which is down-regulated when innervation occurs.

Second, there is co-regulation of these two systems in developing embryonic and adult brains [17, 21, 22]. Stubbs and colleagues [21] found that blood vessels provided a supporting niche in regions of adult neurogenesis. The investigators [21] employed Tbr2-GFP transgenic mice that served as a correlate for the expression of the intermediate progenitor cell (IPC) T-box transcription factor Tbr2, to examine the proximity of dividing cells in the subventricular (SVZ) and VZ of the shaking rat Kawasaki and reeler mutant mouse in relation to blood vessels throughout neurogenesis. Their findings which included the extension of neuritis toward and along labeled blood vessels supported the notion of vascular-neuronal interactions in development. Javaherian and coworkers [22] who likewise studied IPC in the SVZ of embryonic Swiss Webster mouse cortices employed confocal microscopy to image the vast network of capillaries in the SV and SVZ. The authors [22] noted that Tbr2 cells divided near vascular branch-points suggesting
endothelial tip cells contributed to the neurogenic niche for IPC, with ectopic overexpression of VEGF-A in a pattern that followed that of blood vessel development. These findings indicated that the developing cortical vasculature provided a microenvironment within the SVZ in which IPC accumulated and divided during neurogenesis.

Third, a structural and functional BBB complete with TJ appear as soon as cerebral vessels penetrate the CNS parenchyma [23, 24]. Johansson and colleagues [23] explained that the widely held view that the BBB was immature during development stemmed from teleological interpretations and experimental observations of high cerebrospinal fluid protein levels in fetal cerebrospinal fluid and the apparent passive passage of biomarkers during development. Instead, the blood-cerebrospinal fluid barrier, like the BBB is functionally and morphologically mature from very early in development. The authors maintain that inconsistent terminology used in the literature such as leaky, immature, and developing, used to describe the barrier gives a connotation of TJ that are more permeable than their adult counterparts without evidence to support this concept. Mølgård and Saunders [25] noted well-formed complex TJ across cerebral endothelial cells in human embryos and fetuses by freeze fracture and thin section EM by 8 weeks of age, commensurate with the differentiation of brain capillaries. Efflux transporters are likewise expressed in cerebral endothelial and choroid plexus epithelial cells early in the fetal and postnatal rats [31]. Ballabh and colleagues [27] studied the expression and quantification of endothelial TJ molecules including claudin-5, occluding, and JAM by immunohistochemistry and Western blot analysis in blood vessels of germinal matrix, cortex, and white matter of fetuses and premature infants gestational age 16 to 40 weeks. The authors [27] noted no significant decrease in the expression of the endothelial TJ molecules claudin-5, occludin, and JAM-1 as a function of gestational age in germinal matrix compared with cortex and white matter suggesting that they were unlikely to be responsible for germinal matrix fragility and vulnerability to hemorrhage in premature infants. These findings are consistent with the concept that TJ molecules develop and perhaps mature early during human gestation. Ballabh and colleagues [29] observed that a paucity of TJ or pericytes coupled with incomplete coverage of blood vessels by astrocyte end-feet, could instead account for the observed fragility of blood vessels in the germinal matrix of premature infants. Braun and colleagues [29] found pericyte coverage and density that were less in the germinal matrix vasculature than in the cortex or white matter in human fetuses, premature infants, or premature rabbit pups. Although VEGF suppression significantly

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enhanced pericyte coverage in germinal matrix, it remained less than in other brain regions.

**Cellular Elements of the BBB**

**Endothelial Cell Interactions**

The existence of the endothelial cell was first surmised by William Harvey, first observed by Marcello Malpighi in blood capillaries using compound microscopy in the nineteenth century, and later by electron microscopy in the mid-twentieth century, revealing the presence of plasmalemmal vesicles or caveolae. The ability to culture EC later permitted even more detailed investigation of their activation and function *in vivo*. Derived from mesoderm via the differentiation of hemangioblasts and angioblasts, there are a few protein/mRNA marker candidates including platelet/endothelial cell adhesion molecule (PECAM)-1 in monocytes and VE-cadherin in fetal stem cells. Endothelial cells of the BBB not only provide a physical barrier between the systemic circulation and the brain, but assure the selective inward passage of ions, nutrients, and neuropeptides via specialized transport mechanisms. Sodium, potassium, chloride, hydrogen, bicarbonate and calcium ions are transported across the BBB via transporters located mainly along the luminal surface of endothelial cells including, the sodium and potassium adenosine triphosphate (ATP)-dependent transport pump, the sodium-potassium-chloride cotransporter (NKCC), sodium-proton, chloride-bicarbonate, and sodium calcium exchanges that assure optimal levels of brain electrolyte levels and intracellular pH. The transport of essential nutrients is assured by members of the soluble carriers (SLC) superfamily, located variably along the luminal and abluminal membrane including, glucose transporter-1 (GLUT1), monocarboxylic acid-1 (MCT-1); excitatory, organic acid (OAT), cation (OAC), amine and choline transporters (CTL1), respectively to transport lactate and ketone bodies as alternative energy neuronal sources, and sodium-independent or dependent removal of glutamate, aspartate, glutamine, histidine, and asparagine from the interstitial compartment of the brain. Other specific carrier-mediated transporters mediate the passage of transferrin, low-density lipoproteins, leptin, immunoglobulin G (IgG), insulin, and growth factors via receptor-mediated transcytosis via binding of the protein to specific receptors on the endothelial cell surface following by endocytosis of the ligand-receptor complex with passage across
the cytoplasm and exocytosis at the opposite side of the cell [30] and via the formation of caveolae or vesicle formation for the transport of macromolecules [31]. Transmigration of cellular elements across endothelial cells of the BBB during inflammation including leukocytes, neoplastic cells, and pathogenic viruses, bacteria and yeasts, investigated in experimental animal models utilizing HRP, highlighted the role of caveolae as mini-transporters of the CNS [31]. Unique systems of modified caveolae that fuse together forming transendothelial cell channels and later vesiculo-canalicular or vesiculo-tubular structures (VTS) or vesiculo-vacuolar organelles (VVO), appear to be an important gateway to the CNS in damaged endothelial cell populations [31]. Transportation of potentially toxic endogenous or xenobiotic lipid-soluble nonpolar molecules from the brain to the blood are accomplished by transporters located along the luminal membrane such as the ATP binding cassette (ABC) transporter P-glycoprotein 1 (P-gp or Pgp) (multidrug resistance protein 1 [MDR1] or ATP-binding cassette sub-family B member 1 [ABCB1]) respectively important in the distribution of CNS tumor drug therapy [32] and the active efflux of the anti-human immunodeficiency virus type 1 (HIV1) nucleoside drug abacavir at the BBB [33-35]; and breast-cancer resistance protein (BCRP) and multidrug resistance related protein (MRP) 1, 2, 4 and 5 efflux transporter pumps that serve as defense mechanisms and determinate bioavailability and concentration of many CNS drugs important in the treatment of CNS cancers [36] such as the novel tyrosine kinase inhibitor dasatinib [37], and the efflux transportation of the protease inhibitor lopinavir that contributes to its poor oral bioavailability in the treatment of HIV1 [38]. The neuroinflammation and progression of damage associated with focal cerebral ischemia appears to be modulated by upregulation of other MRP protein molecules that activate Toll-like receptor (TLR) signaling contributing to neuroinflammation and progression of ischemic cerebral damage [39].

Transendothelial migration of circulating leukocytes involves a multistep process. Leukocyte adhesion molecules (LAM) expressed on the surface of EC initiate binding of leukocytes as a beginning step in the in their entry in brain tissue which later includes rolling adhesion to EC, firm adhesion, and transmigration. Although less well understood, the molecular mechanism is believed to involve endothelial CAM including CD99, platelet ECAM (PECAM-1)/CD31, vascular CAM-1 (VCAM-1) (important in firm adhesion); junctional adhesion molecule-1; and expression of leukocyte adhesion molecules E- and P-selectin (rolling adhesion); cytokine responsiveness so noted in situ and in cell culture [40, 41] and expression of the integrins alpha4- and beta-2. Inflamed capillary endothelia support transmigration of different
subsets of leukocytes. There are two routes for leukocytes to pass through endothelial cell, the so called paracellular route, or through the endothelial cell itself or transcellular route. The BBB with its abundance of TJ complexes relies primarily on the transcellular route as it does for solute and fluid transport. Neutrophil recruitment is partially dependent ICAM-1, and express L-selectin and lymphocyte function-associated antigen (LFA)-1 but not chemokine C motif receptor 7 (CCR7) may explain why granulocytes roll but do not arrest for transmigrate in high endothelial venules (HEV).

Pericyte Interactions

Brain endothelial cells are exposed to a myriad of pericyte interactions [42] in the regulation of brain angiogenesis, endothelial cell TJ formation, as well as the differentiation, microvascular vasodynamic capacity, structural stability, and neuroimmunologic network operations of the intact BBB [43]. Rouget [44] first ascribed capillary contractility to pericytes but Zimmermann named the cell and described its morphologic aspects [45]. The presence of smooth muscle cells in association with pericytes and the absence of a smooth muscle layer from capillaries and postcapillary venues influenced early views ascribing contractile properties to narrow capillaries hence regulate microvascular flow even though a number of subsequent experimental studies failed to substantiate it [46, 47]. Smooth muscle actin was conclusively demonstrated in pericytes by immunocytochemistry employing smooth muscle α-actin isoform specific antibodies and immunogold labelling in conjunction with electron microscopy noting that smooth muscle α–actin expression in capillaries was limited exclusively to pericytes and not present in endothelial cells [48]. Since the histochemical localization of smooth muscle α-actin is demonstrated in precapillaries and not in midcapillaries, it has been suggested that smooth muscle α-actin containing capillaries are involved in contractility and the control of capillary blood flow in the BBB [49].

Unlike other perivascular cells that lie within the microvessel basal lamina and contribute to its formation, typical CNS pericytes are flattened or elongated stellate-shaped solitary cells with multiple cytoplasmic processes encircling the capillary endothelium and contacting a large abluminal vessel area. Brain pericytes are characterized by granular deposits present in lysosomes that strongly react with acid phosphatase, a finding that led to consideration of a phagocytic role [50]. They rapidly phagocytose an intravenous injection of HRP which can be employed as a pericyte
histochemical stain. The number of granular lysosomes in brain pericytes increases with disruption of the BBB. Several other markers have been employed in the identification of pericytes including, smooth muscle α-actin (SMA), desmin, polydendrocytes (NG2 cells), platelet-derived growth factor receptor (PDGFR)-β, aminopeptidase A and N, regulator of G-protein signaling 5 (RGS5) and the promoter trap transgene XlacZ4 [51].

An active role of pericytes in the BBB was inferred from the localization of γ-glutamyl transpeptidase (GGTP) in brain capillary endothelial cells and pericytes, both in vivo and in vitro [52]. This heterodimeric glycoprotein distributed on the external surface of the cell catalyzes the transfer of γ-glutamyl from glutathione to accept peptides and functionally appears to be concerned with transport of large neutral amino acids across the BBB. Detectable amounts of GGTP are found in other regions of the brain with an intact BBB but not in those that lack one such as the median eminence. Abnormal platelet derived growth factor (PDGF)-B and PDGF-β signaling plays a critical role in the recruitment of pericytes to newly formed vessels, and when deficient, as in knockout of pdgfb and pdbfrb, leads to perinatal death due to vascular dysfunction with associate vascular leakage and hemorrhage.

Pericyte-endothelia cell signaling factors have been identified. Sphingosine-1-phosphate (SIP) signaling triggers cytoskeletal, adhesive, and junctional changes, affecting cell migration, proliferation, and survival [53]. Angiopoietin-Tie2 signaling in the vascular wall involved in reciprocal communication between endothelial cell and pericytes, such as may be seen in ang1- or tie2-null mice deficient in Ang1, which leads to defective angiogenesis and poorly organized BM and reduced coverage and detachment of pericytes. Conversely, overexpression of Ang1 leads to expanded and stabilized, leakage resistant microvasculature [54, 55].

The importance of CNS pericytes has been underscored by their proposed role in neuroimmunological networks associated with BBB function. First, CNS pericytes may be actively involved in the regulation of leukocyte transmigration, antigen presentation, and T-cell activation. They constitutively express low levels of VCAM-1 and ICAM-1, which have costimulatory activity in main histocompatibility cell (MHC)-class II dependent antigen presentation; and leukocytes cluster on pericytes in culture [43] suggesting a role in inflammation. Smooth muscle pericytes present antigen in vivo and differentially activate Th1 and Th2 CD4-T cells. Moreover CNS pericytes produce a number of immunoregulatory cytokines including interleukins (IL) and granulocyte-macrophage (GM) colony stimulatory factor [56].
Transforming growth factor (TGF)-β produced in an active form in pericytes/endothelial cocultures, may function as an endogenous immunoregulator at the BBB [57]. It is therefore of interest that TFG-β1 inhibits cytokine-induced CNS endothelial cell activation in isolated rat CNS microvessels [58]. To further emphasize the importance of pericyte interactions in association with endothelial cells, there are no known genetic human diseases due to pericyte deficiency.

Astrocyte Interactions

The intimate relationship of astrocytes and blood vessels was appreciated by Cajal [59] and Golgi [60] in the late nineteenth century. Since then, ultrastructural studies have shown that astrocytic endfeet in the perivascular astroglial sheath leads to a complete covering of brain microvessels [61]. Signaling at the gliovascular interface is facilitated by astrocyte-specific proteins and channels in astrocyte endfeet including, aquaporin-4, connexin 43, purinergic receptors, and potassium channels [62]. Moreover, ultrastructural studies have demonstrated that processes of vasoactive neurons for the regulation of cerebrovascular tone, in particular those expressing noradrenaline, synapse onto astrocytes rather than directly onto blood vessels [63]. Altogether, these findings support the observation that astrocytes, one of the more numerous cells in the CNS, are important determinants of the intact BBB and crucial as well for ionic homeostasis, neurotransmitter uptake, synapse formation, and neurodevelopment. Zhang and Barres [64] have reviewed the differences in astrocyte morphology, developmental origin, gene expression profile, physiological properties, function and response to injury and disease. Two essential roles of astrocytes, in neurovascular coupling and the regulation of lymphocyte trafficking across the BBB have been extensively studied.

All signaling molecules targeted to the cerebral vasculature must first act on or pass through astrocytes in order to reach smooth muscle cells in the vessel wall. It is now recognized that neurotransmitter-mediated signaling has a key role in regulating cerebral blood flow, and that much of this control is mediated by astrocytes [65], moreover, cerebral blood flow may be controlled by capillaries as well as by arterioles. The glial and neuronal control of cerebral blood flow has been studied in brain slices [66]. Koehler and colleagues [67] demonstrated that electrical field stimulations in brain slices led to an increase in intracellular calcium in astrocyte cell bodies which when
transmitted to perivascular end-feet, was followed by a decrease in vascular smooth muscle calcium oscillations and arteriolar dilation. The increase in astrocyte calcium after neuronal activation was in part mediated by activation of metabotropic glutamate receptors. Calcium signaling *in vitro* was influenced by adenosine acting on A2B receptors and by epoxyeicosatrienoic acids (EET) shown to be synthesized in astrocytes. Moreover, prostaglandins, EET, arachidonic acid, and potassium ions are candidate mediators of communication between astrocyte endfeet and vascular smooth muscle. Astrocytes appear to be capable of transmitting signals to pial arterioles on the brain surface to ensure adequate blood flow to feeding arterioles, therefore these cells play an important role in the coupling of dynamic changes in cerebral blood flow in association with neuronal activity.

Koehler and colleagues [67] have provided insight into the morphological aspects of neurovascular coupling at the capillary level of the BBB. At least one astrocyte endfoot process contacts a blood vessel and those abutting capillaries and larger vessels express connexin-43 and purinergic P2Y receptors, which together permit Ca$^{2+}$ increases to be transmitted 60 μm or more along the abluminal side of the vessel wall. Astrocytic cells are therefore in a unique position for sensing neuronal activity, integrating that information, and communicating with blood vessels in brain parenchyma. While neurons do not directly innervate intraparenchymal vascular smooth muscle, sub-populations of GABAergic interneurons come into close contact with astrocyte foot processes and elicit vasodilation. Such neurons might modulate vascular function through stimulation of nitric oxide (NO) synthase (NOS) activity, release of vasoactive peptides, or an astrocyte signaling mechanism.

Hudson and coworkers [68] studied trafficking of peripheral blood mononuclear cells (PBMC) across feline brain endothelial cells (FBEC) in cell culture system after the addition of combinations of different configurations of astrocytes and microglia in a model of feline immunodeficiency virus. The addition of astrocytes to FBEC significantly increased the adherence of PBMC which was suppressed by the addition of microglia, whereas the latter alone had no effect on PBMC adherence. Whereas all PBMC showed some level of trafficking across FBEC, monocytes and B-cells were significantly increased if astrocytes were present. The exposure of astrocytes notably increased the percentage of trafficking CD8 T-cells from 24% to 64%, while microglia led to a significant reversal in the preferential trafficking of CD8 T-cells in the presence of astrocytes. Astrocytes are capable of secreting various cytokines and chemokines in the upregulation of adhesion molecules and T-cell ligands in intact endothelial cells such as ICAM, VCAM, E-selectin, and PECAM.
Human cocultured human endothelial cells and astrocytes increase the expression of ICAM-1 due to inflammatory activation by hypoxia \textit{in vitro} [69]. Other studies have demonstrated that astrocytes are a source of IL-6, TNF-\(\alpha\), and MCP-1 which contribute to the CNS inflammatory response [70]. Trafficking of PBMC along the endothelial cell of the BBB is a complex mechanism that involves major subsets of immune cells and relies heavily on astrocyte, microglia and endothelial cell interactions, moreover, astrocytes appear to be an active factor in the recruitment of immune cells, while microglia appear to curtail this activity.

**Implications for Lyme Neuroborreliosis**

Interest in concepts of BBB disruption in Lyme neuroborreliosis (LNB) commenced with a decade of experimental and clinical observations in early 1990 focusing on the etiopathogenesis of CNS manifestations, notably encephalopathy in acute and chronic Lyme infection. The mechanisms by which bacteria breached the BBB had been incompletely understood however it had been proposed that during pneumococcal bacteremia, microbial factors acted directly or indirectly to trigger production of endogenous inflammatory mediators that altered endothelial TJ to facilitate bacterial entry.

In 1990, Szczepanski and colleagues [71] studied the emigration of \textit{B. burgdorferi}, the spirochetal agent of Lyme disease, across cultured human umbilical vein EC (HUVEC). Low passage human clinical isolates (HSA1 and HBD1) cultured from skin and blood respectively of patients with erythema migrans (EM), and a tick isolate (T11) from \textit{I. dammini} collected in Montauk, NY, adhered 22 to 30-fold greater than the continuously passaged strain B31 to the subendothelial matrix. Spirochete binding and adherence to the subendothelial matrix was inhibited 48 to 63\% by pretreatment of the matrix with anti-serum to fibronectin, a major component of the matrix produced by cultured EC and a constituent of the basement membrane of blood vessels \textit{in vivo}. The inhibition of spirochete adherence to the matrix by anti-fibronectin indicated that the spirochetes recognized the insoluble matrix form of this glycoprotein. Spirochete migration across endothelial monolayers cultured on amniotic membrane was increased when the monolayers were damaged by chemical or physical means. Electron microscopic examination of spirochete-endothelial interactions demonstrated the presence of spirochetes in the intercellular junctions between EC as well as beneath the monolayers.
Scanning electron microscopy identified a mechanism of transendothelial migration whereby spirochetes passed between cells into the amniotic membrane at areas where subendothelium was exposed. The adherence of *B. burgdorferi* to subendothelial matrix is an important finding since spirochetes must penetrate the subendothelial basement membrane of the BBB to enter the CNS compartment. Spirochete recognition of EC or subendothelial matrix appears to be mediated by separate mechanisms since pretreatment of EC with anti-fibronectin antiserum reduced spirochete adherence to the cells slightly while matrix binding was greatly diminished; moreover little fibronectin is expressed on the surface of EC in culture or in vivo. Spirochete transendothelial migration was facilitated by prior damage of the EC monolayer by physical or chemical injury and spirochete migration at regions where a small gap in the monolayer exposed the underlying connective tissue on scanning electron microscopy, would likewise be expected to occur in vivo for example in areas where EC contraction or damage occurred. A similar sequence of events of attachment to the apical surfaces of cultured cells in intercellular spaced between cells and beneath EC monolayers, and migration via an intercellular route and not by a transcytotic process was described for the transendothelial migration of *Treponema pallidum* spirochete [72].

Grab and colleagues [73] studied the traversal of human brain microvascular EC (BMEC) and HUVEC by *B. burgdorferi* noting facilitation in the former by proteases. The spirochete organism appeared to bind human BMEC by their tips near or at cell borders inducing the expression of plasminogen activators, plasminogen activator receptors, and matrix metalloproteinases (MMP). By comparison to HUVEC, which lacks the TJ complex that is key to the functionality of BMEC as a barrier to pathogen entry into the CNS, the investigators [73] noted that *B. burgdorferi* differentially crosses human BMEC and HUVEC, and that human BMEC formed a barrier to traversal. In comparison to Szczepanski and colleagues [71] who noted a greater than 22-fold greater low-passage *B. burgdorferi* than high-passage strains across the HUVEC, Grab and colleagues [73] noted about a 21-fold more low-passage *Borrelia* crossed HUVEC than BMEC underscoring the importance of extrapolating data concerning *B. burgdorferi* penetration of the BBB from experimental data employing non-brain vascular EC models. The authors [73] hypothesized that *B. burgdorferi* induces the expression of plasminogen activators and MMP and that these enzymes linked by an activation cascade, could lead to the focal and transient degradation of TJ proteins allowing the spirochete organism to invade the CNS, binding via their tips prior to crossing

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the in vitro human BBB model, and doing so presumably without evidence of loss of BBB integrity. To that end, unlike that encountered in purulent bacterial meningitis, *B. burgdorferi* infection usually causes aseptic meningitis in which the permeability of the BBB may not be substantially altered [74]. In a later investigation of the traversal of *B. burgdorferi* across the human BBB using in vitro model systems constructed of HBMEC, Grab and coinvestigators [75] cell monolayers were pretreated with the intracellular calcium chelator BAPTA-AM (1,2-Bis (2-aminophenoxy)ethane-N,N,N',N'-tetra-acetic acid tetrakis(acetoxyethyl ester) and the phospholipase C (PLC) inhibitor U-73122 (1-(6-[[17b]-3-Methoxyestra-1,3,5(10)-tri-en-17-y1amino]heyl)1H-pyrrole-2,5-dione) which inhibited transmigration of *B. burgdorferi*, the former significantly and the latter almost totally blocked as a result of barrier tightening based on electric cell-substrate impedance sensing. These data suggested a role for calcium in CNS spirochete invasion through EC barrier. Nyarko and colleagues [76] noted that *B. burgdorferi* and *Anaplasma phagocytophilum*-infected neutrophils co-incubated with HUVEC and HBMEC was associated with increased blood and tissue spirochete loads and heightened traversal through EC barriers.

Garcia-Monco and coworkers [77] showed early invasion of the CNS in experimental Lewis rats by *B. burgdorferi*, accompanied by increased permeability of the BBB which they measured as the ratio of $^{125}$I-labeled albumin in CSF to that in blood. Dose-dependent BBB permeability changes were noted at 12 hours after inoculation and reversed within a week. Only live, intravenously inoculated organisms produced disruption of the BBB. Moreover, BBB changes were more marked with inoculation of the more recent low-passage strain termed J31 acquired from Long Island than with the original isolate of the B31 strain in long-term in vitro culture from Shelter Island, both of which were grown in serum-free media to log phase. Mild pleocytosis and retrievable spirochetes were noted in the CSF of rats with increased BBB permeability. Specific *B. burgdorferi* antigens were detectable in the CSF of human patients with early Lyme disease by use of murine monoclonal antibodies as probes providing evidence for early CNS invasion.

Garcia-Monco and coworkers [78, 79] described the affinity of the Lyme spirochete for cells of primary neonatal rat brain cultures providing evidence of spirochete binding to cell surfaces and processes of glial fibrillary acidic protein (GFAP)-bearing cells, as well as to the surfaces and processes of myelin basic protein (MBP) and galactocerebroside (GC)-bearing cells and
their extracellular matrix made by the cells visible by microscopy. Given that most of the cells in primary rat brain culture were astrocytes and oligodendrocytes, the investigators [78] suggested that affinity and adherence to these cells and their known proximity to brain capillary EC in the BBB were likely determinants of the initiation of CNS injury and might contribute to the secondary persistence of *B. burgdorferi* in the CNS and the development of cross-reactivity between microbial antigens and neural components. Employing $^{51}$Cr assays for the detection of damage to cells of neural origin, Garcia-Monco and coworkers [79] showed a higher degree of injury in the primary brain than in astroglial cultures on scanning electron microscopy, revealing marked contraction of the membrane sheets and bleb production of oligodendroglia in neonatal rat brain culture after incubation with *B. burgdorferi* while the astroglial layers appeared unharmed. The damage to oligodendroglia was evident on the surface of the cells without detection of intracellular *B. burgdorferi* suggesting that the ensuing morphologic changes were not the result of internalization of spirochetes.

![Figure 3 A-C. Engineering and in situ visualization of infectious fluorescent *B. burgdorferi*. A) The plasmid used to constitutively express green fluorescent protein under the control of the *B. burgdorferi* promoter *flab*. B) Phase contrast and epifluorescent visualization of cultured spirochetes from the mouse ears. C) Visualization of fluorescent *B. burgdorferi* in a living mouse ear by spinning disk confocal intravital microscopy. Reproduced from reference 84, with permission of the publisher.](image-url)

The presence of CNS white matter injury as well as *B. burgdorferi*-specific and autoreactive T-cell lines from the CSF have been described in affected patients with Lyme meningoradiculomyelitis [80, 81], as have

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antibodies to MBP in CSF specimens from patients with chronic meningocerebral manifestations [82] suggesting a role for antibodies to MBP in the pathogenesis of the disease manifestations. However a quarter century later, it is still not known with absolute certainty whether autoreactivity causes tissue damage or is a secondary epiphenomenon [83].

Moriarty and coworkers [84] engineered a fluorescent strain of *B. burgdorferi* which expressed green fluorescent protein (GFP) (Figure 3). Employing real-time 3D and 4D intravital microscopy with quantitative analysis, the investigators studied fluorescent spirochete dissemination noting it to be a multi-stage process that included transient tethering-type associations, short-term dragging interactions, and stationary adhesion (Figure 4). The latter in association with extravasating spirochetes were most commonly observed at endothelial junctions whereas translational motility of spirochetes appeared to play an integral role in transendothelial migration. Stationary adhesions that projected deep into and sometimes through PECAM-1-stained regions of vessels, a phenomenon termed embedding, occurred along the entire length of the spirochete or at one end only, and such spirochetes were frequently observed protruding through both sides of the PECAM-1 signal suggesting migration more deeply into junctions or EC than partially embedded adhesions. This observation appeared to be consistent with early electron microscopic studies that demonstrated that *B. burgdorferi* invaded or was taken up by EC in monolayer cultures [85]. However the investigators did not study aspects of the BBB.

More recently, Brissette and coworkers [86] analyzed the transcriptional responses to the incubation of *B. burgdorferi* in primary cultures with primary human astrocytes and HBMEC over a 72-hour period noting a robust increase in interleukin (IL)-8, CXCL-1, and CXCL-10 chemokines in response to virulent spirochetes. The results were confirmed by ELISA and individual sets of PCR primers. The up-regulation of chemokine receptors from brain microvascular EC and astrocytes have the potential to facilitate entry of neurotoxic neutrophils into the CNS. Non-human primate astrocytes which expressed the neutrophil chemoattractant IL-8 in response to *B. burgdorferi* appeared to contribute to the inflammatory response both *in vivo* and *in vitro* in a macaque model of LNB [87-89]. Similarly, chemokines and their receptors expressed in response to systemic inflammation alter the kinetics of EC TJ in mice after experimental stroke [90].
Figure 4 A-F. Electron microscopic analysis of spirochete migration across EC monolayers. A) Spirochete attachment to the apical surface of an EC. B) Spirochete attachment to the surface of the EC above the region of an intercellular junction. C, D) Spirochetes are located in the intercellular space between adjacent EC. E) Spirochete which has migrated beneath an EC. F) Spirochete penetrating the endothelium at a region near an intercellular junction (arrowhead). (X24,000). Reproduced from reference 71, with permission of the publisher.
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Chapter 4

Non-human Primate Animal Models

Abstract

The rhesus monkey provides a model for *B. burgdorferi* infection in human demonstrating all of the salient phases of the disease, accurately mimicking the microbiological, clinical, immunological, and pathological aspects of human Lyme disease. It is also a useful model to investigate methods of diagnosis, immunoprophylaxis and chemotherapy of human Lyme neuroborreliosis as well as the associated pathogenesis and immunobiology of nervous system involvement.

Introduction

Demonstration of the presence of *B. burgdorferi* the Lyme spirochete in the brain parenchyma or in CSF has rarely been reported [1]. The Lyme spirochete is a fastidious organism that does not culture well even in highly enriched media, localizing primarily to tissue rather than body fluids. Thus very low numbers of spirochetes are predicted in the CSF [2]. With only 50% of *B. burgdorferi* infections leading to clinical disease, it has become increasingly apparent that complex host and spirochete factors play a role in disease manifestations. Heterogeneous strains of *B. burgdorferi* are found not only in different geographic locations but within a given tick. Moreover, cell mediated and humoral host responses appear to play important roles,
promoting the elaboration of circulating proinflammatory cytokines linked to chronic infection. Coyle [3] summarized the background of events leading up to the development of a faithful model of the CNS and PNS manifestations of LNB.

**Background**

Pioneering studies of human LNB by Philipps and colleagues [4] at Tulane Regional Primate Research Center in 1993 employed nonhuman primates (NHP). Six rhesus monkeys in Group 1 were needle-inoculated with the JD1 strain of *B. burgdorferi* while 6 animals comprising Group 2 were exposed to the bite of nymphal *I. dammini* ticks infected by a similar strain of *B. burgdorferi*. A control animal for Group 1 was given a sham needle inoculation while 2 other controls in Group 2 were exposed to the bites of uninfected ticks. The clinical, bacteriological, immunological and pathological signs of infection were investigated 13 weeks after inoculation of the spirochete. Deep dermal perivascular lymphocytic infiltration were observed in the animals with clinical EM, and documented concomitantly with spirochete presence. Spirochete species in cultured samples were confirmed to be *B. burgdorferi* by PCR using the expected species-specific 230-bp fragment of chromosomal DNA amplified in all samples containing cultured spirochetes. Spirochetes were detected in blood by *in vitro* culture until week 6 post-inoculation (p.i.). Two animals developed CNS involvement manifesting lethargy and depression during weeks 2 and 3 p.i. in one animal that resolved spontaneously, and in another animal with multiple episodes of seizure activity while under anesthesia. Signs of PNS involvement such as oculomotor and facial nerve paralysis were not observed. CSF analyzed for the presence of cells, *B. burgdorferi* spirochetes, and anti-*B. burgdorferi* antibodies showed severe pleocytosis in two infected animals of Group II but not in one from Group 1. CSF antibodies to *B. burgdorferi* were demonstrable by Western blot analysis however the antigens recognized did not differ from those in serum. In vitro cultures in CSF were negative for *B. burgdorferi*. Capture ELISA analysis failed to demonstrate intrathecal antibody production. The lack of *in vitro* culture positivity, difference of CSF antigens to serum, and absence of intrathecal antibody production led the authors [4] to doubt the relevance of CSF pleocytosis to active infection.

Two years later, Pachner and colleagues [5] injected rhesus macaques with the N40 strain of *B. burgdorferi* noting that experimentally-inoculated NHP
could serve as models for human LNB. Four untreated animals and one marginally immunocompromised animal were treated with dexamethasone for one week before and another week p.i. with a total of one million spirochetes along their shaved backs. Ten days to 2 weeks p.i. all four immunocompetent NHP displayed characteristic EM, and the three phases of *B. burgdorferi*-specific antibody responses commencing with little or no detectable antibody in the first week, with progressive increase in specific antibody over weeks 2 to 6, followed by a third phase of high-titer antibody. CSF antibody testing showed a pattern of rising antibody reactivity in 3 of 5 animals, while Western blot showed increasing numbers of antigens. CSF PCR correlated with the presence of pleocytosis in 66% of samples, but was negative in 10 cases when pleocytosis was absent yet positive in 17 cases when pleocytosis was present. Pleocytosis disappeared within the second month p.i., then reappeared in the fourth month in all four animals consistent with a relapsing-remitting inflammatory process. Gadolinium-enhanced brain MRI revealed enhancement of the meninges at the base of the temporal lobes on days 32, 46, and 53 p.i. in 1 of 4 animals.

In the same year, Pachner and coworkers [6] reported postmortem examination findings in the 5 earlier described NHP [5]. The authors extended the clinical and laboratory findings in serum and CSF by introducing the intradermal inoculation of a million spirochetes to induce chronic neurological infection as manifested by positive CSF cultures and PCR, with high CSF and serum antibody titers. Postmortem examinations were carried out immediately after death with removal of brain and spinal cord for DNA extraction utilizing the OspA outer surface protein A gene. Hybridization increased the sensitivity of agarose gel PCR reactions by a factor of 10 to 30 allowing detection of *B. burgdorferi* DNA in NHP brain to a higher level of sensitivity. An analysis of extracted DNA from multiple brain and spinal cord locations showed substantial amounts of *B. burgdorferi* DNA in the CNS of all infected animals, with a predilection toward subtentorial structures of the posterior fossa and spinal cord, and dura. Forty-three cultures of tissues from various parts of the brain were negative despite positive CSF samples from 2 of 5 animals previously positive early in CNS invasion [5]. The study of spirochete CNS invasion had benefitted from the development of two independent research advances. First, the advent of PCR identification *B. burgdorferi*-specific DNA sequences in tissue to obviate problems with false positive cultures [7]. Second, the availability of a faithful model of the human Lyme disease in NHP [5] that shared the presence of spirochete organisms in the subarachnoid space, with subarachnoid inflammation, antisyphilitic antibody in the CSF, and

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infection outside of the CNS in skin, blood and heart. CSF lymphocytic pleocytosis in the first few months of infection and variably thereafter were consistent with a meningeal localization. Widespread low density distribution of spirochete DNA throughout the CNS in the infected NHP could be explained, according to Pachner and colleagues [6] by the localization of spirochetes to the region of cerebral vessels and meninges since pia mater penetrated deep into parenchymal structures.

In 1997, England and colleagues [8] performed detailed investigations of the PNS in 8 rhesus monkeys chronically infected with the JD1 strain of B. burgdorferi and compared the results of serial electrophysiological studies to 10 uninfected control monkeys. Four infected and 2 uninfected animals underwent sural nerve biopsy, while 5 infected and one uninfected animal later underwent postmortem examinations. The first observations in the B. burgdorferi-infected animals performed 29 to 32 weeks after infection, and later at 42 to 45 months demonstrated normal results in 3 animals, however 5 others had primarily nerve lesions in mononeuropathy or mononeuropathy multiplex (MNM) patterns. All but one of the monkeys with signs of acquired demyelination had axonal-loss lesions on electrodiagnostic studies. Sural nerve biopsy studies performed on 2 sham-inoculated controls monkeys and in 4 B. burgdorferi-infected animals 40 months after inoculation showed a decrease in the number of myelinated axons without inflammatory cell infiltration. Comprehensive pathological examination of the PNS in the control animal showed no discernible histopathological abnormalities however the B. burgdorferi-infected monkey showed multifocal axonal degeneration as the most common PNS finding with perivascular lymphocytic infiltrates but no vasculitis or vascular thrombosis. There were thinly myelinated regenerating fibers and scattered nerve fibrosis in the chronic stage. Immunohistochemical analysis demonstrated occasional macrophages that exhibited positive immunostaining with anti-B. burgdorferi 7.5-KD lipoprotein monoclonal antibody without evidence of free spirochetal structure. There was an excellent degree of correlation between serial electrophysiological study results and neuropathological findings consistent with axonal multifocal neuropathies.

The finding of inflammatory cell infiltrates observed even in late stages suggested the possibility of active disease in the chronic stage of infection. Indirect evidence of persistent infection discerned by the IgG serum antibody response to B. burgdorferi antigens analyzed sequentially by Western blot over a 54-week period showed a gradual increase in the number of bands up to week 15 p.i. By week 54 all of the animals tested reacted with 5 or more of 10 bands [18, 21, 28, 30, 39, 41, 45, 58, 66 and 93 KD] defined by the Dressler
criteria [9] for the immunodiagnosis of long-term *B. burgdorferi* infection. Those most commonly recognized in the inoculated monkeys were 18, 21, 30, 39, 45, 58 and 93 KD. Together with the reports of Pachner and coauthors [5, 6] that demonstrated CNS infection of rhesus macaques inoculated with the N40 strain of *B. burgdorferi*, the NHP model of Lyme disease appeared to be a major advance in the experimental study of human LNB. The fundamental mechanisms for Lyme-associated peripheral neuropathy were not well appreciated but the multifocal nature and perivascular infiltrates suggested an immune-mediated inflammatory etiopathogenesis with associated angiopathy of epineurial, perineurial, and endoneurial blood vessels. A mechanism of molecular mimicry was suggested by the prior finding of dorsal root ganglia that stained positively with the highly specific anti-*B. burgdorferi* 7.5-KD lipoprotein monoclonal antibody [10]. A minor role for direct PNS infection was suggested by the finding of several intraneural macrophages that immunostained positively with highly specific anti-*B. burgdorferi* 7.5-KD lipoprotein monoclonal antibody.

Roberts and colleagues [11] studied PNS manifestations of early and late LNB in rhesus macaques infected with the JD1 strain of *B. burgdorferi* in which infection was proven by culture or PCR analysis of skin biopsies or indirectly by p.i. Western blot analysis. Three months after infection, neuritis involving endoneurial vessels of multiple nerves were the most consistent PNS manifestation with both macrophage and B-cell but not T-cell cellular infiltrates. There were features of demyelination and axonal phagocytosis with immunohistochemically visualized *B. burgdorferi* antigens present within macrophages. Forty-six months after infection, the most common nerve features were aberrant nerve regeneration, irregularly sized myelinated fibers and fibrosis. However, even at this late stage, *B. burgdorferi* were found in macrophages in endoneurial nerve lesions. Schwann cells that stained positively for anti-tumor necrosis factor (TNF)-α and anti-nitrotyrosine antibodies appeared to be the focus of an autoimmune attack or participated in immunomodulatory phenomena, manifested by the production of interleukin (IL)-1, IL-6, and TNF-α cytokines. Persistence of *B. burgdorferi*, most often noted in infiltrating macrophages in early dissemination, and in isolated foci in the late phase of infection, was considered necessary to initiate and sustain active inflammatory lesions.

Pachner and coinvestigators [12] compared the ability of PCR and culture to detect the presence of spirochetes in the CSF and brain tissue of infected NHP employing the mouse infectivity test (MIT). Two NHP were treated orally with 2 mg/kg of body weight of dexamethasone for 1 week then 1

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mg/kg/day for 9.5 weeks after infection, a dosage considered low to moderate to render them immunocompromised, while two other NHP remained immunocompetent. The two immunocompetent underwent postmortem examination 9.5 p.i. while the immunocompetent NHP were studied at 13 weeks p.i. The MIT, culture, PCR and antibodies studies of the CSF obtained at the time of inoculation prior to infection were negative in all animals. A previous study determined that CNS invasion did not occur before 3 weeks p.i. in NHP [5] but was positive for infection at 3.5 weeks with one or more of the four tests in 14 of 16 (88%) among all four animals, and notably positive in 7 of 8 (88%) CSF specimens from immunocompromised NHP. The MIT test was the only positive test in 2 of 8 (25%) CSF samples from the immunocompromised NHP. CSF antibody measured by ELISA was negative in the CSF of the immunocompromised NHP despite a low level of specific antibody that appeared in the blood and steadily rose by the time of postmortem examination. None of the immunocompromised NHP CSF displayed lymphocytic pleocytosis although sera from the immunocompromised NHP had a number of *B. burgdorferi*-specific IgG bands on Western blot. None of the CSF samples had 5 or more IgG-specific bands fulfilling the criteria for positive seroconversion in CSF. *Borrelia*-specific antibody was present in the CSF of immunocompetent NHP 5.5 weeks p.i. with CSF pleocytosis, and much high serum antibody levels, with IgG and IgM Western blot positivity in the serum. The MIT outperformed culture in samples of CNS tissue in the immunocompromised NHP, while MIT and PCR were concordant for the majority of tissue samples. Thus, in the immunocompetent NHP, *B. burgdorferi*-specific antibody in the CSF was a successful assay for detection of CNS invasion however false negatives were frequent especially early in the course of the infection or with transient immunosuppression. The latter can occur with Lyme coinfections. In immunocompetent NHP, the window of opportunity for CNS invasion prior to development of CSF antibody was brief and the chance of detection of spirochetes by any of the three techniques was low. In this group, measurement of CSF antibody was generally diagnostic. In immunocompromised NHP, intrathecal antibody production was delayed and thus falsely negative, requiring PCR, culture or MIT for diagnosis at 3.5 to 9.5 weeks after infection. The appearance of anti-*B. burgdorferi* antibody in the CSF can be delayed when there is interference by anti-*B. burgdorferi* immune responses. The chance of detecting spirochetes by culture, PCR and MIT was least likely to be successful when the anti-*B. burgdorferi* antibody was present.
To better understand the relationship between chronic infection, antispirochetal immunity and inflammation in LNB, Pachner and colleagues [13] measured spirochetal density in CNS and PNS tissues by PCR and correlated them to anti-*B. burgdorferi* antibody in the serum and CSF, and to inflammation in tissues of 8 rhesus monkeys with experimental LNB. Their findings identified the CNS and PNS as major reservoirs of spirochetal infection and demonstrated that a strong, well-developed anti-*B. burgdorferi* humoral immune response did not clear spirochetes from the animal during the months of infection, especially in the nervous system. Spirochetal presence was a necessary but not sufficient condition for inflammation. Despite the presence of spirochetes in CNS tissue, there was little inflammation and no intrathecal antibody production in the CNS in contrast to strong systemic antibody production and marked inflammation in cardiac and skeletal muscle tissue. Two methods of spirochete inoculation, by needle injection of 1 million N40Br strain spirochetes and feeding of infected ticks were found to be comparable in establishing infection. Transient immunosuppression was employed to maximize the yield of infection in some of the NHP. The presence of spirochete infection was demonstrated by identifying *B. burgdorferi* DNA in multiple tissues by PCR, with a load that varied in nervous system tissue. The authors [13] constructed at least three important hypotheses which were tested in this NHP model of LNB. First, that spirochetes would be found by PCR diffusely throughout the CNS and PNS, and this was supported by the results. The density of spirochetes per milligram of tissue or microgram of extracted DNA was found to be lower in the immunocompetent NHP relative to immunosuppressed NHP but spirochete densities were uniform in all tissues with CNS loads essentially identical to other tissues, and at levels close to those in immunosuppressed NHP. This provided evidence that spirochetes in CNS tissue were protected from immune-mediated clearance relative to spirochetes in the peripheral nerve and skeletal muscle [14, 15].

Second, the authors [13] hypothesized that the spirochete load correlated with the anti-*B. burgdorferi* antibody response and that the former drove the latter, however there was no consistent correlation of the two as measured by ELISA. The amplitude of the IgG response by ELISA and the number of *B. burgdorferi* protein bands identified on Western blot increased with increasing duration of infection. The height and complexity of the IgG response in NHP LNB was likely important for spirochete clearance in the CNS. In other experiments, NHP treated with corticosteroids that mounted a high titer IgM response with a negligible IgG response had very high levels of spirochetes in
tissues other than CNS relative to animals who mounted a typically strong IgG response to disseminated infection [16]. Corticosteroid treatment resulted in interference with isotype switching in NHP providing evidence that anti- *B. burgdorferi* IgG antibody was more effective than IgM antibody in decreasing the spirochetal load in infected animals.

A third hypothesis was that inflammation was highly correlated with the presence of spirochetes. This was not entirely accurate as the cerebrum had a high load of spirochetes relative to other tissues but no inflammation whereas other organs such as skeletal muscle had very high levels of inflammation with spirochete loads similar to that of the brain.

A fourth hypothesis was that selective intrathecal IgG antibody production would not be prominent despite the presence of large amounts of *B. burgdorferi* within the CNS, and that proved to be accurate. The intrathecal IgG antibody production of the N40Br *B. burgdorferi* sensu stricto strain of the pathogen was not prominent at all [17] in contrast to the pathogen of Lyme meningitis in Europe, *B. garnii* in which selective intrathecal IgG antibody was a consistent feature [18]. In light of the similar values of serum and CSF IgG, when each were adjusted to the same concentration of IgG and there was no evidence of intrathecal antibody synthesis, it was conjectured that diffusion from the serum into the CSF accounted for the impression of selective intrathecal production in a prior study [5]. In another set of NHP experiments, Cadavid and colleagues [19] employed the PCR-ELISA of the OspA gene followed by immunohistochemistry to study the localization and numbers of spirochetes in CNS and PNS tissues in three groups of animals: infected immunosuppressed, infected immunocompetent, and uninfected controls. An unexpired finding was that treatment with corticosteroids resulted in persistence of high levels of specific antibodies of the IgM isotype, which were unable to eradicate the infection from any of the tissues examined. OspA was down-regulated early after infection and OspC was down-regulated in chronic infection while expression of flagellin was maintained. Employing PCR-ELISA, their investigations were able to detect as little as 10 to 30 fg of *B. burgdorferi* DNA per 500 ng of tissue DNA. The amount of OspA DNA was significantly higher in all tissues from immunosuppressed animals compared with immunocompetent animals. PCR-ELISA showed that *B. burgdorferi* DNA was present in most tissues examined from all inoculated animals but highest in the immunosuppressed group. Spirochetes were found in the leptomeninges, motor and sensory nerve roots and dorsal root ganglia but not in spinal cord parenchyma, with the highest counts in the lumbar roots and cauda equina. Spirochetes were also found in the perineurium,
endoneurium, and epimysium and endomysium, as well as, in leptomeninges and nerve roots but not in CNS parenchyma consistent with the usual presentation of headache, meningismus or radiculopathic symptoms. The localization of spirochetes in CNS of humans has rarely been described using nonspecific silver impregnation [20, 21, 22] revealing a positivity of PCR-ELISA in samples from cerebrum and brainstem were likely to be leptomeningeal rather than parenchymal in localization.

Bai and colleagues [23] provided additional insight into spinal cord involvement in the NHP model of LNB in a study of 25 adult rhesus monkeys inoculated with *Bb* sensu stricto strains N40 or 297 by needle or tick inoculation, or *Bb* genospecies *B. garinii* Pbi, 793, or Pli, by needle. Two of the NHP were immunocompetent; all others were immunosuppressed with dexamethasone transiently during the first month of infection or permanently for the duration of infection lasting 2 to 4 months before postmortem examination. Spinal cord tissue was processed for RNA extraction and histology, and comparisons of spirochetal load in different regions of the spinal cord including meninges, dorsal root ganglia and motor and sensory nerve roots. Serum ELISA, Western blots, and immunohistochemistry were performed for detection of *B. burgdorferi* using hyperimmune serum from a rabbit persistently infected with *B. burgdorferi* strain N40 using negative controls, and rabbit polyclonal antibody antihuman immunoglobulins, B- and T-cells, C1q and C5b-9. Total RNA was extracted and reverse transcription (RT) was performed as well as PCR amplification for rhesus genes using Taq DNA polymerase and human C1q beta chain sequences. DNA sequencing was performed after RT-PCR amplification using *Borrelia* Taqman probes. Viable spirochetes were identified only in tissue culture from immunosuppressed NHP but not in any of the cultures from transiently immunosuppressed or immunocompetent NHP. Serum ELISA showed that all NHP inoculated with *B. burgdorferi* sensu stricto strain N40 by syringe or tick developed specific antibody, compared to only one of the two NHP inoculate with *B. burgdorferi* strain 297 and 4 of 10 NHP inoculated with *B. garinii* strains. All immunocompetent and transiently immunosuppressed NHP inoculated with N40 or 297 showed positive Western blot results. One animal inoculated with the *B. garinii* strain Pbi showed mild mononuclear meningoaradiculitis with inflammatory infiltration along the entire neuraxis including the brain, brainstem, spinal cord, spinal nerve roots and dorsal root ganglia, however microscopic examination failed to reveal inflammation in the remaining 24 NHP. The inflammatory cell infiltrate in that animal was composed of T-cells and plasma cells, rare B-cells and macrophages, with local deposition of IgM.
IgG, and C1q in meninges and spinal nerve roots. Spinal cord and brain tissue of this animal showed local production of C1q mRNA in areas of CNS inflammation. Spinal cord tissue examined for spirochetes by immuno-histochemistry and TaqMan RT-PCR, and microscopic examination of spinal cord immunostained with hyperimmune rabbit specific serum to B. burgdorferi strain N40, revealed spirochetes only in tissues from permanently immunosuppressed NHP with localization in the dura mater, leptomeninges, dorsal root ganglia and motor and sensory spinal nerve roots but not in the cord parenchyma. In no case were spirochetes observed in the spinal cord of immunocompetent or transiently immunosuppressed NHP implying that if immunocompetent or transiently immunosuppressed had persistent infection, then spirochetal load was probably very low. There was variation in spirochete load in the immuno-histochemical reaction and TaqMan RT-PCR analysis between animals and along regions of the spinal cord, although the majority of spirochetes were in meninges, spinal nerve roots and dorsal root ganglia rather than in cord parenchyma. The ratio of spirochete load in leptomeningeal, spinal nerve root and dorsal root ganglia compared to the CNS parenchyma varied from 12 and 185 to (mean ratio 100). The finding of immunoglobulin deposits and C1q indicated activation of the humoral arm of the immune response to infection and a potential mechanism of infection-related and immune complex-mediated tissue injury with potential induction of chemokines and cytokines, and the recruitment of inflammatory cells.

Primary cultures of normal rhesus brain astrocytes and microglia incubated with live B. burgdorferi were used to quantify Toll-like receptor (TLR) activity and to study its involvement in mediating the expression of cytokines and chemokines in response to in vitro B. burgdorferi. TLR have a purported role in innate immune responses against microbial pathogens including B. burgdorferi through the recognition of highly conserved structural motifs or pathogen-associated molecular patterns. Of the ten TLR types displaying distinct ligand specificities, Bernardino and colleagues [24] hypothesized that TLR1, -2, -5, and -9 might be involved in the innate response of Bb in the CNS. After incubation with live B. burgdorferi, TLR and cytokine mRNA expression were measured and TLR and cytokine transcripts quantified by reverse transcriptase PCR (qRT-PCR). Expression of TLR proteins were assessed by Western blotting demonstrating upregulation of TLR1, -2 and -5. The correlation of upregulated levels of mRNA encoding TLR types with increased receptor protein expression in microglia was assessed by flow cytometric analysis of CD45 and CD11b cell markers. Confocal microscopy was used to further verify the expression of TLR1, -2
and -5 on microglia and astrocytes and to assess their localization in microglia. There was upregulation in microglia that contained spirochetes or spirochetal fragments. Microglia that had not internalized spirochetal components expressed much lower levels of TLR1 and -5 and no TLR-2 however, TLR1, -2 and -5 proteins were constitutively expressed by astrocytes by spirochete stimulation. These findings contrasted with previously reported production of TNF-α by astrocytes stimulated with L-OspA [25] suggesting that there might have been microglia contamination. While B. burgdorferi spirochetes localized primarily to the meninges in the CNS of rhesus monkeys, the authors [25] suggested that they probably migrated to the CNS parenchyma wherein they elicited TLR-mediated proinflammatory responses in glial cells that might be an important factor in the pathogenesis of human LNB.

Embers and colleagues [26] studied persistence of B. burgdorferi in rhesus monkeys after antibiotic treatment of disseminated infection. The authors contended that in comparison to control animals, a few spirochetes grew in cultures of organ tissues collected at postmortem examination from animals treated with varying regimens of parenteral and oral antibiotics. These included parenteral ceftriaxone for 4 days followed by doxycycline for 8 days during the late disseminated phase of infection in Experiment 1, and doxycycline alone for 28 days in the early disseminated phase of infection in Experiment 2. However the investigators were unable to subculture any spirochetes from either treated or untreated animals. Metabolically active spirochetes were indicated by the presence of B. burgdorferi DNA and RNA in experiment 1, while spirochetes were recovered by xenodiagnoses with confirmation by analysis of OspA gene transcription in experiment 2. The authors contended that it was unclear whether spirochetes remained pathogenic after antimicrobial therapy. Wormser and colleagues [27] argued that insufficient attention was placed on documenting the blood levels, pharmacokinetics (PK) and pharmacodynamics (PD) parameters for the antibiotics used in this host. Therefore it was not possible to conclude that doxycycline or ceftriaxone were inefficacious in the treatment of rhesus monkey LNB or by extension in humans. Antibody responses to the B. burgdorferi-specific C6 peptide employed in the study by Embers and colleagues [26] that showed longitudinal decline in the treated animals were less variable than other recombinant antigens suggesting that it had the potential to detect infection throughout all disease phases.
Conclusion

Pachner [28] observed that the diagnosis of human LNB is made clinically by the appearance of neurological involvement in association with prototypical serum and CSF Borrelia-specific serology. Measurement of B. burgdorferi-specific antibody during the early phase of infection is commonly negative. The amplitude of the antibody response in the serum varies with time making it most useful with increasing time post-infection. This maturation in the antibody response is evident an increased in amplitude of antibody and increase in the number B. burgdorferi-specific proteins recognized by Western blotting. The intrathecal antibody response has not been shown to be useful or predictive in the early phases of neurological involvement in NHP. Immunosuppression favors the recovery of spirochetes in neural tissue notably the meninges, dorsal root ganglia and nerve roots. Infected NHP manifest persistence of spirochete infection for months to years after infection, although with very low pathogen load. The most dramatic features of LNB appear to be due to extensive chronic inflammation which does not appear to exist without infection. The converse also occurs, that is infection in the absence of local inflammation particularly in the CNS of NHP. Infection is a necessary but not sufficient cause for inflammation. Spirochete-mediated activation of the immune system includes systemic upregulation and intrathecal synthesis of complement proteins, activation of TLR determinants, and the proliferation of proinflammatory cytokines and chemokines that mitigate inflammatory responses to spirochete components.

References


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Clinical Aspects

Abstract

The neurological clinical aspects of Lyme disease encompass three major systems, the CNS composed of the brain and spinal cord, the PNS constituted by exiting cranial and spinal nerve that coalesce to form distal peripheral nerves, and the autonomic nervous system that comprises a complex network of central and peripheral fibers that commence in the brain hypothalamus and terminate in the individuals organs and tissues it innervates. Lyme disease can affect one or all of these systems resulting in characteristic clinical features. In addition to serologic studies in the blood and CSF to identify past or present infection by *B. burgdorferi*, neurological laboratory studies can be performed to identify central, peripheral and autonomic nervous system and enhance the diagnosis and differential diagnosis of affected patients. This chapter reviews the clinical aspects of Lyme neuroborreliosis.

Introduction

LNB is the preferred term for the neurological complications of Lyme disease caused by *B. burgdorferi* sensu lato worldwide and *B. burgdorferi* sensu stricto [1], the vector of Lyme disease in North America. CNS manifestations result from involvement of the brain and spinal cord that presents as meningitis, encephalitis and encephalopathy. PNS manifestations result from involvement of large caliber, named peripheral nerves that present
as cranial neuritis, radiculoneuritis, MNM, distal polyneuropathy, and painful small fiber neuropathy. Autonomic nervous system (ANS) manifestations result from autonomic neuropathy and ganglioneuritis that presents as orthostatic intolerance and postural orthostatic tachycardia syndrome.

Even before recognition of the responsible spirochete, European investigators described EM in 1922 [2] and a year later, the neurological triad of meningitis, cranial neuritis, and painful radiculitis [3]. European physicians familiar with the disorder emphasized both neurological and rheumatic involvement [4] and routinely treated it with penicillin. In 1977, EM rash was later described in the U.S. in conjunction with childhood arthritis in near epidemic proportions in towns surrounding Lyme Connecticut [5]. Detailed epidemiological studies demonstrated that the disorder occurred in children with a history of *Ixodes* tick bites and EM. Two years later, a triad of neurological sequela was described similar to those in Europe years before [6]. Subsequent studies in the U.S. [7-9] and Europe [10, 11] led to the isolation and identification of the causative *B. burgdorferi* spirochete and the neurological sequela thereof. This chapter reviews the clinical aspects of LNB.

**Epidemiology**

With an incidence of 1 in 2719 persons, and involvement of the nervous system in 12 to 15% of individuals infected by *B. burgdorferi* in the U.S. [12], reported cases of Lyme disease have been rising steadily for the past three decades. This underreported disease has a 15-year mean annual rate for all states ranging from less than .01 cases per 100,000 persons of Montana and Colorado, to 74 cases per 100,000 in Connecticut. The vast majority of statistically affected individuals are white, children of age less than 15 years, and adults of either gender of more than 30 years of age. Since the responsible spirochete is transmitted almost exclusively by the bite of infected hard-shell back legged *Ixodes* tick that occur in specific locations in which appropriate hosts are available, residence in or visitation to endemic areas during the spring and summer months are useful in determining the likelihood of contracting the illness.
Etiology

The life cycle of the causative *B. burgdorferi* spirochete is well understood. Larval ticks transmitted by the bite of infected *Ixodes* ticks hatch uninfected and feed upon small mammals such as white footed field mice, catbirds, squirrels, opossum, and other small mammals, and then mature into nymphs. If the initial host is infected so will be the nymph tick which can transmit the disease. For the second host, perhaps human, two other conditions must be met before contraction of the disease. First, spirochetes must proliferate in the tick gut prompted by ingestion of blood with subsequent dissemination to tick salivary glands. Second, the infected tick must attach to the host for a relatively prolonged period typically 24 to 48 hours. Endemic cycles have been established in discrete areas of North America, Europe, and Asia. Along the eastern coast of the U.S. from Maryland to Massachusetts, infected *I. scapularis* ticks, known as deer ticks, are widely prevalent. The same ticks are also found in Minnesota and Wisconsin where they are known as bear ticks. In general, causative ticks reside where animal hosts are widespread inhabiting underdeveloped areas to wealthy exurban regions beyond the suburbs of a city. Some mammalian species are poor Lyme disease hosts because they infect few of the ticks that bite them, or kill the ticks when they groom their fur. Habitats high in biodiversity lead to an overall reduced risk of Lyme disease to its inhabitants because ticks feed both upon efficient hosts such as field mice and less efficient ones such as squirrels and opossum.

Clinical Manifestations

Clinical manifestations of this infection differ in other parts of the world such as Europe where cutaneous abnormalities such as acrodermatitis chronica atrophicans and lymphocytoma cutis, which have rarely been recognized in the U.S., while joint and cardiac appear less commonly in European patients. Several factors appear to contribute to these apparent differences including bias of ascertainment; and significant differences in the prevalent strains of infecting *Borrelia* organisms in different parts of the world, each with tropisms for different organ involvement leading to clinical differences. Bias of ascertainment may be influenced by rheumatologists who have traditionally treated the disease in the U.S., while in Europe it has been historically treated by neurologists. There are more common European strains besides *B.
*burgdorferi* including *B. garinii*, responsible for most neurological diseases, and *B. afzelii*, that causes acrodermatitis. There are differences in tick vector species around the world with *I. ricinus* noted in Europe, and *I. persulcatus* predominating in Asia.

Although the causative pathogen has only been characterized in the past three decades, many of the clinical disorders it causes have been well known for many years. After acute exposure via prolonged tick attachment, most but not all patients develop a characteristic EM rash. This slowly enlarging erythoderm, which may reach many inches in diameter, typically develops over days to weeks at the site of the tick bite and attachment, which may be unapparent. One prospective study noted occurrence of an EM rash in up to 90% of affected children [13], however that appears to be excessive. In some patients the spirochetes disseminate early and cause multifocal EM. There is often concomitant fever, arthralgia, malaise, and flu like illness but not so in the sense of a typical upper respiratory or gastrointestinal infection. However, as both the tick bite and rash may be asymptomatic and occur on parts of the body not easily seen, affected patients may be unaware of either.

Three neurological syndromes clearly attributed to Lyme disease include lymphocytic meningitis, painful meningoradiculoneuritis, and encephalomyelitis. They occur relatively acutely providing pathognomonic recognition of the disease. They were collectively recognized by U.S. investigators [9] as a triad more than fifty years after the initial European description [3]. Two others, peripheral neuropathy and encephalopathy, that present variably early or late in the disease course, have been ascribed to chronic infection [14]. Lymphocytic meningitis is clinically indistinguishable from viral or aseptic meningitis, occurs several weeks after the skin rash, and is characterized by varying degrees of headache, neck stiffness, and photophobia; whereas, radicular involvement, especially evident in painful polyradiculoneuritis, is typically asymmetric, of sudden onset, and localized close to the bite with a burning quality and nocturnal exacerbation [15]. Weakness appears over several days with cranial nerve involvement, especially the facial nerve, although this may occur as part of the PNS disorder in the absence of meningitis [16, 17]; and tendon reflexes, which may be reduced or absent suggesting Guillain-Barré syndrome. Lyme encephalomyelitis [18-20], which occurs in 0.1% or less of untreated patients in Europe and North America with a presentation of symptoms and signs appropriate to the site of inflammatory focal white matter brain and spinal cord involvement, typically manifests focal cerebral deficits or progressive gait disorder with sphincter dysfunction.
There have been arguments forwarded on both side for the connection between Lyme disease and motor neuron disease (MND). One is that improvement of MND with treatment for Lyme is evidence of the presence of Lyme disease. A second is the increased frequency of positive serologic testing for Lyme disease in those with MND. Halperin and colleagues [21] performed a variety of serological studies on 56 patients with MND from a highly endemic area for Lyme disease noting 9/19 (47%) who tested positive for Lyme compared to 4/38 (11%) without MND. Quereshi and colleagues [22] employed ELISA and Western blot testing at the Massachusetts General Hospital noting 4/414 (0.97%) of amyotrophic lateral sclerosis (ALS) patients to be positive for Lyme disease by the two-tier method, thus similar to the background rate of positive Lyme tests in the northeastern U.S. Although exceedingly rare, spontaneous remissions of MND do occur in those not taking any antibiotics [23, 24]; while those reported to be cured with antibiotics occurred in patients with atypical presentations. One such patient with cervical myeloradiculopathy had lower motor neuron signs in the arms and upper motor neuron signs in the legs [25]. Notwithstanding, it would be appropriate to consider Lyme testing in patients with atypical MND accompanied by rash, headache, stiff neck, fever, reversible facial nerve palsy, dermatomal pain and sensory loss, especially those from Lyme-endemic areas, according to the CDC criteria and to treat rationally according to validated guidelines if Lyme was diagnosed [26]. Compelling arguments can be proposed for two other relatively uncommon disorders that may coexist with LNB without true causality, namely Parkinson disease and MS.

**Diagnostic Approach**

The systematic approach to the investigation of the CNS, PNS and ANS function in LNB employing strict CDC criteria for case selection has been reviewed [27].

Suspected patients with endemic exposure to the causative organism, *B. burgdorferi*, with or without known tick attachment or EM rash, should undergo two-tier serological testing. First-tier screening is performed by an ELISA which should be performed in all suspected patients that becomes informative 3 to 4 weeks after initial exposure, when Lyme-specific IgM serology is also detectable followed months later by a Lyme-specific IgG response. During the early phase of exposure, serological testing may be uninformative and falsely negative. Second-tier confirmatory Lyme IgM and

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IgG Western blot or immunoblot, should be obtained with borderline and reactive first-tier test results. The Lyme IgM Western blot response is the first to appear after initial exposure to *B. burgdorferi* and is comprised of specific and non-specific bands. This is followed by the Lyme IgG Western blot response of specific and non-specific band reactivity months later. Consensus criteria have been developed for Western blot confirmation which have very high specificity such that a positive Lyme Western blot IgM provides compelling confirmation that an acute disorder may indeed due to *B. burgdorferi* infection, and a positive Lyme Western blot IgG provides strong support of more long-standing *B. burgdorferi* infection. This approach which is recommended by most authorities [28-30] and the CDC have certain limitations. First, the sensitivity and specificity of the serological assessment of Lyme disease has not been determined in representative cohorts. Second, early treatment may lead to falsely negative serological test results as may also occur in immune-compromised individuals and immune-competent patients with true infection that are tested before a Lyme IgM Western blot immune response is mounted.

A spinal tap for CSF studies should be considered in all patients with CNS involvement for routine studies and paired serum for *B. burgdorferi* serology including diagnostic levels of intrathecal *B. burgdorferi*-specific IgM and IgG antibodies with derived comparison to serum in paired specimens. This derived Lyme index is indicative of intrathecal secretion of anti-*Bb*-specific antibody. A *B. burgdorferi*-specific intrathecal response is rarely found by coincidence and in the presence of concomitant inflammation has a diagnostic predictive value of >95% [31]. CSF inflammation and intrathecal section of anti-*B. burgdorferi*-specific antibody are currently the best laboratory evidence of active LNB [32]. Specific CSF antibodies appear during the second week after onset of neurological symptoms [33, 34]. In Europe where investigators required demonstration of intrathecal synthesis of specific antibody to render a diagnosis, the sensitivity was by definition 100% [35] in comparison to 50% in a large U.S. study of Lyme disease [36]. Isolation studies for *B. burgdorferi* by PCR, while insensitive, provides additional confirmation of exposure to *B. burgdorferi*. Common CSF findings in LNB include elevated protein content, normal glucose, lymphocytic pleocytosis, and humoral immune response composed of intrathecal immunoglobulins that depend upon the stage and activity of the disorder [37]. There may rarely be CSF lymphoid pleocytosis suggestive of cerebral or cerebromeningeal malignancy due to activation and transformation of T- and B-lymphocytes as a result of antigenic stimulation by *B. burgdorferi* [38]. In individuals with an
MS-like illness or otherwise prominent intrathecal IgG synthesis, measuring *B. burgdorferi*-specific intrathecal antibody production should be highly reliable [35]. CXCL13 is one very important specific chemokine biomarker of acute untreated LNB that appears in the early stages of the disease when the *Bb*-specific antibody index is still negative. Stromal cell-derived factor-1, a ligand of the chemokine receptor CXCR4 known as fusin, appears also to be expressed in CSF and blood of patients with various stages of Lyme disease. In addition to studies for *B. burgdorferi*, the CSF should be screened for viral serology, other bacterial, tuberculosis and fungal organisms, and a panel of immunological determinants including CSF IgG, oligoclonal bands, panels of autoantibodies appropriate to the neurological diagnosis, and cytology.

MRI of the brain and spinal cord should be considered in all patients with LNB employing T<sub>1</sub> and T<sub>2</sub>-weighted sequences to quantify the structural integrity of the brain and search for white matter changes (WMC) and gray matter atrophy, as well as, other disease associated lesions. Conventional MRI shows subcortical white matter lesions indistinguishable from MS in T<sub>2</sub> and fluid-attenuated inversion recovery (FLAIR) imaging. Multi-parametric magnetization transfer and diffusion tensor MRI [39] and high-field 3-D <sup>1</sup>H+MR spectroscopy can be coupled with non-localizing proton MR spectroscopy (1H-MRS) and three dimensional (3D) <sup>1</sup>H-MRS at 3 tesla (T) to examine an array of key brain metabolites including N-acetylaspartate (NAA), an indicator of neuronal integrity, creatine (Cr) corresponding to cellular energy reserves, choline (Cho) and phosphocholine indicative of the levels of membrane turnover, and myoinositol (mI) an astrocyte marker [40, 41]. Preliminary studies of 3D-<sup>1</sup>H-MRS (TR/Te=1800/35ms) in patients with CDC confirmed Lyme disease and CNS manifestations [41] found no differences between affected cases and controls in absolute NAA, Cr, and mI content in whole brain or when gray and white matter were considered separately within the voxels of interest using a mixed model of analysis of variance (ANOVA). Brain and cervical spinal cord T<sub>1</sub>- and T<sub>2</sub>-weighted WMC so noted in three patients with LNB were not associated with the structural or metabolic changes seen in MS lesions [39], including one patient who had resolution of WMC after treatment with intravenous ceftriaxone.

Nuclear medicine cerebral perfusion with single photon emission computed tomography (SPECT) reveals various patterns of potentially reversible cortical hypoperfusion in various stages of Lyme encephalopathy (LE) due to infectious or inflammatory disruption of the BBB [42]. Logigian and colleagues [42] derived a perfusion deficit index (PDI) based upon acquired Tc-99m hexamethylpropyleneamine oxime (HMPAO) activity
reflective of the number and severity of focal perfusion defects, notably in subcortical frontal and other cortical regions. Mean PDI values were highest in those with definite LE (255, range 193-354) characterized by either *B. burgdorferi* intrathecal antibody production or spirochetal DNA in CSF, or serological evidence of exposure by ELSIA and Western blotting in serum with evidence of neuropsychological testing abnormalities. Mean PDI were intermediate in possible LE (198, range 136 to 275) and significantly lower in normal controls (136, range 67 to 354). Six months after treatment with intravenous ceftriaxone, the PDI declined in all of patients with definite LE. The authors concluded that LE patients could be distinguished by the presence of hypoperfusion of frontal subcortical and cortical structures that partially reversed after intravenous antibiotics, however SPECT could not be used alone to diagnose LE or determine the presence of active CNS infection. There has not been a systematic analysis of the sensitivity or specificity of nuclear medicine brain SPECT in LNB nor has it been adequately studied in well-defined cohorts of LNB. Most authorities consider nuclear medicine SPECT a useful predictor of BBB integrity due to either acute, subacute or chronic infection, or the sequela of post-infectious autoimmunity. Patients with presumptive or definite LNB may be distinguished by a variety of cortical hypoperfusion deficits that may be generalized or heterogeneous in both hemispheres, localized to a single hemisphere, cortical or subcortical region (Figure 1).

Brain Fluorodeoxyglucose proton-emission tomography (FDG-PET) may be useful in the evaluation of cognitive and memory disturbances in affected patients with LE. It has the capacity to reveal cerebral metabolic changes. Hypometabolism in the temporal lobes was noted in 17/23 (74%) of patients, 12 of which were bilateral, 7 of whom had diffuse cortical hypometabolism that include frontal and parietal lobes [43]. Thus two patterns in patient with LE were specific temporal lobe hypometabolism or a diffuse cortical hypometabolism, in which temporal lobe involvement was an important associated feature of the memory disturbance. LE was evaluated in a case-controlled study quantifying global or topographic distribution of regional cerebral blood flow (rCBF) or cerebral metabolic rate (rCMR) in 35 affected patients [44]. There was no difference between study patients and controls in measurements of resting rCBF and rCMR except after hypercapnia challenge. The close coupling between rCBF and rCMR suggested that the observed regional abnormalities were primarily metabolically driven. MRI can be combined with PET imaging to more effectively display the area of hypometabolism (Figure 2).

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Figure 1. Nuclear medicine cerebral perfusion (brain) single photon emission tomography (SPECT). A 17-year-old girl with Lyme encephalopathy and memory disturbance. There is hypoperfusion of the right parietotemporal regions (*).

Nerve conduction studies and electromyography of the affected limbs and regions employing standard techniques [45] should be considered in clinically symptomatic patients to investigate the commonest causes of neuromuscular symptoms in LNB including painful polyradiculoneuritis [15], cranial neuritis [17], and peripheral neuropathies [16, 46] the commonest of which are MNM and sensory axonal polyneuropathy. Less likely neuromuscular associations in patients with LNB have included a motor neuron syndrome [21], acute [16, 47] and chronic demyelinating neuropathy [48, 49], demyelinating neuropathy with multifocal conduction block [50, 51], and myositis [52-54], further separable by electrodiagnostic studies.

Quantitative sensory testing for heat pain perception thresholds and epidermal nerve fiber density through a 3 millimeter punch skin biopsy are tests for small fiber neuropathy, which is often reported as burning or shooting sensations in association with coldness of the limbs [27]. Cutaneous sensory nerve biopsy can be useful in documenting the type and severity of neuropathy, and whether it is likely to require adjunctive therapy with immune modulatory therapies [15]. Quantitative sudomotor axon reflex testing, beat-to-beat blood pressure and heart rate responses to head-up tilt, deep breathing, and Valsalva maneuver are useful measures in those with suspected
dysautonomia [55], the symptoms of which typically consist of palpitation, dizziness, headache, and lightheadedness in association with orthostatic intolerance and postural orthostatic tachycardia syndrome [20, 56-58].

Figure 2. Magnetic resonance imaging (MRI) combined with Fluorodeoxyglucose positron emission tomography (FDG-PET). A 12-year-old girl with memory disturbance. There is hypometabolism of the left temporoparietal regions (*).

Vestibulonystagmography [59] may be appropriate in symptomatic patients with Lyme disease, vestibular cranial neuritis and vertigo, alone or associated with facial palsy [60, 61] or concomitant brainstem and vestibular nerve root involvement [62].

**Etiopathogenesis**

The etiopathogenesis of nervous system damage in LNB are not well understood but are likely related to aspects of the infectious process and associated autoimmune host factors; and the two processes may overlap in a given patient. The factors that influence the infectious process include tropism of the *B. burgdorferi* pathogen for particular areas of the CNS and PNS, the duration of tick attachment, dose of spirochete inoculum, the particular organism strain, and persistent atypical cystic and granular spirochete forms.
The factors that influence the host response to *B. burgdorferi* infection may include a history of prior history of infection, the status of host immune competence, the innate host major histocompatibility complex (MHC) or human leukocyte antigen (HLA) system that resides on chromosome 6 encoding antigen-presenting proteins and other essential elements of cell-mediated and humoral immune host responses; and concomitant tick-borne opportunistic co-infections. CNS vasculitis, while exceedingly uncommon, was associated with true vasculitis [63], accounting for 0.3% of cases of LNB in three East German neurological departments located in regions endemic for Lyme disease [64]. Patients with LNB may present with cerebral infarction, intracerebral or subarachnoid hemorrhage and transient ischemic attack due to CNS vasculitis [65-70]. The possible mechanisms of inflammation in LNB are reviewed elsewhere [71, 72].

Human autopsy studies in LNB are sparse [73, 74] but have shown parenchymal inflammation of nerve roots and spinal ganglia likely due to their proximity to inflamed and Bb-containing CSF. Non-human primate models have offered some clues to the etiopathogenesis of CNS and PNS associated with *B. burgdorferi* [75-79]. Since *B. burgdorferi* is primarily a human pathogen, animal models have generally required immunosuppression. Immune-suppressed non-human primates inoculated with the N40 strain of *B. burgdorferi* developed infection of multiple tissues including the CNS and PNS [78] with higher numbers of spirochetes than immune-competent and uninfected controls along the leptomeninges, nerve roots and dorsal root ganglia, but not CNS parenchyma suggesting spirochete tropism for those areas [79]. Although frank CNS inflammation is not observed, one reported primate model of LNB [80] demonstrated *B. burgdorferi* penetration into freshly collected slices with increased overall expression of inflammatory intermediates and their transcripts employing DNA microarray analysis, as well as, increased expression of TNF-α, interleukin (IL)-6, IL-8, and CXCL13 in glial cells in situ by immunofluourescent staining and confocal microscopy. Mitogen-activated protein kinase (MAPK) which inhibits p38 and Erk1/2 MAPK and diminishes TNF-α production in rhesus astrocyte cultures infected with *B. burgdorferi*, and may be one strategy to control inflammation and apoptosis in CNS LNB [81]. The expression of toll-like receptor (TLR), in particular TLR 1, -2, and -5, that play a major role in innate immune responsiveness against microbial pathogens and recognize a variety of highly conserved structural motifs and pathogen-associated molecular pattern (PAMO), was enhanced in the phagocytosis of *B. burgdorferi* in primary cultures of rhesus microglia and astrocytes [82]. Three patients with
neuropathologically-confirmed LNB demonstrated atypical and cystic spirochete forms with nuclear fragmentation and apoptosis of infected astrocytes employing deoxynuclerotidyltranferase (TdT)-mediated dUTP nick end labeling (TUNEL), induced in vivo and in vitro by following infection of primary chicken and rat neurons, as well as rat and human astrocytes [83].

Lyme meningoradiculoneuritis, which similarly combines aspects of direct spirochete infection with host autoimmunity, offers additional clues to the etiopathogenesis of injury in LNB. Reported patients develop early CNS and PNS involvement related 18 days to 2 months after EM manifested respectively by transient encephalopathy, and objective distal sensory involvement in a radicular or diffuse sensory neuropathy type [11]. CSF analysis in such patients shows lymphocytic pleocytosis of up to a few hundred cells, with mildly elevated protein and normal glucose content, with isolation of B. burgdorferi in up to 10% of CSF cultures [84], and demonstrable intrathecal antibody in up to 90% of CSF specimens [85]. Sural nerve biopsy in several such patients showed axonal and secondary demyelination mediated by endoneurial and epineurial lymphocytic invasion forming perivascular cuffs, in agreement with tandem electrophysiological studies [11, 15].

Lyme encephalomyelitis and the far more common chronic encephalopathy, originally considered a peculiarly American phenomenon, was later observed in European patients [86]. Both demonstrate abnormalities on neuroimaging studies and CSF studies, including intrathecal antibody production, and responsiveness to antimicrobial therapy [87, 88]. Affected patients with serologically-proven and previously treated late LNB, manifested neuropathological abnormalities including distal polyneuropathy and mononeuritis multiplex with mild axonopathy and secondary demyelinating changes that resolved with higher doses or a more prolonged regimen of parenteral penicillin and ceftriaxone suggesting insensitivity of the spirochete or inadequate antibiotic penetration across the BBB and blood-nerve barrier. Affected patients with chronic polyneuropathy and detectable B. burgdorferi DNA by PCR amplification had demonstrable C5b-9 membrane attack complex (MAC) deposits in biopsied cutaneous sensory nerve tissue, and improvement of neuropathic symptoms after parenteral ceftriaxone [46] suggesting induction of immune injury by B. burgdorferi even in the late stages of LNB. One patient with serologically-proven LNB and polyradiculoneuritis had demonstrable CD8+ suppressor T-cell infiltrates in association with deposits of MAC in biopsied sural nerve tissue and improved with a second course of parenteral ceftriaxone [15] suggesting participation of
both cell- and humoral-mediated immune injury as a likely consequence of *B. burgdorferi* infection. A variety of candidate antigens that cross-react with constituent peripheral nerve molecules were found in sera and peripheral nerve of affected patients with LNB [51, 89].

Acquired autonomic neuropathy in association with LNB, in which autonomic fibers are selectively or disproportionately affected leading to orthostatic intolerance and other dysautonomic syndromes, are also presumably of autoimmune cause [27, 57] as suggested by the occurrence of autonomic neuropathy after Lymerix and Connaught vaccination [90, 91].

**Conclusion**

The abrupt onset of acute meningoradiculoneuritis with CSF pleocytosis and a triad of meningitis, radiculitis, and cranial neuritis in an affected patient, weeks to months after a spreading erythoderm, so called Garin’s triad, should prompt a vigorous search for the causative agent of Lyme disease, *B. burgdorferi*. Occasional patients present with CNS involvement including encephalopathy long after initial treatment that may be due to mild encephalitis. Accurate diagnosis of CNS infection requires paired serum and CSF specimens, measurement not just of CSF anti-*B. burgdorferi*-specific antibody but actual intrathecal antibody production. Non-human primate models have contributed to our understanding of the etiopathogenesis of nervous system injury, providing clues to the complexity of clinical presentation in human including tandem autoimmune mechanisms of injury. The tandem occurrence of other rare degenerative or inflammatory neurologic disorders may require further investigation to establish causality.

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Clinical Aspects


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Serological Testing

Abstract

The laboratory evaluation methodologies supportive of the diagnosis of human Lyme disease are in general indirect based on serological assays because of the inherent difficulty in demonstrating *B. burgdorferi* by direct techniques such as culture and PCR. Moreover, the direct detection of human Lyme antigens has not been reliable. This chapter reviews current approaches to the serological assessment of Lyme neuroborreliosis.

Overview

The laboratory diagnosis of Lyme disease and LNB has traditionally relied on the demonstration of peripheral blood immunoreactivity against the causative organism. Microbiologic culture has not been widely used for several reasons. The spirochete, *B. burgdorferi* is fastidious, requiring specific media [1-3], which is not commonly stocked by most hospitals and commercial laboratories. The bacterial load is rather small except in skin lesions, for which microbiologic diagnosis is usually superfluous, giving rise to problems of sampling and limiting sensitivity [4]. The organism grows slowly *in vitro* sometimes requiring up to 16 weeks of incubation before being considered clearly positive or negative. As a result, cultivation of *B. burgdorferi* for diagnostic purposes is utilized only for selected patients.
Two-Tier Elisa and “Western” or Immunoblot Assays

The concept of two-tier testing employing a screening ELISA which when positive was reflexively confirmed by immunoblot or Western blot assay in convalescent sera was developed for epidemiological surveillance of suspected cases in association with EM [5, 6] and was not intended to be used for clinical diagnosis. The kinetics of the appearance and evolution of antibodies to B. burgdorferi-specific antigens have been studied in disease populations from areas where Lyme disease is endemic, comprised of culture-positive erythema migrans rash employing IgG-IgM ELISA and separate IgM and IgM Western blot assay techniques [7, 8]. Such studies demonstrated early dissemination of infection and a humoral antibody response to B. burgdorferi-specific antigens in 89% of patient sera by serum ELISA and in 80% by IgM Western blot assay following EM rash of 7 to 14 days duration according to guidelines of CDC [9]. Both assays peaked at about 20 days post-baseline, with 38% of IgM Western blot still positive at one year post-baseline despite antibiotic therapy. The persistence of IgM Western blot positivity was directly related to disease duration and dissemination prior to treatment. Although 89% of patient sera showed IgG-specific antibodies, less than one-quarter of patient sera were positive by formal IgG Western blot criteria [10].

However there is an implicit tradeoff between sensitivity and specificity in the serologic assessment of Lyme disease skewing the results towards the latter at the expense of the former. One review of commercially available ELISA tests found that sensitivities ranged from 29% to 68% with specificities of 96% to 100% [11]. Eighty-nine serum samples from 59 clinically well-defined patients with clinically suspected Lyme disease (skin manifestations in 8; neurological symptoms in 26, arthritis in 11, ocular symptoms in 4, and other in 10); 14 healthy controls, and 16 in patients with a high possibility for cross-reacting antibodies due to syphilis or Mycoplasma pneumonia infection, were studied by eight different ELISA systems based on whole-cell antigens, whole-cell antigens supplemented with variable major protein-like sequence-expressed (VlsE) and assays using exclusively recombinant proteins. The number of IgM or IgG-positive ELISA results among those with suspected Borrelia infection ranged from 34% to 59%, with cross-reactivity occurring in 0% to 38% [12]. Aggregating the results from IgM and IgG tests and assessing them using a kappa statistic to determine agreement between the ELISAs, yielded kappa values of 0.41 (moderate agreement) to 0.79 (substantial to
good agreement), emphasizing the differences between the ELISAs. Moreover, the percentage of positive blots following a positive ELISA result depended heavily upon the choice of ELISA-immunoblot combination. A criticism of that study voiced by other investigators [13] was the design such that their results could have been foreseen in the absence of clinical information comprising the patient cohort of suspected *Borrelia* infection and their comparison of good with bad tests that could have resulted in the observed kappa values.

Serological testing has other inherent limitations. First, after the immune system is exposed to a novel antigen such as *B. burgdorferi*, it takes time for a specific detectable antibody to develop, typically three to six weeks, during which even a very sensitive antibody-based assay will be negative. Second, since antibody responses in general persist for an extended period of time, a single positive serological test can at best be taken as evidence of prior exposure and not necessarily indicative of active infection. Although in other infections it is commonplace to look at evolving titers using an acute change as evidence of active infection, this had generally not be possible in Lyme disease except in very early infections in which an acute and convalescent titer may be compared. However it is critical in light of the variability of test results not only between laboratories but within the same laboratory at two different time points, to hold the acute serum until the convalescent one is drawn and then process them both at the same time.

The Lyme ELISA can also result in false-positive results due to cross-reactivity with antigenically similar organisms such as other spirochetes including syphilis, relapsing fever, and periodontal disease from *Treponema denticola*. Other cross-reactions may be due to nonspecific polyclonal B-cell activation in the setting of bacterial endocarditis, parvovirus infection, or infectious mononucleosis. False-negative ELISA assays can occur in early Lyme disease when the antibody response is not robust enough to be detected, in cases in which early antibiotic treatments have been administered, even if the antibiotics were not sufficient to fully eradicate the infection, and in others with impediments to mounting a normal immune response such as those receiving immunosuppressive therapy and others already immune-deficient. Some patients with well-established Lyme disease may remain seronegative due to capture of free antibody in antigen-antibody complexes. One can apply immune complex dissociation measures to these sera, resulting in release of free antibody which can then be detected; however this is not commercially available.
PCR and Culture Techniques

A shortcoming of the serological approach to the diagnosis of Lyme disease is that indirect methodology does not recognize the presence of the spirochete itself, rather the infected host’s response. Therefore much research has been performed on direct detection assays. According to Aguero-Rosenfeld and colleagues [7], of the four different approaches used in the clinical laboratory, namely microscope-based assays, detection of *B. burgdorferi*-specific-proteins or nucleic acids, and culture, the latter offers the best confirmation of active infection.

Over the past three decades, recovery of *B. burgdorferi* has been reported from the blood in patients with early Lyme disease [14-17] employing the original Kelly [18] and BSK I and II media [1-3], and the standardized BSK-H medium [19] that permitted growth from an inoculum of as few as one to five organisms and permitted the inoculum to double in 10 to 12 hours reaching densities of at least 10^8 organisms per ml. The results of further culture media modifications were reported in later clinical studies of early Lyme disease with characterization of the isolates by indirect IF assay [14,15, 20, 21] and PCR amplification, with reported specificity of 96% and sensitivity of 40% to 59% [17, 22]. Only recently with the commercial availability of BSK-H has there been a renewed interest in the applicability of culture in routine clinical practice as a diagnostic test. However limitations include the labor-intensive aspect of the methodology, increased expense, and slower incubation times requiring up to 16 weeks before being considered negative. One recently reported methodology [23] that employed a modified BSK-H media (mBSK-H) for short term cultures with the addition of the matrix protein collagen to long-term cultures, improved the growth of spirochetes, later defined by IFA, PCR amplification and nucleotide sequencing using primers to amplify *Borrelia* 16S ribosomal ribonucleic acid (RNS) subunit or cytidine-5-prime-triphosphate (CTP) synthetase gene locus. Overall, the proposed technique [23] led to a positive culture result in 47% of sera at 6 days, 83% at 8 weeks, and 94% at 16 weeks in 72 CDC-seropositive, symptomatic adult Lyme disease patients. As of this writing, this method has not been confirmed by other investigators nor has a more vigorously defined patient population been studied. Perhaps if this method is confirmed, and the time necessary to detect a positive culture shortened, then this assay may become a more relevant diagnostic tool.

PCR, which detects genetic material from one or a small number of organisms, can be reasonably sensitive when testing synovial fluid yielding
rates of 42% to 100%, and in biopsies of the EM rash wherein 36% to 88% show positivity. However, this method is notoriously insensitive when testing human blood. Reporting on three studies of PCR assays for the detection of *B. burgdorferi* DNA, Aguero-Rosenfeld and colleagues (5) found that the median sensitivity of peripheral blood, plasma and serum in the U.S. was 18% and in Europe 10% for an overall median of 14%. Human plasma may contain inhibitors to DNA amplification and the presence of host DNA may lead to uninterpretable PCR results. There is the yet unresolved dilemma of which DNA primer sets to use, how many loci to be included, and how large a segment from each locus needs to be analyzed. Such reasons among others may account for the poor performance of this method in testing blood samples and demonstrates why PCR is not routinely used.

Antigen-based assays have demonstrated quite variable results and none to date have stood the test of time [19]. One advanced experimental method described by Eshoo and colleagues [24] employing 1.25 ml of whole blood with a novel pre-enrichment of the entire specimen extract for *Borrelia* DNA prior to multifocal PCR and electrospray ionization mass spectrometry detection assay. Among 21 endemic area patients who had both physician-diagnosed EM and positive two-tiered serology at initial visit or follow-up after three weeks of antibiotic therapy, DNA analysis showed detection of *B. burgdorferi* in the blood of 13 of 21 (62%), and in most cases provided the *B. burgdorferi* genotype. However, this method has not been fully verified, requires complex, expensive laboratory instruments and dedicated personnel making it unlikely to be introduced into routine clinical practice in the near future.

### Cerebrospinal Fluid Testing

The concept of LNB remains a matter of definition. For example, should it be defined based simply upon demonstration of intrathecal *B. burgdorferi* specific antibody production alone regardless or do we define it as the presence of this organism in the CNS, regardless of CSF serology? According to the 2010 European Federation of Neurological Societies (EFNS) guidelines [25], the diagnosis of Lyme neuroborreliosis is based on the patient’s medical history, clinical findings and analysis of CSF with confirmation by culture, PCR or specific anti-*Borrelia* antibody index (AI). A few mainly European studies have investigated CSF by the immunoblot methodology [26-28] demonstrating the appearance of IgG antibodies in 99% and IgM antibodies in...
60% of CSF specimens, with IgM antibodies occurring early after infection and IgG predominating in late neuroborreliosis, and the number of bands increasing with the duration of infection [44, 46]. Lakos and colleagues [26] noted that the immune response in the CSF directed to other antigens than those in the serum reflected intrathecal antibody production.

In comparison to the first generation serology based on whole-cell sonicates [5, 29] performed in serum and CSF that typified LNB testing over the preceding two decades [30-32] with resulting poor specificity due to cross-reacting antibodies to other spirochete infection including syphilis, the serologic diagnosis of LNB has become easier and potentially more informative with development of second and third-generation B. burgdorferi-specific antibody tests.

Second generation antibody tests based on native Borrelia antigens such as the flagella protein improved the test specificity [29] and third-generation antibody tests based instead on synthetic peptides and recombinant antigens [5] have the potential to improve the sensitivity and specificity in establishing the diagnosis of LNB in suspected patients especially those with CSF pleocytosis and others with atypical presentations.

Two studies recent studies [33, 34] compared commercially-available first, second and third generation ELISA-AI in adults and children with LNB employing stepwise diagnostic procedures similar to serum in determining the ELISA-AI followed by Western blot confirmation. Wutte and colleagues [33] studied 50 patients including 27 adults and 23 children for the presence of intrathecal Borrelia-specific antibodies by flagellum ELISA-AI (fIELISA), recombinant ELISA-AI (rELISA) and by Western blot. Of the 50 patients, 29 (58%) were diagnosed conclusively with rELISA-AI and confirmed by Western blot in two-thirds, while 17 (34%) were diagnosed by fIELISA-AI of which 15 (88%) were confirmed by Western blot. While only 20 (40%) were diagnosed by Western blot, in 4 of 8 patients with negative AI, IB showed many detectable bands both in CSF and serum. rELISA-AI testing was the most sensitive followed by fIELISA-AI. Henningsson and colleagues [34] compared the performance of commercially available fIELISA-AI and rELISA-AI tests noting an overall sensitivity of 88% and specificity of 99% for the native flagellum antigen test, and an overall sensitivity of 100% and specificity of 97% for the recombinant test concluding that they performed equally well in specificity but there was improved diagnostic sensitivity with the recombinant B. burgdorferi test.

Returning to the original problem of the definition of the EFNS definition of LNB, the requirement of laboratory confirmation of B. burgdorferi has led
to the potential for circular reasoning which may yet be misleading. For example, in standard clinical practice, PCR analysis is rarely performed and when done, usually uninformative. Culture of this fluid is not commercially available. Therefore the overwhelming majority of testing involves serological testing. So if one defines LNB as a typical clinical presentation plus intrathecal antibody production, then it cannot be stated with full assurance that all patients with LNB will be accounted due to positive CSF serology. Should one argue that what is equally important is whether the infecting organism is present in CNS tissues or not? In that regard, Luft and colleagues [35] who performed a prospective study of CNS involvement in acute disseminated *B. burgdorferi* infection PCR assay with comparison to standard serological tests found that among 12 patients with acute disseminated Lyme borreliosis and less than two weeks of active disease, 4 of 6 study patients with EM and 4 of 6 with cranial neuritis without EM, demonstrated evidence of CSF *B. burgdorferi*-specific DNA, compared to none of 16 control samples. However, in as much as only 4 of 8 study patients found to have spirochetal DNA in their CSF had complaints referable to the CNS, and 3 of the 8 PCR-positive CSF samples had no other noted abnormalities, they concluded *B. burgdorferi* invaded the CNS early in the course of infection and that CSF analysis could be misleading.

Two reported patients [36, 37] exemplify the limits of intrathecal studies in the definition of LNB. In 1989, Pfister and colleagues [36] described a patient with a history of multiple tick bites, elevated serum IgG anti-*Borrelia* antibody titers, *B. burgdorferi* in the CSF and intrathecal antibody production without concurrent inflammatory signs consistent with a phase of latent LNB in which no tissue infection or reactive had yet occurred. Several years later, Lawrence and colleagues [37] described a patient with evidence of *B. burgdorferi* infection who experienced repeated neurologic relapses despite aggressive antibiotic therapy each course of which was associated with a Jarisch-Herxheimer-like reaction. Although the patient never had detectable free antibodies to *B. burgdorferi* in serum or spinal fluid, the CSF was positive on multiple occasions for complexed anti-*B. burgdorferi* antibody, nucleic acid and free antigen.

The apparent lack of sensitivity of detecting intrathecal *B. burgdorferi* antibody production in patients who otherwise harbor CNS infection probably parallels the insensitivity of currently accepted blood serological criteria in those nevertheless infected however this is difficult to establish with certainty. It may therefore be useful to reconsider the definition of LNB. In addition, one could assume that in instances where CNS dissemination and invasion by *B.
burgdorferi occurred early, that full doses of antimicrobial agents would thus be warranted.

**CXCL13 Chemokine**

Among the postulated useful biomarkers for the diagnosis of LNB, the chemokine CXCL13 has been widely investigated. Its concentration in CSF rapidly declines during antibiotic therapy making it useful as a followup parameter. Rupprecht and colleagues [38] studied 179 patients who were not previously treated with antibiotics with suspected acute LNB and analyzed for routine parameters including the production of *B. burgdorferi*-specific antibodies and CXCL13. Employing a cut-off level for acute Lyme neuroborreliosis for CXCL13 of 250 pg/mL, the authors [38] noted a sensitivity of CXCL13 of 100% compared to 87% *B. burgdorferi*-specific antibodies, both with comparable specificity of 99%. Intrathecal measurement of CXCL3 detected acute LNB in two patients without CSF pleocytosis in whom the diagnosis of LNB was serologically later confirmed. Schmidt and colleagues [39] studied 192 CSF samples of patients with untreated LNB and CSF pleocytosis with a cutoff of 1,229 pg/mL, noting similar specificity of 96.1% but sensitivity of 94.1% for CXCL13 compared to 85.7% for AI. Moreover, CXCL13 was helpful in clinically atypical cases and in particular in early stages of the disease when *B. burgdorferi*-specific AI was still negative.

**C6 ELISA**

The public health service recommendations, which provide support for two tier testing [40], also stipulated the development of alternatives to one or both steps provided that equal or better performance was demonstrated by such alternative methods. Wormser and colleagues [41] postulated that based upon past experience [42-54] an ELISA of sufficiently high specificity and sensitivity might be provided in the highly conserved 25-amino acid peptide C6 which is derived from the sixth invariant region of this protein [55]. Using a reference standard of two-tier testing, they studied the C6 peptide ELISA as a single step serologic diagnostic test [41]. In over 2,200 blood donors, patients with other conditions, and Lyme disease vaccine recipients the respective concordance between the C6 ELISA and the CDC-defined two-tier
standard were 98.9% and 99.5%. This statistical comparability between these two methods was maintained in patients with early neurological manifestations and arthritis. The notable difference between the C6 ELISA and the two-tier method occurred in early Lyme disease patients who had an EM rash present at the time of blood draw. Here, the C6 assay performed significantly better, with a sensitivity of 66.5% as opposed to 35.2% for the two-tiered test.

The increased sensitivity of the C6 ELISA in early disease might prove useful if someone presented with an atypical rash, as this single-step assay is simpler to implement in the clinical laboratory. However as discussed earlier, just as in serological testing in general, the same limitations of reduced sensitivity and less than optimal specificity in this indirect test, apply to the C6 assay as well.

**Conclusion**

The serological assessment of Lyme disease continues to be problematic making it important to place equal reliance on clinical findings and to use serological studies to support the diagnosis while not regarding them as absolute indicators of disease or absence thereof. A suite of available tests may need to be performed including not only routine serological and CSF testing but also assays employing *B. burgdorferi*-specific native and recombinant antigens and when available, PCR, culture and chemokine measurements.

**References**


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Chapter 7

Treatment

Abstract

Physicians caring for patients with LNB must choose the appropriate drug, route of administration and duration of treatment for their patients without the benefit of randomized control trials. The majority of confirmed cases of Lyme disease present early in the infection with easily recognizable erythema migrans rash. Such patients have an excellent response to antibiotic therapy. The most effective antibiotics including doxycycline, amoxicillin, and cefuroxime, and ceftriaxone respectively for localized early or late stage systemic and nervous system manifestations are all relatively inexpensive and rarely associated with serious adverse side effects. Medical and public health efforts are important in the prevention, early diagnosis and treatment of early Lyme disease to avert nervous system dissemination and the resulting clinical involvement that occurs in a minority of confirmed cases.

Introduction

General guidelines for the treatment of Lyme disease were developed by the Infectious Diseases Society of America [1] while those for LNB were developed by the American Academy of Neurology (AAN).
The first of two evidenced-based Practice Parameters published by the AAN in 1996 [2] was based upon a literature search that yielded fifty-nine relevant articles, only a few of which compared different treatment regimens for LNB [3-5]. The authors concluded that CNS involvement probably required parenteral antimicrobial therapy with a third generation cephalosporin, while limited European data [6] suggested that oral regimens might be equally efficacious in acute meningitis.

The literature search forming the second Practice Parameter [7] published in 2007 disclosed thirty-seven articles with assessable data, eight of which [6, 8-14] provided data on patients with definite LNB, and all were European-based. An aggregated analysis failed to demonstrate any difference in outcome whether patients received oral doxycycline or parenteral beta-lactam therapy. With an overall response rate of doxycycline to parenteral penicillin or ceftriaxone of 98.6% and associated narrow confidence intervals, there were no apparent clinically or statistically significant differences between the oral and parenteral regimens.

However, there were obvious problems in this analysis that pointed to a lack of epidemiological rigor and generalizability of their findings from the European to the U.S. patients. First, all of the eight studies [6, 8-14] were Class III or Class IV with regard to clinical outcome according to a four-tiered classification-of-evidence scheme [15]. In that classification [15], Class I studies were judged to have a high quality and low risk of bias; Class II were of moderate quality and risk of bias. Class III studies had a high risk of bias. Class IV studies were those with very highest risk of bias. French and Gronseth [15] and the Editors of the journal Neurology [16] recognized the importance of this evidence-based classification noting that the evidence classification did not relate to the studies per se, but rather to the questions addressed by them. Thus, as the same study could contain a high level of evidence (Class I) for one question but a low level (Class III or IV) for another, two of the eight European studies [6, 12] neither of which were Class I or II with regard to clinical outcomes, were both Class II in at least one of their predetermined objective measures of disease activity. With low levels of evidence for the European studies suggesting a high risk of intrinsic bias, it would be appropriate to question the validity of a meta-analysis claiming comparable response rates for doxycycline to parenteral antibiotic therapy for the outcome of neurological disease. While U.S. patients with the same manifestations of neuroborreliosis might be similarly responsive to
doxycycline, differences between U.S. and European \textit{B. burgdorferi} strains and species and their initial clinical presentation might not make the data fully applicable to U.S. cases \cite{2}. Treatment responses might be expected to be comparable in U.S. and European cases, but this too had not yet been confirmed \cite{2}.

**Recommendations**

At the nexus of the yet resolved debate is still the appropriate drug, dose, route, and duration of initial antimicrobial therapy for LNB which varies even among contemporary experts. The 1996 Summary Statement of the Quality Standards Subcommittee of the AAN and the related Practice Parameter \cite{2} asserted that causally-related neurologic disease which included lymphocytic meningitis and encephalomyelitis, and encephalopathy associated with memory or cognitive dysfunction and abnormal neurologic examination, MRI or CSF abnormalities, warranted treatment with 2 to 4 weeks of parenteral beta-lactam antimicrobial therapy. A caveat was that limited European data suggested oral regimens might be effective in acute meningitis \cite{6}. Steere and colleagues \cite{17} called for early treatment of EM, flu-like symptoms and co-infections with oral therapy beginning with doxycycline for 10 to 20 days noting the limited utility of serological testing in the first 2 weeks of infection. However cardiac and nervous system organ dissemination and clinical involvement warranted parenteral therapy with ceftriaxone as first choice therapy. Acute facial cranial neuritis was treatable with oral therapy similar to early infection. The latest Practice Parameter \cite{7} called for initial treatment of Lyme meningitis, any neurological syndrome with CSF pleocytosis, peripheral radiculopathy, diffuse neuropathy, MNM and cranial neuropathy with oral doxycycline for 10 to 28 days allowing further treatment with parenteral therapy in relatively severe cases, as well as those unresponsive to an initial course of oral doxycycline, and others with CNS parenchymal involvement or encephalopathy. Treatment regimens for childhood and adult LNB, which can be found in standard textbooks of neurology \cite{18-19}, are shown in Tables 1 and 2.
Table 1. Suggested Treatment Based On Neurological Syndrome

<table>
<thead>
<tr>
<th>Syndrome</th>
<th>Treatment</th>
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<tr>
<td>Meningitis</td>
<td>IV ceftriaxone or cefotaxime or penicillin G</td>
</tr>
<tr>
<td>Encephalomyelitis</td>
<td>IV ceftriaxone or cefotaxime or penicillin G</td>
</tr>
<tr>
<td>Encephalopathy</td>
<td>treat as encephalomyelitis if CSF abnormal; IV or PO antibiotics if CSF normal</td>
</tr>
<tr>
<td>Radiculopathy, neuropathy, MNM, cranial neuritis</td>
<td>Oral antibiotics or IV if treatment failure or severe</td>
</tr>
</tbody>
</table>

1Adapted from reference 18.

Table 2. Suggested Treatment Regimens

<table>
<thead>
<tr>
<th>Line</th>
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<th>Parenteral Adult Regimen</th>
<th>Oral Pediatric Regimen (in children ≥ 8 years of age)</th>
<th>Parenteral Pediatric Regimen</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>First Line</strong></td>
<td>100-200 mg bid</td>
<td>ceftriaxone 2 g IV daily</td>
<td>4-8 mg/kg/d in divided doses, max 200 mg/dose</td>
<td>50-75 mg/kg/d in 1 dose; max 2 g</td>
</tr>
<tr>
<td><strong>Second Line</strong></td>
<td>500 mg TID</td>
<td>cefotaxime 2 g IV Q8H</td>
<td>50 mg/kg/d in 3 divided doses; max 500 mg/dose</td>
<td>150-200 mg/kg/d in 3-4 divided doses; max 6 g/day</td>
</tr>
<tr>
<td><strong>Third Line</strong></td>
<td>500 mg BID</td>
<td>penicillin G 18-24 MU/d, divided doses Q4H</td>
<td>30 mg/kg/d in 2 divided doses; max 500 mg/dose</td>
<td>penicillin G 200-400U/kg/d in divided doses Q4H; max 18-24 MU/day</td>
</tr>
</tbody>
</table>

1Adapted from reference 18.

Immunological Aspects

To further complicate management of disseminated Lyme disease has been the recognition that the immune system play an important role is disease
progression and recovery. With the general recognition that early in the disease process there is the potential for infectious-related toxic, metabolic, and autoimmune manifestations that can contribute to disease progression and clinical manifestations, the avenues for treatment can be conceptually broadened. Steere and colleagues [20] called attention to concomitant immune processes in infectious Lyme disease in the investigation of treatment-resistant Lyme arthritis, a complication rarely noted in Europe. With only about 10% of patients presenting with persistent joint inflammation for months to years after standard courses of antibiotic treatment, Steere and colleagues [21] studied the binding of OspA and human lymphocyte function-associated antigen 1 (hLFA-1) peptides to 5 major MHC molecules noting the OspA (163-175) identified the critical epitope in triggering antibiotic treatment-resistant Lyme arthritis. The hypothesis of infection-induced autoimmunity [22] was based on T-cell epitope mimicry between a spirochetal and host protein of bystander activation of a T-cell response to a self-epitope unrelated to spirochetal proteins. Either way, the T-cell response or linked antibody response to the self-protein could stimulate persistent synovial inflammation. Only some MHC molecules bound particular autoantigens accounting for the HLA association with autoimmune diseases, which made it all the more important that most patients with treatment-resistant Lyme arthritis were found to have HLA-DRB1*0401 or HLA-DRB1*001 alleles, and to a lesser degree, the HLA-DRB1*0404 allele. These three alleles, which have a similar sequence in the third hypervariable region of the HLA-DRB1 chain were are also associated with increased severity of adult rheumatoid arthritis. However, in a study of European Lyme disease [23] there was no noted association among 283 patients between HLA determinants and any of the various early or late infectious manifestations. There is very limited information on post-infectious autoimmune syndromes of the nervous system caused or mediated by the Lyme spirochete. However early susceptibility and protracted involvement, combined with the presence of serological markers of altered immunity in affected patients with LNB [24], should lead to a consideration of appropriate oral or parenteral immune modulatory immunotherapy to enhance recovery and treat autoimmune disease manifestations.

**Conclusion**

LNB is amenable to treatment with oral and parenteral antibiotic in uncomplicated patients, and combined with immune modulatory therapy for
those most affected and complicated cases with evidence of altered immunity. Early treatment and prevention are the mainstays of treatment in those with early Lyme disease to forestall neurological involvement.

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