TURUN YLIOPISTON JULKAISUJA
ANNALES UNIVERSITATIS TURKUENSIS

SARJA - SER. D OSA - TOM. 854

MEDICA - ODONTOLOGICA

BORRELIA BURGDORFERI EVADES THE EFFECTS OF CEFTRIAXONE TREATMENT IN A MOUSE MODEL OF LYME BORRELIOSIS

by

Heta Yrjänäinen

TURUN YLIOPISTO
Turku 2009

FROM THE DEPARTMENT OF MEDICAL MICROBIOLOGY AND IMMUNOLOGY, UNIVERSITY OF TURKU

Supervised by

Professor Matti K. Viljanen, MD
Department of Medical Microbiology and Immunology
University of Turku
Turku, Finland

Docent Jarmo Oksi, MD
Department of Medicine
University of Turku
Turku, Finland

Reviewed by

Associate Professor Brian Fallon, MD, MPH, MEd
Director of Lyme and Tick-borne Diseases Center
Columbia University
New York, United States

Professor Hilpi Rautelin, MD
Clinical Bacteriology
Department of Medical Sciences
University of Uppsala
Uppsala, Sweden

Dissertation opponent

Docent Pekka Lahdenne, MD
Helsinki University Central Hospital
Hospital for Children and Adolescents
Helsinki, Finland

ISBN 978-951-29-3938-1 (PRINT)
ISBN 978-951-29-3939-8 (PDF)
ISSN 0355-9483
Painosalama Oy – Turku, Finland 2009

Sisters are doin' it for themselves

ABSTRACT

Heta Yrjänäinen

***Borrelia burgdorferi* evades the effects of ceftriaxone treatment in a mouse model of Lyme borreliosis**

Department of Medical Microbiology and Immunology, University of Turku
Turku, Finland 2009

Lyme borreliosis (LB) is a tick-borne infectious disease with variable symptoms. The most common manifestation of LB is an expanding rash on the skin but frequently arthritic or neurological and occasionally cardiac or ocular symptoms are seen. If detected early, most patients are cured with antibiotic treatment but some patients have persistent symptoms after recommended antibiotic treatment. The proportion of these patients has been estimated to be as high as 10 % of all LB patients. Thus far, different theories for the etiology of treatment-resistant LB have been proposed (e.g. infection-induced autoimmunity or chronic infection) but none of them has gained undisputed acceptance.

The aim of this study was, using a mouse model, to investigate the pathogenesis of treatment-resistant LB. Ceftriaxone treatment administered at the early stage of the disease (at two weeks of infection) diminished infection-induced joint swelling and inhibited the growth of *B. burgdorferi* spirochetes from tissue samples. However, the *B. burgdorferi* specific IgG antibody levels remained highly elevated and, further, DNA of *B. burgdorferi* could be detected in a number of tissue samples. When mice were treated later in the infection (at 18 weeks or more), the results were largely similar as those obtained after early treatment. The main difference was that ceftriaxone did not have any effect on joint swelling of the mice treated at later stages of the infection. This resembles human treatment-refractory Lyme arthritis in which the symptoms and manifestations continue but the infectious agent can not be cultivated from the tissues of the patients.

Anti-TNF-α has been approved for treatment of rheumatoid arthritis because of its anti-inflammatory action. Therefore, its effect on persisting joint swelling after antibiotic treatment was evaluated in the mouse model. Anti-TNF-α treatment had no effect on joint manifestations but, surprisingly, a number of mice converted culture positive after the treatment. Thus, it is clear that the *B. burgdorferi* spirochete is able to evade the effect of ceftriaxone by hiding in a protective niche or by transforming to a metabolically inactive form that can not be eradicated by the antibiotic treatment.
Various tissue samples were analyzed by PCR to study possible niches where *B. burgdorferi* could hide to avoid the effects of the antibiotic. In infected and ceftriaxone treated mice, DNA of *B. burgdorferi* was almost exclusively found in joint tissue samples. However, it is still unclear in what state the *B. burgdorferi* spirochetes remain in tissues after ceftriaxone treatment in our mouse model.

keywords: *Borrelia burgdorferi*, C3H, persistent infection, Lyme disease, arthritis, ceftriaxone, anti-TNF- α

TIIVISTELMÄ

Heta Yrjänäinen

***Borrelia burgdorferi* infektoitujen hiirten antibioottihoidon jälkeinen oireilu**

Lääketieteellinen mikrobiologia ja immunologia, Turun yliopisto
Turku, Suomi 2009

Lymen borrelioosi on puutiaisten välittämä monimuotoinen infektiotauti, jonka tunnetuin oire on ns. vaeltava ihottuma eli erythema migrans. Muita tavallisia ilmentymiä ovat erityisesti nivel- ja hermosto-oireet sekä harvemmin sydän- ja silmäoireet. Suurin osa potilaista paranee täysin terveeksi antibioottihoidon avulla, mutta jopa 10 % borrelioosiin sairastuneista oireilee suositusten mukaisesta hoidosta huolimatta. Pitkittyneen oireilun on ajateltu johtuvan mm. infektion laukaisemasta autoimmuunitaudista tai kroonisesta infektiosta, mutta teorioiden tueksi ei ole kyetty esittämään kiistattomia todisteita. Onkin todennäköistä, että antibioottihoidon jälkeisen oireilun takana on useampia mekanismeja eikä yksi teoria selitä kaikkien potilaiden oireilua.

Tässä väitöskirjatyössä on tutkittu hoidonjälkeistä borrelioosia hiirimallin avulla. Varhaisvaiheessa (2 viikkoa infektoinnin jälkeen) annettu antibiootti vähensi hiirten nivelturvotusta ja esti *B. burgdorferi* – bakteerin kasvun kudoksista otetuista näytteissä. Hoidettujen hiirten *B. burgdorferi* -spesifiset IgG-luokan vasta-aineet pysyivät kuitenkin koholla ja osasta kudosnäytteistä löytyi *B. burgdorferi*:n DNA:ta PCR-tutkimuksen avulla. Mikäli hiiret hoidettiin myöhäisessä vaiheessa (yli 18 viikkoa infektoinnista) tulokset olivat muuten samanlaiset, mutta keftriaksoni ei vaikuttanut nivelturvotukseen. Näin hiirissä oli aikaansaatu tilanne, joka on hyvin samankaltainen ihmisen hoitoresistentin borreliaartriitin kanssa: oireet jatkuvat, mutta taudinaiheuttajaa ei saada esiin.

Inflammaatiota vaimentavaa anti-TNF-α:a on käytetty nivelreuman hoidossa menestyksekkäästi huonosti muuhun hoitoon reagoivilla potilailla ja siitä syystä sen ajateltiin voivan vaikuttaa suotuisasti myös *B. burgdorferi* -infektoitujen hiirten hoidonjälkeiseen nivelturvotukseen. Sillä ei kuitenkaan ollut vaikutusta nivelturvotukseen, mutta yllättäen hoidon jälkeen osa hiirten kudosnäytteistä osoittautui viljelypositiivisiksi. On siis ilmeistä, että hiirimallissamme osa *B. burgdorferi* spirokeetoista pystyy välttämään keftriaksonihoidon vaikutuksen joko hakeutumalla elimistössä kudokseen, jossa antibiootin pitoisuus ei nouse riittävän korkeaksi, tai ne kykenevät muuntautumaan metabolisesti inaktiiviin tilaan eikä mikrobilääke yhdessä immuunipuolustuksen kanssa onnistu tappamaan niitä. Jatkotutkimuksissa selvitimme *B. burgdorferi* -spirokeetan mahdollista piilopaikkaa tutkimalla antibioottihoidon jälkeen useita eri kudoksia PCR-menetelmällä. Tulosten perusteella spirokeetta näyttää suosivan nivelkudosta tai soluja, joita esiintyy nivelessä runsaasti. On kuitenkin edelleen epäselvää, missä muodossa *B. burgdorferi* –spirokeetat säilyvät kudoksessa antibioottihoidon jälkeen.

avainsanat: *Borrelia burgdorferi*, C3H, krooninen infektio, borrelioosi, niveltulehdus, keftriaksoni, anti-TNF- α

TABLE OF CONTENTS

ABBREVIATONS

ACA	acrodermatitis chronica atrophicans
anti-TNF-α	anti-tumor necrosis factor α
BSK-II	Barbour-Stoenner-Kelly (cultivation medium)
CNS	central nervous system
CSF	cerebrospinal fluid
EM	erythema migrans
IFN	interferon
IgG	immunoglobulin G
IgM	immunoglobulin M
IL	interleukin
JRA	juvenile rheumatoid arthritis
LA	Lyme arthritis
LB	Lyme borreliosis
MHC	major histocompatibility factor
Osp	outer surface protein
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RA	rheumatoid arthritis
s.l.	sensu lato
s.s.	sensu stricto
Th1, Th2	T helper cell type 1; T helper cell type 2

LIST OF ORIGINAL PUBLICATIONS I-III

This thesis is based on the following papers which are referred to in the text by the Roman numerals (I to III).

I Heta Yrjänäinen, Jukka Hytönen, Karl-Ove Söderström, Jarmo Oksi, Kaija Hartiala, Matti K. Viljanen: Persistent joint swelling and borrelia-specific antibodies in *Borrelia garinii*-infected mice after eradication of vegetative spirochetes with antibiotic treatment. Microbes and Infection, 2006 Jul; 8(8); 2044-2051

II Heta Yrjänäinen, Jukka Hytönen, Xiao-yu R. Song, Jarmo Oksi, Kaija Hartiala, Matti K. Viljanen: Anti-TNF-α-treatment activates *Borrelia burgdorferi* infection in ceftriaxone treated C3H/He mice. Journal of Infectious Diseases, 2007 May 15; 195(10):1489-1496.

III Heta Yrjänäinen Jukka Hytönen, Jarmo Oksi, Pauliina Hartiala, Matti K. Viljanen: Detection of borrelial DNA, but not cultivable spirochetes, in the joints of *Borrelia burgdorferi* infected mice several months after ceftriaxone treatment (manuscript submitted)

The original communications are reprinted with the permission of the copyright holders.

1. INTRODUCTION

Lyme disease or Lyme borreliosis (LB) was described in 1977 by Allen Steere et al. after unexplained arthritis occurred in many residents of the town of Lyme. It was soon reported that the symptoms of the disease were not limited to the joints. An expanding rash on the skin, erythema migrans (EM), after a tick bite, became a hallmark for the disease. Manifestations of the nervous system and heart were also often noticed as signs of LB. Controversies in the treatment of Lyme disease occurred early, with Mast and Burrows arguing that the erythema chronicum migrans and systemic symptoms should be treated with antobiotics, whereas Steere et al., impressed with the arthritic component of the disease and the occurrence of arthritis in some patients despite antibiotic treatment, was less certain as to the need for antibiotic treatment. Lyme disease was later shown to be caused by a spirochete *Borrelia burgdorferi* for which antibiotic treatment was indicated.

Lyme arthritis (LA) usually appears months or even years after EM. It is not known where the spirochetes remain during the non-symptomatic phase. After antibiotic treatment, most patients are cured but approximately 10 % of them continue to have intermittent attacks of joint symptoms. However, in some of these treatment-refractory LA patients the symptoms will resolve over time, whereas some have continuous pain and joint dysfunction. (Steere, Schoen et al. 1987)

The reason for persistent symptoms is not known. A hypothesis of infection-induced autoimmunity underlying the treatment-resistant LB has been proposed. This hypothesis is based on a finding that patients with this disease manifestation have MHC II alleles that are associated with rheumatoid arthritis (RA) (Steere, Gross et al. 2001). However, another study found no association between MHC II alleles or genotype and post-Lyme disease syndrome (Klempner, Wormser et al. 2005). On the other hand, *Borrelia burgdorferi* spirochetes have been detected in the patients' tissues after antibiotic treatment (Haupl, Hahn et al. 1993; Hudson, Stewart et al. 1998; Oksi, Marjamäki et al. 1999), which suggests that the symptoms may be due to a persistent infection.

2. REVIEW OF THE LITERATURE

2.1. Lyme borreliosis

2.1.1. History of Lyme borreliosis

In 1965 Mrs. Polly Murray, a resident of Lyme, Connecticut, USA, started to observe unusual and mysterious symptoms in herself and her family. In 1975, a cluster of juvenile rheumatoid arthritis cases was detected in her hometown. Mrs. Murray contacted the Connecticut State Health Department which was also contacted at the same time by another mother, Judith Mensch, because of similar symptoms in her family and surroundings. Their relentless work trying to find a reason for their and other families' illness initiated a medical investigation. The list of children diagnosed with juvenile rheumatoid arthritis was presented to a young rheumatologist Allen Steere. He recognized that the disease was not juvenile rheumatoid arthritis but obviously an infectious disease caused by a microbe transmitted by an Ixodes tick. In addition to arthritis, an expanding skin rash with central clearing, appearing days or weeks later at the site of the tick bite, was a typical manifestation of the disease. In 1977, Lyme disease was described as a clinical entity (Steere, Malawista et al. 1977). In 1981, the causative agent was isolated and identified by William Burgdorfer (Burgdorfer, Barbour et al. 1982; Steere, Grodzicki et al. 1983). Soon after, the spirochetes were isolated from blood, skin, and cerebrospinal fluid of a number of LB patients in the USA and Europe (Benach, Bosler et al. 1983; Pfister, Einhaupl et al. 1984; Asbrink and Hovmark 1985; Stanek, Wewalka et al. 1985).

Identification of the causative agent of Lyme disease made it possible to reveal that *Borrelia burgdorferi* infection was the etiology of different symptoms and syndromes that had been described in Europe for decades. These symptoms included both dermatological and neurological manifestations. An atrophic skin lesion, acrodermatitis chronica atrophicans (ACA), was described in Europe in 1883 by Buchwald. A slowly expanding skin rash with central clearing, erythema migrans (EM), was first described by a Swedish dermatologist Afzelius in 1908. Meningoradiculitis appearing after a tick bite was first described by French scientists Garin and Bujadoux in 1922, and the infectious etiology of the disease was suspected in 1950 (Binder, Doepfmer et al. 1955; Sonck 1965). Penicillin was successfully used for the treatment of skin manifestations long before the discovery of *B. burgdorferi* (Scrimenti 1970). The various clinical manifestations caused by *B. burgdorferi* are mostly called borreliosis or Lyme borreliosis (LB) in Europe and Asia and in North America, Lyme disease (Rosa, Tilly et al. 2005).

2.1.2. *Borrelia* spirochetes

The causative agent of LB, bacterium *B. burgdorferi* sensu lato (s.l.), belongs phylogenetically to the *Spirochaetes* phylum, *Spirochaetales* order and *Spirochaetaceae* family (Paster, Dewhirst et al. 1991; Rosa, Tilly et al. 2005). Another important human pathogen of this family is the causative agent of syphilis, *Treponema pallidum*. All members of the family share a spiral morphology and flagellae that function as motility organs. Borrelia spirochetes are divided into species that are associated with different human diseases: *B. recurrentis* causing louse-borne relapsing fever and *B. burgdorferi* s.l. causing LB. *B. burgdorferi* s.l. is further divided into different genospecies of which three have been identified as major human pathogens: *B. burgdorferi* sensu stricto, *B. garinii* and *B. afzelii* (Baranton, Postic et al. 1992; Marconi and Garon 1992; Canica, Nato et al. 1993). All the three species can be found in Europe and Asia whereas in the USA only *B. burgdorferi* s.s. occurs. The genome of *B. burgdorferi* consists roughly of 1,5 x 10^6 base pairs comprising a linear chromosome and 21 linear and circular plasmids, containing altogether 1780 genes (Barbour 1988; Casjens, Palmer et al. 2000). The number of plasmids and their gene order varies substantially between different genospecies and even among individual strains (Terekhova, Iyer et al. 2006). The linear structure of the chromosome and extensive amount of plasmids of *B. burgdorferi* are characteristics that are rather unusual among bacteria. The benefits of these exceptional properties for the bacterium are not fully known but the large number of plasmids may enable an extensive antigen variation capacity and other means for adaptation to the different environments the bacterium may encounter.

2.1.2.1. Structure and morphology

B. burgdorferi spirochetes are thin and corkscrew like organisms composed of 3 to 10 loose coils. The genospecies vary in length (8 to 30 µm) and diameter (0,2 to 0,5 µm) though the cell length may also be a function of the age of the cultures (Barbour and Hayes 1986). The cell surface of *B. burgdorferi* is slimy and easily disrupted. The next structure under the surface is the highly flexible trilaminar outer membrane. It contains many polypeptides, of which the most extensively studied are the outer surface proteins (Osp). The most inner part of *B. burgdorferi* is called the protoplasmic cylinder.

The periplasmic space is the space between the outer membrane and the protoplasmic cylinder. In the periplasmic space 7 to 11 flagellae are located that impart the helical morphology of *B. burgdorferi* (Rosa, Tilly et al. 2005). The spirochetal motility results from rotation of the flagella and it is important for the pathogenesis of this organism allowing *B. burgdorferi* to stay motile in a viscous medium (Sadziene, Thomas et al. 1991; Sadziene, Thompson et al. 1996; Charon and Goldstein 2002). Each flagellum is attached to only one end of the cylinder, flagellar insertion points are located near the

termini of the spirochetes and flagellae are long enough to overlap in the centre of the cell (Wolgemuth, Charon et al. 2006).

Outer surface proteins exposed on the cell surface interact with the host and thus contribute to the pathogenesis of LB. Their expression depends on the environment; some are expressed only in ticks and others only in mammalian hosts. The purpose of this differential expression is presumably to aid the adaptation of the spirochete to different environments.

2.1.2.2. Cultivation

B. burgdorferi can be grown *in vitro* under microaerophilic conditions in various modifications of liquid medium. The composition of the commonly used Barbour-Stoenner-Kelly medium is complex. It includes components such as CMRL-1066 (a chemically defined medium developed in the late 1950 by Connaught Medical Research Laboratories), bovine serum albumin fraction V, N-acetylglucosamine, rabbit serum, citrate, and puryvate. The optimal temperature for multiplication is from 30° to 34°C. Incubation at temperatures of 39°C or higher may reduce or prevent growth (Barbour 1984). The generation time is 7 to 20 h *in vitro* and it is influenced by nutrients and culture conditions (Barbour 1984). Cultures are usually incubated for up to 6 weeks, which is much longer than the incubation time of most other human bacterial pathogens. Detection of growth is accomplished by periodic examination of cultures using dark-field or phase-contrast microscopy. *B. burgdorferi* spirochetes can easily be identified based on their unique coiled/helical shape and screw-like motility.

2.1.3. Epidemiology of Lyme borreliosis

The association between EM and joint symptoms suggested that the disease was spread by arthropods, and further epidemiological studies revealed *Ixodes* ticks as vectors of the illness (Burgdorfer, Barbour et al. 1982). The vector ticks in the USA, Europe and Asia are *Ixodes damminii*, *I. ricinus* and *I. persulcatus*, respectively. The ticks feed exclusively on vertebrate blood but live most of their life off their host animals (Anderson and Magnarelli 2008). Natural hosts are mostly small mammals, especially rodents, and birds (Humair and Gern 2000).

LB is the most prevalent tick-borne disease in the northern hemisphere but there is a large geographical variation in its incidence (Wilske 2005). The geographical distribution of LB reflects the areas in which *Ixodes* ticks live and in Europe it has been reported in nearly all countries. In Finland, 1276 cases were reported in the year 2008; Southern Finland and particularly the Åland Islands representing highly endemic areas (National Institute for Health and Welfare/Register for Infectious Diseases).

A. B.

Figure 1. A. Ixodid ticks in different development stages: the nymph, adult male, adult female and larva. On the background paper each square is 1 mm. B. Attached adult tick. Pictures Heta Yrjänäinen.

2.1.4. Lyme borreliosis in humans

2.1.4.1. Pathogenesis and clinical manifestations

Transmission of *B. burgdorferi* from the tick into the skin

The spirochetes are transmitted from the reservoir animals into the skin of a (human) host through the bite of an infected tick. During the blood meal, *B. burgdorferi* migrate from the mid-gut of the tick into its salivary glands and, as the feeding continues, the spirochetes move along the flow of saliva into the skin. Tick saliva contains factors that reduce clotting, increase vasodilatation and impair the action of neutrophils (Ribeiro, Weis et al. 1990; Ribeiro and Francischetti 2003; Montgomery, Lusitani et al. 2004). Tick bites are usually painless and not itchy. Infective doses of *B. burgdorferi* spirochetes are usually transferred after 24 hour attachment of a tick (Kahl, Janetzki-Mittmann et al. 1998). If undisturbed, ticks feed on host skin on average for 3 to 12 days depending on their developmental stage (Anderson and Magnarelli 2008).

The antigenic composition of *B. burgdorferi* changes drastically depending on the environment. During the blood meal of an infected tick, spirochetes change the expression of a number of genes including the downregulation of outer surface protein A (OspA), which in turn leads to detachment of the spirochetes from the tick mid-gut. Simultaneously, the expression of a colonizing factor, OspC, is upregulated. In the skin, the first host factors to encounter the bacteria are components of the complement system and host immune cells. EM lesion consists of perivascular infiltrates of lymphocytes, dendritic cells, macrophages and a small number of plasma cells (Mullegger, McHugh et al. 2000).

From the site of the tick bite the spirochetes may spread to other tissues and organs through the bloodstream, lymphatics or directly through soft tissues (Shrestha, Grodzicki et al. 1985; Nadelman, Pavia et al. 1990; Hansen and Lebech 1992; Goodman, Bradley

et al. 1995; Sood, Salzman et al. 1997; Straubinger 2000). As a mechanism to facilitate spreading, *B. burgdorferi* binds host plasminogen and plasminogen activators to its surface (Klempner, Noring et al. 1995). The spirochete also adheres to various receptor molecules, including integrins, glycoproteins and proteoglycans of the host cells and extracellular matrix. The genome of *B. burgdorferi* encodes proteins for the attachment to decorin (Guo, Norris et al. 1995), fibronectin (Probert and Johnson 1998), fibrinogen and vitronectin (Coburn, Magoun et al. 1998). Decorin binding activity has an important role in borrelial pathogenesis. The activity is carried by decorin binding proteins A and B (DbpA and B) on the surface of the spirochete (Guo, Norris et al. 1995).

Primary skin lesion erythema migrans

EM lesions have cellular infiltrations mainly consisting of lymphocytes and macrophages and a small number of plasma cells (Mullegger, McHugh et al. 2000). EM is found in 51 to 92 % of LB patients (Berglund, Eitrem et al. 1995; Huppertz, Bohme et al. 1999; Oksi, Marttila et al. 2001; Priem, Munkelt et al. 2003). EM should be distinguished from local reaction to the tick bite. Only half of EM patients recall a preceding tick bite that probably transmitted the infection (Kuiper, Cairo et al. 1994; Lipsker, Antoni-Bach et al. 2002). The incubation time from the tick bite to the appearance of EM is commonly 1 to 2 weeks but can vary between one day and four months (Nadelman and Wormser 1995). EM is classically homogenous or ring-like but atypical forms such as those with blisters (Oksi, Marttila et al. 2001) or a purpuric appearance (Berger 1989) have been reported. The diameter of the skin lesion is typically more than 5 cm and the lesion usually remains flat. The majority of patients in the USA have symptoms resembling those of viral infection including malaise, arthralgias, fatigue, headache, or neck pain simultaneously with EM (Nadelman and Wormser 1995; Nadelman, Nowakowski et al. 1996). However, prominent respiratory or gastrointestinal symptoms are atypical to LB and should raise suspicion of an alternative diagnosis (Nadelman and Wormser 1995). European patients with EM have systematic symptoms less frequently than patients in the USA (Strle, Nelson et al. 1996; Strle, Nadelman et al. 1999; Lipsker, Antoni-Bach et al. 2002). Without treatment, EM lesions resolve spontaneously within 4 to 12 weeks (median four weeks) (Steere, Hutchinson et al. 1983).

Different stages of disseminated Lyme borreliosis

The clinical course of LB is often divided into three stages. In reality, borreliosis follows a variable course with a wide variety of symptoms. A patient may not have to go through every phase and some patients already have symptoms of disseminated infection in the early stage of the disease. The infection may affect several organs: the musculo-skeletal system, nervous system, the skin, eye, the heart and vessels. The involvement of other organs, e.g. the liver and spleen, is possible but rarely reported (Ilowite 1995; Dadamessi, Brazier et al. 2001). Further, spontaneous recovery might be possible also after years of infection (Steere, Schoen et al. 1987) and different treatments. However, some patients

have persisting symptoms despite prolonged treatment with recommended antimicrobial drugs. In addition to EM, LB patients usually have symptoms primarily from one organ, e.g. only arthritis or only neuroborreliosis (Priem, Munkelt et al. 2003).

A. B.

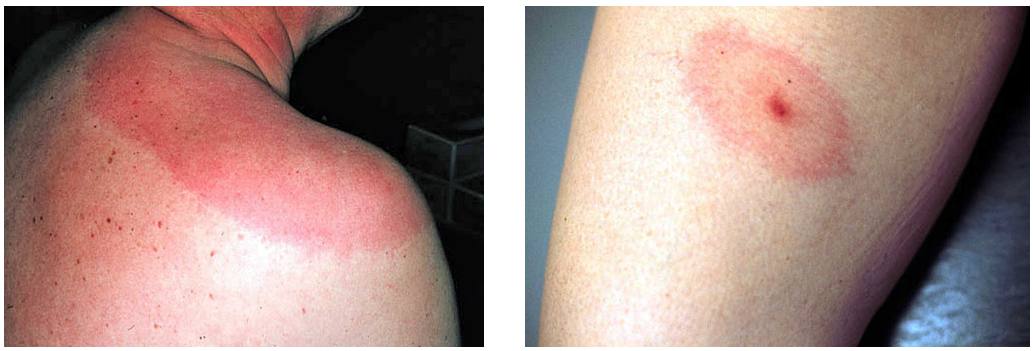


Figure 2. A: Homogenous EM on the shoulder. B: Ring-like EM and the site of the tick-bite in the middle. Pictures Jarmo Oksi.

Skin manifestations of disseminated Lyme borreliosis

A European entity in cutaneous borreliosis is borrelial lymphocytoma that usually appears close to the site of the tick bite. It is a benign B-cell lymphoproliferative process that represents an immunologic reaction to *B. burgdorferi* in the skin (Asbrink and Hovmark 1988). Chronic skin manifestations are quite common in untreated patients in Europe but rare in the USA (Franz and Krause 2003). ACA occurs more than 12 months after the initial infection. It usually develops on the extensor sites of the distal extremities without simultaneous systemic symptoms and is noticed as livid red color changes and a doughy swelling of the involved skin (Weber, Schierz et al. 1984; Asbrink 1985; Asbrink and Hovmark 1988). ACA is preceded by an EM lesion in 10 to 20 % of patients in the same location several months to many years earlier (Mullegger 2004). Activation of the secretion of many cytokines has been found in EM patients with a predominant expression of IFN-γ and IL-10 whereas ACA patients mostly expressed TNF- α and IL-4 (Mullegger, McHugh et al. 2000).

Arthritic manifestations of Lyme borreliosis

Arthritis is one of the most common and clinically important manifestations of disseminated LB and usually appears weeks or months after the onset of infection. The mechanism by which arthritis is induced by *B. burgdorferi* is not completely understood. *B. burgdorferi* s.s. seems to invade the joints more often than other genospecies, since arthritis seems to be more common in the USA than in Europe or Asia (Stanek and Strle 2003). *B. burgdorferi* does not cause a classic bacterial septic arthritis with rapid joint destruction but rather a RA like lesions in synovial tissue (Steere, Duray et al. 1988).

The natural history of LA was described by Steere et al. before the antibiotic treatment of LB was established (Steere, Schoen et al. 1987). In the study, 55 untreated patients who had EM were observed from 1976 to 1979. About 60 % of the patients began to have intermittent attacks of joint swelling and pain, mainly in the large joints. Additionally 20% of patients had arthralgia without objective signs of inflammation. The symptoms tended to be migratory, with onset from 1 day to 8 weeks (mean 2 weeks). Of the 55 patients, six developed chronic synovitis in one to three large joints. Of these patients, three were treated successfully with parenteral antibiotics and another three recovered spontaneously. Today, it is known that a variety of host innate defense mechanisms work together to limit the dissemination of *B. burgdorferi* and initiate an adaptive immune response. After spirochetes have disseminated to the joint, the synovial tissue is infiltrated by mononuclear cells, including macrophages, T cells, B cells and plasma cells (Steere, Duray et al. 1988). A large number of neutrophils, immune complexes, components of complement and inflammatory cytokines are found in the synovial fluid of Lyme arthritis patients (Hardin, Steere et al. 1979; Beck, Benach et al. 1989; Miller, Lynch et al. 1993; Yin, Braun et al. 1997).

B. burgdorferi induced arthritis is monoarticular or oligoarticular affecting most typically the knee, and further, the elbow, ankle, shoulder, wrist, hip, and the temporo-mandibular joints are commonly involved (Stanek and Strle 2003). Arthralgia is mostly described as mild or moderate and the joints are tender and warm. The course of LA is very variable. It is usually recurrent and can last for several years. If no spirochetes are found in the synovial fluid or tissue samples of the patients by culture or polymerase chain reaction (PCR), there are no specific findings for *B. burgdorferi* induced arthritis. However, it is easily distinguished from classic bacterial arthritis by its relapsing/remitting nature and the absence of typical septic symptoms. All inflammatory arthritides result in synovial hyperemia and there are no specific findings for LA in the radiographic presentation (Lawson and Steere 1985; Ecklund, Vargas et al. 2005). Magnetic resonance imaging has revealed that joint effusion, synovial hypertrophy, and enhancement are seen in both septic arthritis and LA at least in children (Ecklund, Vargas et al. 2005). Histologically, there are many neutrophils in synovial fluid whereas synovial tissue is infiltrated mostly with mononuclear cells (Steere, Schoen et al. 1987). The prevalent alteration is non-specific hypertrophic synovitis (Johnston, Duray et al. 1985). It is constructed of papillary fronds of synovial stroma lined by hyperplastic synovial cells forming a thicker layer than the healthy two-cell thick synovial surface (Johnston, Duray et al. 1985; Duray and Steere 1986). Without treatment the symptoms usually last for years and positive, although sporadic, PCR results for *B. burgdorferi* indicate that spirochetes may be present in the joint for a long period (Nocton, Dressler et al. 1994).

A. B.

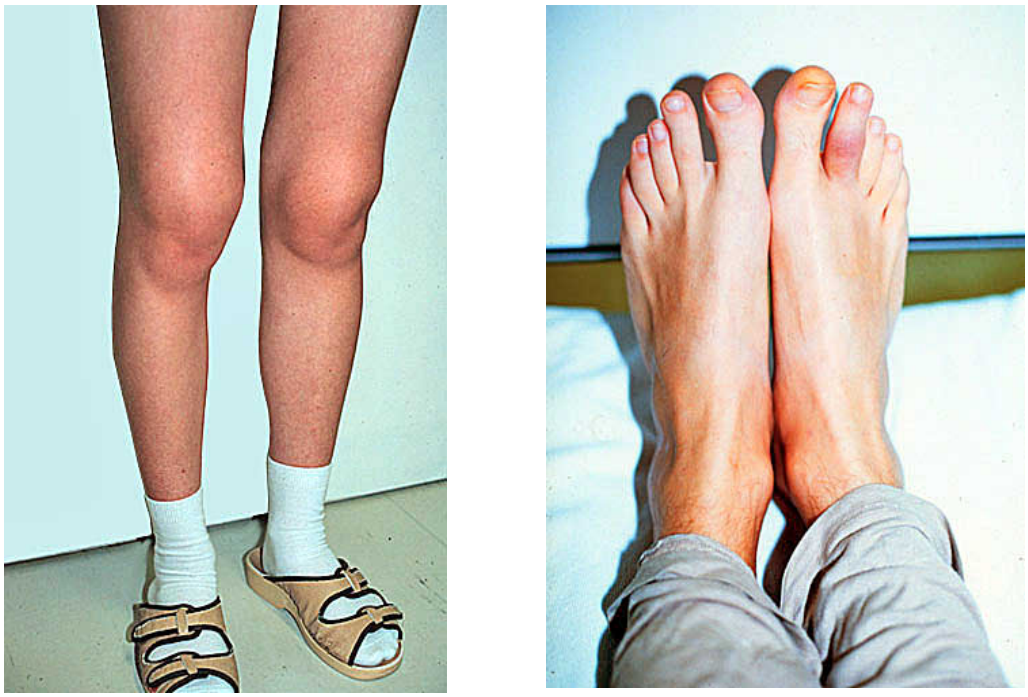


Figure 3. A. Lyme arthritis causing notable joint swelling in both knees. B. Arthritis affecting only the toe in another patient. Pictures Jarmo Oksi.

Neurological manifestations of Lyme borreliosis

The neurological symptoms of LB are caused by direct invasion of *B. burgdorferi* into the central nervous system (CNS) and peripheral nervous system or by toxic or metabolic effects of infection (Halperin 1997). The CNS is reached either through the circulation or along other structures such as the peripheral nerves (Rupprecht, Koedel et al. 2008). The exact mechanism how spirochetes enter the bloodstream and evade the circulating immune cells are not known. After this step, the spirochetes still have to pass the blood-brain barrier. They may penetrate between the endothelial cells (Szczepanski, Furie et al. 1990; Grab, Perides et al. 2005) or use a transcellular passage (Comstock and Thomas 1989). Neuroborreliosis is usually acute or subacute and symptoms are consistent with mild to moderate inflammatory involvement. The disorder occurs in 10 to 15 % of infected patients and there seems to be a difference between the European and North-American disease: in the United States, most of the patients suffer from meningeal symptoms whereas in Europe they are rarely reported. In contrast, painful radiculitis is common in Europe and only occasionally seen in the United States (Donaldson and Lewis 1983; Hansen, Rechnitzer et al. 1987).

B. burgdorferi is able to cause meningopolyradiculoneuritis (Bannwarth´s syndrome), cranial nerve paresis, meningitis, radiculitis, and polyneuritis/polyneuropathy (Halperin

2008). Further, encephalomyelitis, radiculomyelitis and chronic meningitis are rare manifestations of chronic *B. burgdorferi* involvement (Stanek, O'Connell et al. 1996). Unfortunately, neurological examination does not reveal any symptoms absolutely specific for neuroborreliosis. Patients have characteristic effects of inflammation in the meninges, spinal cord root and cranial nerves. In Europe, Bannwarth's syndrome is a common manifestation of acute *B. burgdorferi* infection in adults (Kaiser 1998; Pfister and Rupprecht 2006). About 60 % of Bannwarth's syndrome patients have cranial nerve paresis that may occur as a sole symptom or combined with other pareses. Of the cranial nerves, the facial nerve is most commonly affected and in children facial nerve paresis and meningitis are the most common manifestations of neuroborreliosis (Christen 1996; Kaiser 1998). Diplopia, facial numbness or pain, dizziness and hearing impairment have been described following the involvement of other cranial nerves. Based on detailed neurophysiological and pathological studies as well as studies in the rhesus monkey model, it has been suggested that virtually all cases of peripheral nervous system involvement are due to a mononeuropathy multiplex, regardless of clinical presentation (Halperin 1998). Patients with meningitis usually have mild or intermittent headache. Even after a long period without symptoms, a patient may have progressing encephalomyelitis, atypical paresis, epileptic seizures or encephalopathy.

Cardiac manifestations of Lyme borreliosis

Lyme carditis occurs typically within weeks of initial infection. Approximately 4 to 10 % of patients with untreated LB in the USA and 0,3 to 4 % in Europe develop carditis (Fish, Pride et al. 2008). *B. burgdorferi* can affect all layers of the heart and reside between muscle fibers and in the myocardium (de Koning, Hoogkamp-Korstanje et al. 1989; Duray 1989; van der Linde 1991). Early in the disease process, small inflammatory nodules composed primarily of neutrophils and macrophages have been reported (Duray 1989). This is followed by infiltration of lymphoid cells that create a characteristic plaque-like pattern. The principal manifestation is self-limited conduction derangement, most commonly involving the atrioventricular node. Temporary pacing may be necessary in up to one third of patients but permanent heart block rarely develops (Fish, Pride et al. 2008). Pericarditis, endocarditis, myocarditis, pericardial effusion, myocardial infarction, coronary artery aneurysm, QT-interval prolongation, tachyarrhythmia, and congestive heart failure have been reported. Further, vasculitis involving the small and large intramyocardial vessels can occur. Valvular dysfunction is extremely rare though it is quite often seen in rheumatic myocarditis (Sigal 1995).

Ocular manifestations of Lyme borreliosis

LB rarely affects the ocular system although this rare manifestation may easily remain undiagnosed (Karma and Mikkila 1996). Intraocular inflammation, external ocular disease and neuro-ophthalmic disorder are characteristic for ocular LB (Mikkilä 1998).

2.1.4.2. Microbiological diagnostics

In most countries, the presence of a typical EM is sufficient for confirmation of the diagnosis of acute LB. In the southern part of the United States, however, a new tick-borne illness has been described. In this disease, a rash virtually identical to the Lyme rash is seen but the causative agent is uncertain (Masters, Grigery et al. 2008). For disseminated LB the diagnosis must rely on a combination of history, clinical examination, antibody studies of serum and CSF, and routine CSF analyses. In some cases, analysis of samples by culture and PCR may also assist the diagnosis. However, the diagnostics is challenging, especially at the late stages of the disease.

Though culture is the gold standard of diagnostics in bacterial infectious diseases, the demonstration of *B. burgdorferi* by cultivation is difficult, time-consuming, and of low sensitivity. On the other hand, the sensitivity of PCR in detection of *B. burgdorferi* in clinical samples has varied widely in separate studies. Both cultivation and PCR have proven to be most sensitive in the detection of the spirochete in skin manifestations of LB (Aguero-Rosenfeld, Wang et al. 2005).

The demonstration of the presence of anti- *B. burgdorferi* antibodies is the most often used laboratory method in the diagnostics of LB. Today, the use of two-tier testing is recommended to improve test accuracy. Enzyme-linked immunosorbent assay is used as a screening test, as it gives quantitative information about the amount of antibodies. It is recommended that positive results be further tested using the Western immunoblotting method. It shows the proteins of the spirochete against which the antibodies are directed. The antibodies start to develop 2 to 4 weeks after the primary infection and rise slowly. Therefore, serology is not very useful in early LB. To confirm a disseminated or a late LB, elevated levels of IgG, and sometimes IgM, antibodies against *B. burgdorferi* are required. There is considerable variation in the sensitivity and specificity between commercial kits for antibody testing and, unfortunately, none of the existing tests is completely accurate. Usually, the patients with disseminated infection have elevated antibody levels (Kalish, Kaplan et al. 2001) but there are also reports showing that LB patients may totally lack the humoral immune response to *B. burgdorferi* (Dejmkova, Hulinska et al. 2002; Harrer, Geissdorfer et al. 2007; Holl-Wieden, Suerbaum et al. 2007).

An enzyme-linked immunosorbent assay can produce slightly elevated antibody results because of cross-reactive antibodies in patients with other spirochetal infections (syphilis, relapsing fever, leptospirosis), certain viral infection (e.g. Epstein-Barr virus infection), certain autoimmune diseases (e.g. systemic lupus erythematosus) or because of rheumatoid factor (Steere, Grodzicki et al. 1983; Craft, Grodzicki et al. 1984; Russell, Sampson et al. 1984). On the other hand, there are patients who have clearly elevated antibody levels without signs or symptoms of LB and, therefore, elevated antibody level does not always indicate an active disease (Feder, Gerber et al. 1992; Glatz, Golestani

et al. 2006). In a follow-up study, 67 % (26/39) of LA patients still had elevated levels of IgM or IgG antibodies against *B. burgdorferi* 10 to 20 years after primary infection (Kalish, McHugh et al. 2001). All patients were found to have good overall health without signs of active infection. In a vaccine trial, 11 % (30/269) of unvaccinated control subjects were classified as having asymptomatic seroconversion in antibodies to *B. burgdorferi* (Steere, Sikand et al. 1998; Steere, Sikand et al. 2003). Thus, serological test results should always be considered in connection with the clinical picture and no follow-up testing is recommended.

In rare cases, *B. burgdorferi* spirochetes can also be visualized in tissue samples using microscopic examination of preparations stained immunohistochemically or with silver impregnation techniques. However, the recognition of the spirochete in tissue sections is difficult because of its length and spiral shape. These recognition difficulties may be solved by a new technique, focus floating microscopy (Eisendle, Grabner et al. 2007). This is a modified immunohistochemical method in which tissue sections are stained with a *B. burgdorferi* antibody and scanned simultaneously horizontally and vertically.

In all suspected cases of neuroborreliosis, CSF analysis is necessary. A positive analysis result is characterized by a lymphocytic pleocytosis with a cell number up to 1000 per microliter and elevated protein levels (1 to 2 g/l). The diagnosis can be confirmed by demonstration of *B. burgdorferi* specific intrathecal antibody synthesis. It is noteworthy that intrathecal antibody production can persist for many years or even decades (Halperin 1998; Pfister and Rupprecht 2006). However, in early cases of neuroborreliosis, lymphocyte numbers and protein concentrations are usually moderately elevated whereas intrathecal antibodies may still be negative. In late neuroborreliosis patients, lymphocyte numbers are usually normal or slightly elevated whereas protein concentration may be persistently elevated. Demonstration of intrathecal antibody production is usually the basis of the diagnosis of late neuroborreliosis.

2.1.4.3. Treatment and prevention

Reducing exposure to ticks is the most effective preventive measure against *B. burgdorferi* infection. If one has to enter area with a lot of ticks, insect repellents and suitable clothing (light-colored clothes and long sleeves, long pants, and long socks) are recommended. Further, a daily tick check should be performed and any attached tick should be removed. According to national and international recommendations, tick bite is not an indication for antibiotic treatment but the skin should be monitored for the potential development of an EM rash.

The choice of antibiotic regimen in the treatment of borreliosis can not be based on susceptibility testing and, in that sense, is empiric. In the early stage of the disease, oral amoxicillin or doxycycline for 2 to 3 weeks are recommended as the first choice.

In Finland, the disseminated disease is usually treated with intravenously administered ceftriaxone for 3 weeks. Ceftriaxone inhibits bacterial cell wall synthesis by binding to one or more of the penicillin-binding proteins. Ceftriaxone has an exceptionally long half-life of approximately 8 hours which results in therapeutic serum and synovial fluid concentrations up to 24 hours after a single intravenous dose (Gnann, Goetter et al. 1982; Morgan, Paull et al. 1985).

2.1.5. Chronic and treatment-refractory Lyme borreliosis

Most LB patients are cured after the recommended antibiotic treatment. However, there are patients with persistent symptoms despite proper or prolonged treatments (Steere, Levin et al. 1994). This number has been estimated to be as high as 10 % of the treated patients. However, there is a lot of confusion concerning this patient group and especially about its treatment strategies since it consists of patients having very different conditions from non-specific signs to objective manifestations. Therefore, it has been suggested that the symptoms of these patients are not caused by the presence of the spirochete in their body (Seidel, Domene et al. 2007). However, some studies have shown that *B. burgdorferi* can be cultivated from patients who have received the recommended antibiotic treatment (Preac-Mursic, Weber et al. 1989; Viljanen, Oksi et al. 1992; Oksi, Marjamäki et al. 1999; Hunfeld, Ruzic-Sabljic et al. 2006). The mechanisms underlying persistent symptoms after antibiotic treatment remain to be discovered. It is probable that there are many factors influencing the disease outcome. Post-treatment symptoms may be due to persistent infection, infection induced autoimmune reaction, the presence of an unrecognized coinfection, postinfective fatigue syndrome, or even incorrect diagnosis (Fallon, Keilp et al. 2008; Marques 2008). The most common symptoms are neurological or musculoskeletal symptoms, which are often non-specific, such as fatigue, and further, arthralgia, myalgia, headache, paresthesias, sleeping disorders, irritability, and difficulty with memory, word finding and concentration (Nowakowski, Nadelman et al. 2003; Wormser, Ramanathan et al. 2003; Picha, Moravcova et al. 2006).

Peripheral blood mononuclear cells of late stage LB patients have decreased IL-4 and increased IFN-γ production spontaneously or when stimulated by *B. burgdorferi* (Oksi, Savolainen et al. 1996). Since protection against *B. burgdorferi* is considered to be antibody mediated, the possible down-regulation of Th2 cells may lead to impaired humoral immune response that offers an explanation for the long duration of the disease. Patients with antibiotic-refractory arthritis have high levels of inflammatory chemokines and cytokines in their synovial fluid throughout the illness (Shin, Glickstein et al. 2007). In the same study, the leukocyte count of synovial fluid was shown to be similar in antibiotic-responsive and antibiotic-refractory patients. Comparison of antibody responses to *B. burgdorferi* in patients with antibiotic-refractory, antibiotic-responsive or untreated arthritis showed that increasing antibody titers usually suggest the presence

of live spirochetes, whereas declining titers suggest that the spirochetes have been eradicated (Kannian, McHugh et al. 2007).

2.2. Animal models for Lyme borreliosis

To gain a better understanding of pathogenesis of LB, animal models have been developed. Various animals, e.g. mice, rats, rabbits, dogs and monkeys, develop symptomatic *B. burgdorferi* infection (Barthold, Moody et al. 1988; Barthold, Moody et al. 1988; Schaible, Kramer et al. 1989; Barthold, Beck et al. 1990; Moody, Barthold et al. 1990; Barthold, de Souza et al. 1993). Unfortunately, there is no animal model which completely mimics human LB.

2.2.1. Murine borreliosis

In mice, the severity of the disease depends on which *B. burgdorferi* strain is used, the amount of injected bacteria, infection route, age and genetic background of the animals (Schaible, Gay et al. 1990; Barthold 1991; de Souza, Smith et al. 1993; Barthold and de Souza 1995; Weis, Yang et al. 1997; Wang, Ojaimi et al. 2002). Murine borreliosis exhibits manifestations similar to those seen in human disease but there are also differences. Infection resistant mice, e.g. C57BL/6, have spirochetes in their joints but develop minimal or no arthritis (Ma, Seiler et al. 1998). Intermediately susceptible mice, e.g. BALB/c and SJL, develop mild to severe joint inflammation depending on the bacterial dose. Susceptible C3H/He mice develop clear joint swelling that continues months and mice remain culture positive for their lifetime (Barthold, de Souza et al. 1993). The difference of the disease outcome between the different mouse strains has been suggested to depend on the Th1/Th2 immune response balance (Keane-Myers and Nickell 1995; Matyniak and Reiner 1995). Th1 cells are involved in cell-mediated immunity by activating macrophages and cytotoxic T cells and they secrete cytokines such as IL-2 and IFN-γ. Th2 cells are involved in humoral immunity and they secrete IL-4 and IL-10. Lymphocytes from infected C3H/HeJ mice produce high levels of IFN-γ and low levels of IL-4 when restimulated with borrelial sonicates, and neutralization of IFN-γ during infection attenuates the initial severity of arthritis in these mice (Matyniak and Reiner 1995). In contrast, antigen stimulated lymphocytes from resistant BALB/c mice produce high levels of IL-4 and lower levels of IFN-γ than the cells of C3H/HeJ mice. Further, neutralization of IL-4 in vivo significantly worsens the symptoms of early disease in BALB/c mice. Thus, Th1 dominance is associated with susceptibility to *B. burgdorferi* infection and disease severity in mice.

Today, most of the animal studies are performed using the susceptible C3H/He mice and *B. burgdorferi* sensu stricto N40 strain. For some reason, mice do not develop EM lesions despite the presence of *B. burgdorferi* in their skin (Barthold, Beck et al. 1990).

Joint swelling and arthritis develop to susceptible mouse strains when infected with *B. burgdorferi*, but they do not usually have clear neurological manifestations though meningitis in a mouse model has been described (Garcia-Monco, Miller et al. 1997).

Joint swelling has been considered as an indicator of arthritis in mice. It usually starts to develop as soon as 5 days after infection, being significant at 1 to 2 weeks. However, the course of swelling does not correspond directly to the course of actual histopathological changes in the joint because they are regulated in different gene loci (Weis, McCracken et al. 1999). After *B. burgdorferi* inoculation, the bacteria can be detected in many tissues, including joint, heart, bladder and spleen, within one week of infection (Yang, Weis et al. 1994). Within one month, mice have acute inflammation in the joints, bursae, tendon sheats, ligaments and tendons (Barthold, de Souza et al. 1993). Further, the synovium and periarticular connective tissue are affected.

A. B.

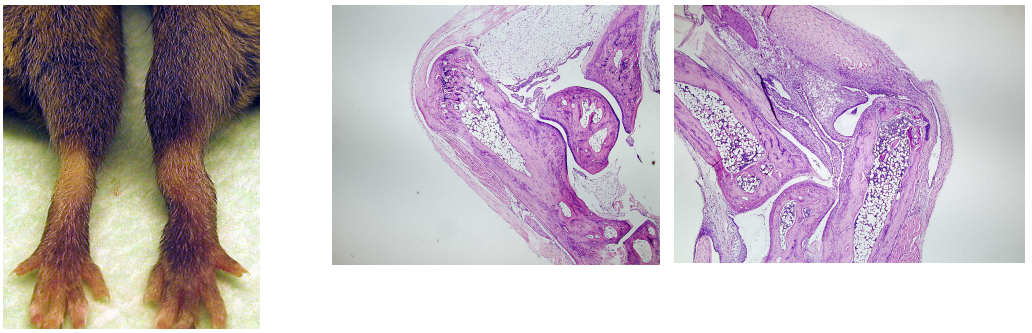


Figure 4. A. Tibiotarsal joint swelling: on the left a non-infected control mouse and on the right an infected mouse. B. Histology of the tibiotarsal joint of a control (on the left) and infected mouse (on the right).

2.2.2. Other animal models

Rabbits develop EM but no other signs of the disease (Krinsky, Brown et al. 1982; Benach, Bosler et al. 1984; Burgdorfer 1984; Kornblatt, Steere et al. 1984). Outbred Hartley guinea pigs develop a widely disseminated infection with major histopathologic changes in the heart and urinary bladder tissues (Sonnesyn, Manivel et al. 1993). Hamsters develop arthritis when *B. burgdorferi* spirochetes are inoculated in the footpad and the animals are irradiated; without irradiation only acute transient synovitis is present (Schmitz, Schell et al. 1988). Laboratory rats can remain persistently infected with *B. burgdorferi* having fluctuating joint swelling and also nonsuppurative myocarditis (Moody, Barthold et al. 1990). Experimental borreliosis has also been reported in pathogen-free beagle dogs (Appel, Allan et al. 1993). They developed clinical disease that can be confirmed by seroconversion, and *B. burgdorferi* can be detected in them by culture and PCR. The dominant clinical signs in dogs are acute recurrent lameness and fibrinopurulent arthritis,

the symptoms which are also reported in pet dogs suffering from LB (Skotarczak 2002; Littman, Goldstein et al. 2006). Experimental studies on neuroborreliosis have been performed with rhesus monkeys (Pachner 1995; Pachner, Delaney et al. 1995; Pachner, Amemiya et al. 2001; Pachner, Gelderblom et al. 2001). In addition to peripheral and CNS disease, monkeys develop EM, uveitis, and myocarditis (Pachner, Gelderblom et al. 2001). Inflammation in the heart has been most consistently observed in this model but only immunosuppressed monkeys develop cardiac fiber degeneration and necrosis (Cadavid, O'Neill et al. 2000).

2.2.3. Efficacy of antimicrobial treatment in *B. burgdorferi* infected animals

Various antimicrobial drugs and treatment regimens have been tested in the treatment of mice infected with *B. burgdorferi* (Moody, Adams et al. 1994; Kazragis, Dever et al. 1996; Pavia, Wormser et al. 2001; Pavia, Inchiosa et al. 2002; Sicklinger, Wienecke et al. 2003). When mice are treated with ceftriaxone at the early stage of the disease, their joint lesions are eliminated and tissue samples are culture negative (Moody, Adams et al. 1994; Kazragis, Dever et al. 1996). Antibiotic therapy leads to a rapid decline in antibody titers but the levels still remain elevated (Moody, Adams et al. 1994; Bockenstedt, Mao et al. 2002). In one study, *B. burgdorferi* was detected by xenodiagnosis after ceftriaxone treatment (Bockenstedt, Mao et al. 2002). The spirochetes isolated from the ticks used in xenodiagnosis were shown to be attenuated (non-dividing) and noninfectious.

Viable spirochetes after antibiotic treatment have been detected in experimental studies with dogs and mice (Straubinger, Summers et al. 1997; Bockenstedt, Mao et al. 2002; Hodzic, Feng et al. 2008). In an experimental study of canine borreliosis, dogs were infected using ticks carrying wild-type *B. burgdorferi* and treated at 2 months after tick exposure with high doses of amoxicillin or doxycycline for 30 days (Straubinger, Summers et al. 1997). The treatment prevented the joint symptoms and signs, only mild lesions were seen in the joint histopathology in one of the 12 dogs after a follow-up period of 2 or 6 months. However, multiple skin punch biopsy samples remained PCR positive up to 6 months after treatment. Further, two dogs were culture positive at 6 months after doxycycline and one dog at 2 months after amoxicillin treatment. In another canine borreliosis study, the dogs were infected as mentioned above, treated at 120 days after tick exposure with ceftriaxone, azithromycin or doxycycline, and half were further treated with prednisone for two weeks at 420 days, just before they were killed (Straubinger, Straubinger et al. 2000). *B. burgdorferi* DNA but not viable spirochetes was found in skin biopsy samples more than 360 days after the antibiotic treatment.

2.3. Immunomodulatory treatment

Immunomodulatory therapies are used in several human diseases. Many commonly used drugs have immunomodulatory effects though they are not classified as immunomodulators. For example, corticosteroids have an effect on immunological cells. They are widely used in several inflammatory and autoimmune diseases. Novel immunomodulatory substances target more specific components of the immune system than corticosteroids. Most of these therapeutic agents have been developed for the treatment of autoimmune diseases such as RA, multiple sclerosis, Crohn's disease and psoriasis. However, the increased risk of infection has been considered to be a major side effect of these therapies. Therefore, their use needs special awareness of activation of latent infections.

2.3.1. Anti-TNF- α treatment

Tumor necrosis factor α (TNF-α) is one of the most important pro-inflammatory cytokines produced by a variety of cell types including monocyte/macrophages and T cells. Blocking its effects by specific antibodies ameliorates collagen induced arthritis in mice (Williams, Feldmann et al. 1992). Anti-TNF-α antibodies and TNF-α receptor antagonists have been approved for the treatment of rheumatoid and psoriatic arthritis as well as inflammatory bowel disease (Elliott, Maini et al. 1993; Elliott, Maini et al. 1994; Feldmann, Brennan et al. 1996). Since Th1 dominance is associated with susceptibility to *B. burgdorferi* infection and with disease severity in mice, antagonizing the effects of TNF-α may, in theory, be beneficial in the treatment of joint manifestations refractory to antibiotic therapy.

However, the use of anti-TNF-α treatment carries the concomitant risk of activation of chronic infections. Several patients with activation of tuberculosis during anti-TNF-α treatment have been reported (Keane, Gershon et al. 2001; Gardam, Keystone et al. 2003). Further, invasive pulmonary aspergillosis, listeria meningitis, cytomegalovirus retinitis and intramyocardial inflammatory process induced by *Staphylococcus aureus* have been associated with anti-TNF-α treatment (Reichardt, Dahnert et al. 2002; De Rosa, Shaz et al. 2003; Bowie, Snella et al. 2004; Haerter, Manfras et al. 2004).

3. AIMS OF THE STUDY

The aim of the study was to develop a mouse model to examine the following aspects of Lyme borreliosis:

- The dynamics of *B. burgdorferi* infection and infection-induced joint manifestations;
- The effects of ceftriaxone treatment on the infection and its manifestations;
- The effect of anti-TNF-α treatment on the infection and its joint manifestations;
- The mechanisms and possible niches used by *B. burgdorferi* to evade the effects of antibiotic treatment.

4. MATERIALS AND METHODS

4.1. *Borrelia spirochetes* and infection in mice (I, II, III)

The low-passage strain (less than 10 passages) of *B. garinii* Å218 (a Finnish tick isolate) and *B. burgdorferi* s.s. N40 (originally a tick isolate, kindly provided by Sven Bergström, University of Umeå, Sweden) were used to infect the mice. Stock cultures of the bacteria were aliquoted and stored at -70°C. For culture, frozen aliquots were thawed and cultivated in modified Barbour-Stoenner-Kelly II (BSK II) medium at +34°C without antibiotics. Spirochetes were counted using a Neubauer counting chamber and dark-field or phase-contrast microscopy. Mice were infected into the lower back by intracutaneous syringe inoculation of 10^6 spirochetes in 100 µl of BSK II medium or phosphate buffered saline (PBS) solution. Control animals were inoculated with an equal volume of the culture medium or PBS.

4.2. Mice (I, II, III)

Female mice were used in all studies. SJL mice were obtained from the Central Animal Laboratory, University of Turku, Finland, and C3H/He mice from M & B A/S, Ry, Denmark and from Harlan, Netherlands (Horst, the Netherlands). Mice were 3 to 4 weeks of age at the beginning of each experiment. They were bred and raised in pathogen-free conditions in cages in groups of five and provided with food and water *ad libitum*. All experimental protocols were approved by the Animal Ethics Committee, University of Turku (permission numbers STO991, 1359/03, 1656/06 and 2008-06959).

4.3. Ceftriaxone and anti-TNF-α treatment (I, II, III)

Ceftriaxone (Rocephalin®, Roche, Grenach-Wyhlen, Germany) was administered 50 mg/kg intraperitoneally as a single daily dose for 5 or 18 days. A rat murine chimeric TNF-α antibody of IgG2ak isotype was obtained from Centocor Inc. (Malvern, PA, USA) (Marini, Bamias et al. 2003). It was given intraperitoneally (10mg/kg/mouse/week) once a week for four weeks. When given during the same day with ceftriaxone, it was injected 4 to 6 hours after ceftriaxone to avoid possible interference of different drug substances.

4.4. Experimental design

4.4.1. *Borrelia garinii* Å218 infection in SJL and C3H/He mice

Twenty-five SJL mice were infected with *B. garinii* Å218 as described above, and another 25 mice served as non-infected controls. The medio-lateral diameter of the hind

tibiotarsal joints was measured using a metric caliper weekly or every second week. The measurer was blinded to the group's identity. The mice were also weighed at the time of joint measurement. At 2, 4, 8, 16 and 39 weeks of infection, 5 mice from both groups were killed to examine their infection status. Tissue samples from tibiotarsal joint, urinary bladder and pinna were cultured for *B. burgdorferi* by placing a dissected sample into a culture tube. Blood was collected to measure IgG antibody levels against *B. burgdorferi*, and other tibiotarsal joint was prepared for the histological specimen.

Next, 20 C3H/He mice were infected with *B. garinii* Å218, and five mice served as controls. At 5 weeks of infection, ear punch biopsy specimens were taken and cultured. At 18 weeks of infection, 10 infected mice were treated with ceftriaxone and the residual 10 mice with saline, and their joint status was monitored for further 19 weeks. Finally, the animals were killed, and their tissue samples were collected as in previous experiments.

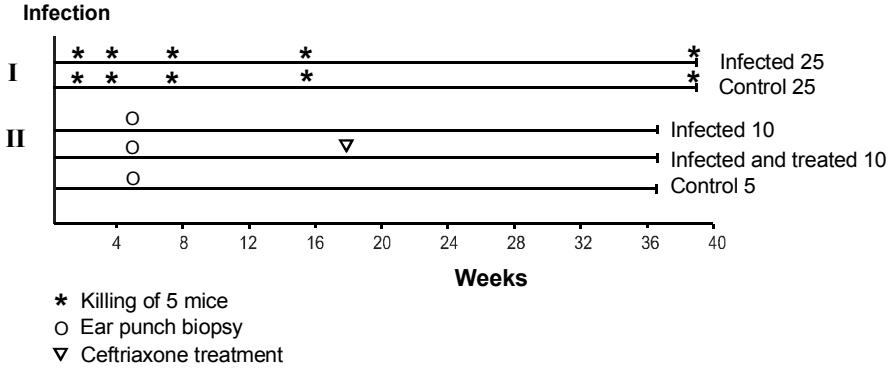


Figure 5. Scheme of the experimental design of the study presented in 4.4.1.

4.4.2. The effect of ceftriaxone and anti-TNF-α treatment in a mouse model for chronic borreliosis

Anti-TNF-α treatment was tested separately in both *B. garinii* Å218 and *B. burgdorferi* s.s. N40 infected mice. Forty-five C3H/He mice were infected in each study. Mice were divided into six groups. At two weeks of infection, 10 mice were treated with ceftriaxone only (group cef) and another 10 with anti-TNF-α only (group aT2). In one group of 10 mice, anti-TNF-α was given simultaneously with ceftriaxone (group cef+aT2) and in another group of 10 mice (group cef+aT6) four weeks after ceftriaxone (six weeks of infection). In both studies, five infected control mice (group inf+) were sham-treated with equal volumes of saline, and five mice served as non-infected controls (group inf-). At 14 weeks of infection, the mice were killed to examine their infection status by culturing samples from tibiotarsal joint, urinary bladder and pinna for *B. burgdorferi*. Bladder samples were also analyzed using polymerase chain reaction (PCR) for detection of

flaB genes of *B. burgdorferi* DNA. Blood was collected to prepare serum specimens for antibody measurements.

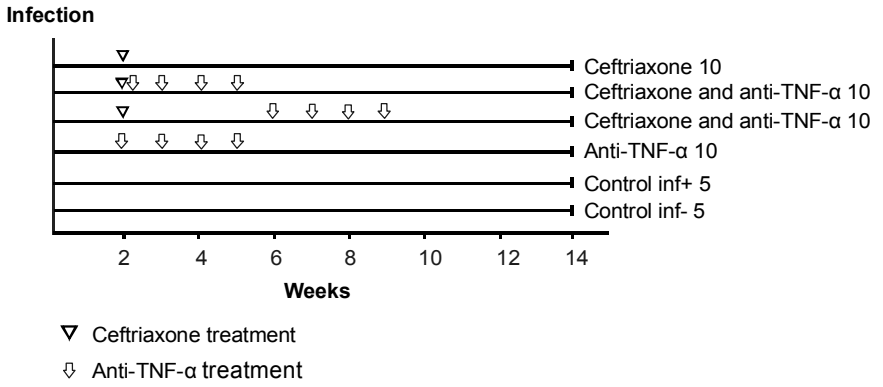


Figure 6. Scheme of the experimental design of the study presented in 4.4.2.

4.4.3. Detection of *B. burgdorferi* DNA after antibiotic treatment

Possible hiding sites of persisting *B. burgdorferi* were examined. Two separate studies were carried out, one focusing on early and the other on late treatment in our mouse model. In the early treatment study, 44 mice were infected and seven mice served as non-infected controls. At two weeks of infection, 30 mice were treated with ceftriaxone and 14 infected control mice were sham-treated as in previous experiments. Ten weeks after treatment, tissue samples of tibiotarsal joint, bladder and pinna were collected for *B. burgdorferi* culture. Of the joint and bladder, samples were also collected for PCR tests of *flaB* and *ospA* genes of *B. burgdorferi.* Blood was taken for anti-borrelia IgG antibody assay. From a subgroup of 32 mice (20 infected and ceftriaxone treated, 9 infected, 3 non-infected controls) a wider tissue sample repertoire was collected for the culture and PCR tests. In addition to the three tissues mentioned above, samples of the heart, brain, kidney, mesenteric lymph node, spleen, liver, and eye ball were studied. With the same study set-up the effect of an 18-day long ceftriaxone treatment was examined in a group of 5 mice.

In the late treatment study, 20 mice were infected and five mice served as non-infected controls. At 18 weeks of infection, 10 infected mice were treated with ceftriaxone and 10 were sham-treated. After treatment, mice were monitored for 21 weeks and killed at 39 weeks of infection. Tissue samples of the tibiotarsal joint, bladder and pinna were collected for *B. burgdorferi* culture. Like in the previous study, the joint and bladder samples were also collected for two different PCR tests. Blood was taken for serum anti-borrelia IgG antibody assay.

4.5. Bacterial culture (I, II, III)

The infection status of the mice was assessed by culturing the samples of tibiotarsal joint, urinary bladder, pinna, heart, brain, kidney, mesenteric lymph node, spleen, liver, or eye ball. Before tissue collection, the mice were killed with CO_2 and flushed externally with 70 % ethanol. When tissues samples were prepared, all instruments were disinfected in ethanol between the dissections of the different samples. The samples were grown in 6 or 10 ml of BSK II medium supplemented with phosphomycin (Sigma, Steinheim, Germany) and rifampin (Sigma) at +34°C for a maximum of 8 weeks, and the cultures were examined for spirochetes using dark-field microscopy.

4.6. Polymerase chain reaction (I, II, III)

DNA was isolated from the tissue samples with a commercial DNA Isolation Kit for Blood/Bone Marrow/Tissue (Roche Diagnostics GmbH, Mannheim, Germany) according to the instructions of the kit manufacturer. A nested PCR was performed with primer sets targeting the chromosomal flagellin gene (*flaB*) of *B. burgdorferi*. The specificity of PCR products was confirmed by sequencing. Real-time PCR was performed using the LightCycler apparatus (Roche Applied Sciences) and Taqman principle amplifying a 102 base pair product of the *ospA* gene according to the method described by Ivacic et al. (Ivacic, Reed et al. 2007). As above, all runs included a positive and a negative control.

The nested PCR (Schmidt, Muellegger et al. 1996) was performed with primer sets (Eurogentec S.A Seraing, Belgium) targeting the chromosomal flagellin gene (*flaB*) of *B. burgdorferi.* The outer primer set, BBSCH31 (5'-CACACCAGCATCACTTTCAGGGTCT-3') and BBSCH42 (5'-CAACCTCATCTGTCATTGTAGCATCTTTTATTT-3'), was designed to amplify a 437 bp fragment and the inner primer set, FL7 (5'-GCATTTTCAATTTTAGCAAGTGATG-3') and FL59 (5'-TTTCAGGGTCTCAGGCGTCTT-3'), a 277 bp fragment of the gene. PCR was performed in a reaction volume of 50 µl containing 10 mM of Tris hydrochloride (pH 8,3), 50 mM KCl, 0,01% gelatin, 3,5 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate (dATP, dCTP, dTTP, dGTP), and 1 U of *Taq* DNA polymerase (Amplitaq Gold, Roche, Basel, Switzerland). Of each primer, 20 pmol was used. After initial denaturation at 94°C for 10 min, PCR was run with 25 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 10 min. Five microliters of the product of the first PCR was used as template DNA for the second amplification. After initial denaturation at 94°C for 10 min, PCR was run with 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s, extension at 72°C for 30 s, and final extension at 72°C for 10 min. All PCR reactions were run in a thermal cycler (GeneAmp 9700, Applied Biosystems, Foster City, CA, USA). Each PCR experiment included both negative reagent and blank sample controls, with water replacing template DNA, and a positive control containing 1 ng of purified DNA from *B.*

burgdorferi ATCC 35210. Amplicons were visualized on a 1,5 % agarose gel stained with ethidium bromide and photodocumented using a gel documentation system (GDS8000, UVP, Cambridge, UK). To avoid the risk of contamination, amplification products were analyzed in an area isolated from that where the PCR was performed. The specificity of PCR products was confirmed by sequencing.

4.7. Antibody assays (I, II, III)

Borrelia-specific IgG antibodies were measured using an in-house enzyme immunoassay (EIA) (Viljanen and Punnonen 1989). An extract removed by sonication from whole cells of the strain *B. burgdorferi* B31 (ATCC 35210) was used as antigen. Of this solution, 200 µl (protein concentration 20 µl/ml) per well was incubated on microtiter plates (Microtiter Assembly strips, Thermo Electron Corporation, Vantaa, Finland) at room temperature overnight. The plates were washed three times with a detergent solution (H_2O supplemented with 0.05 % Tween 20). Of the serum sample, 100 µl was added to duplicate wells at a dilution of 1:200 in 1 % BSA-PBS. The plates were incubated at 37 °C for 1 h and washed three times with the detergent solution. Of goat anti-mouse IgG antibodies (Southern Biotechnology Associates, Inc, Birmingham, AL, USA), 100 µl diluted 1:16000 or 1:32000 in PBS, respectively, were added to the wells. The plates were incubated at 37 °C for 1 h and washed three times as above. 100 µl of substrate solution (ortho-phenylene-diamine, DAKO, Glostrup, Denmark) was incubated in the wells at 37 °C for 15 min, and 100 µl of 1 M HCl was added to stop the reaction. The binding of anti-borrelia antibodies was detected by measuring absorbance at 492 nm using a spectrophotometer (Multiskan, Labsystems, Helsinki, Finland). The results are expressed as optical densities (OD) ×1000 at this wavelength. The mean OD values of the sera of uninfected mice groups remained below 500. This OD value was then used as the positive cut-off.

4.8. Virulence testing (II)

Three C3H/He mice were infected with *B. burgdorferi* s.s. N40 spirochetes isolated from a ceftriaxone and anti-TNF-α treated mouse. Two animals served as uninfected controls. Ear punch biopsy samples were taken for culture at two weeks of infection. At seven weeks of infection, the mice were killed and samples from their tibiotarsal joint, urinary bladder and pinna were taken for culture.

4.9. Susceptibility testing (II)

The minimal inhibitory concentration of ceftriaxone was determined by a broth microdilution method with 96-well round-bottom microtiter plates (Nunclon microwell plate, A/S NUNC,

Roskilde, Denmark). Two-fold dilutions of ceftriaxone were prepared in BSK II medium covering a concentration range of 0.002 to 0.5 µg/ml. Of each ceftriaxone concentration to be tested, 100 µl was dispensed into three parallel wells. For growth and negative controls, 100 µl of BSK II medium alone was used. All wells, except the negative control wells, were inoculated with 10 µl of actively growing borreliae (10^6 bacteria/ml). The plates were incubated at 34 °C for 72 h. Phase-contrast microscopy was used to detect the growth of *B. burgdorferi*.

4.10. Plasmid screening (II)

DNA was isolated from bacteria using a commercial nucleic acid extraction kit (Wizard Genomic, DNA Purification Kit, Promega Corporation, Madison, WI, USA). Amplification and detection of 12 linear and 9 circular plasmids were performed as described by Elias et al. (Elias, Stewart et al. 2002).

4.11. Statistical analysis (I, II, III)

Analysis of joint swelling data was carried out using the statistical software package SPSS 12.0. The repeated measures analysis of variance was used to calculate significances of the group effect, time effect and interaction effect. Huynh-Feldt corrected F-tests were used if Mauchly's test indicated that the sphericity assumption was violated. Simple contrasts were used to indicate which comparisons of time points showed statistical differences between the groups. Simple contrasts (Bonferroni corrected) were used to compare time points between groups. One-way analysis of variance was used to test group differences in each time point. Multiple comparisons were done with Tukey HSD or Tamhane's test. If only one interesting pair of groups was available for testing, it was tested using the two-sample t-test.

To analyze the differences of IgG antibody levels, two-tailed Mann-Whitney U test or Student's t-test was used. When studying various tissue samples by PCR, the McNemar test was used in the statistical comparison of test results.

With all tests, observed significance levels <0.05 were considered to indicate a statistically significant difference.

5. RESULTS

5.1. *B. burgdorferi* induced infection and joint swelling in mice (I,II,III)

SJL or C3H/He mice were infected using *B. garinii* Å218 or *B. burgdorferi* s.s. N40. In total 65 mice were infected and sham-treated in the separate experiments. All were culture-positive for *B. burgdorferi* in at least two of the three most commonly studied tissue samples (tibiotarsal joint, urinary bladder and pinna) at different time points ranging from 2 to 52 weeks of infection. In two experiments (III), several other tissue samples, in addition to those mentioned above, were cultured: the heart, brain, kidney, mesenteric lymph node, spleen, liver, and eye ball. Only the liver specimen remained culture negative in every mouse tested (n=5), all other tissues were positive in 2 to 9 of 9 mice studied (Table 1: sham-treated mice). Joint, bladder, pinna, heart and eye ball were all culture positive. All uninfected control mice were culture negative in every study.

In the first experiment (I), IgG antibodies against *B. burgdorferi* were monitored at several time points during the follow up of *B. garinii* infected SJL mice. IgG antibody levels were clearly elevated already at 2 weeks of infection (OD value > 500) and they peaked at 8 weeks. IgG antibody levels remained highly elevated until the end of the follow-up of 39 weeks (Figure 7A.). In the further experiments (II, III), the antibody levels were determined only at the end of the follow-up. They were highly positive in every experiment independently of the *B. burgdorferi* (*B. garinii* Å218, *B. burgdorferi* s.s. N40) or mouse strain (SJL, C3H/He) used (Figure 7B.).

Table 1. Culture and PCR findings of different tissue samples of infected and ceftriaxone or sham-treated mice. Mice were treated with ceftriaxone for 5 days at two weeks of infection and were killed after follow-up of 10 weeks. The number of ceftriaxone treated mice was 20, but liver samples were taken only from 10 of the mice. The number of sham-treated mice was 9, but liver samples were only taken from 5.

	Treatment	joint	bladder	pinna	heart	brain	kidney	lymph node	spleen	liver	eye ball
Culture	Ceftriaxone	0/19[a]	0/20	0/19[a]	0/19[a]	0/20	0/19[a]	0/18[b]	0/19[a]	0/9[a]	0/19[a]
	Sham	9/9	9/9	9/9	9/9	2/9	8/9	6/9	8/9	0/3[b]	9/9
flaB PCR	Ceftriaxone	15/20	0/20	0/20	2/20	1/20	0/20	0/20	0/20	0/10	0/20
	Sham	8/9	6/9	7/9	8/9	0/9	1/9	4/9	0/9	0/5	0/9
ospA PCR	Ceftriaxone	11/20	0/20	0/20	1/20	0/20	0/20	0/20	0/20	0/10	0/20
	Sham	6/9	4/9	1/9	5/9	0/9	1/9	1/9	0/9	0/5	0/9

[a] one sample excluded due to contamination

[b] two samples excluded due to contamination

Joint status was assessed by measuring the medio-lateral diameter of the hind tibiotarsal joints. The mice were 3 to 4 weeks of age at the time of infection, and as a result of their natural growth, the diameter of the joints of both infected and control mice increased similarly at the beginning of the experiment. Usually, clear joint swelling of the infected animals was observed at two weeks of infection, and persisted without regression until the end of the follow-up of up to 52 weeks (I). The difference in joint diameter between control and infected mice was statistically significant already at two to three weeks of infection ($p < 0.05$). Otherwise, the infected mice were in good condition during the experiments and their weight gain was similar to that of the control mice. No increased mortality, signs of disease except joint swelling, or neurological findings, such as abnormal behavior, were observed among the infected mice.

These results clearly show that *B. garinii* Å218 and *B. burgdorferi* s.s. N40 cause a chronic infection in these mice: the mice developed persistent joint swelling, all the infected mice were culture positive for *B. burgdorferi* and their IgG antibodies against *B. burgdorferi* were significantly elevated.

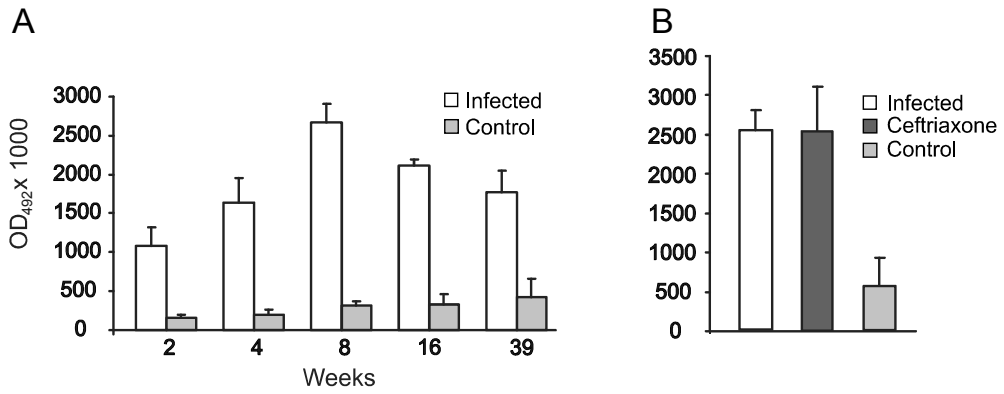


Figure 7. A. Development of borrelia-specific IgG antibodies in *B. garinii* Å218 infected SJL mice at different time points. Each column represents the mean IgG antibody level of five mice, and the bars indicate the standard deviation of the mean values. **B.** IgG antibody levels of *B. garinii* Å218 infected (n=9), infected ceftriaxone-treated (n=10) and non-infected (n=5) C3H/He mice at 37 weeks of infection. The ceftriaxone group was treated at 18 weeks of infection. The bars indicate the standard deviation of the mean values.

5.2. The effect of ceftriaxone treatment in *B. burgdorferi* infected mice (I,II,III)

The effect of antibiotic treatment on *B. garinii* or *B. burgdorferi* s.s. induced infection and joint swelling was evaluated at both the early (2 weeks) and late stage of infection (8 or 18 weeks). At two weeks of infection, all mice had developed clear joint swelling.

The tibiotarsal joint, urinary bladder and pinna samples were cultured for *B. burgdorferi* from all ceftriaxone treated mice (n=83). Further, in a subgroup of mice (n=20) the samples of the heart, brain, kidney, mesenteric lymph node, spleen, eye ball and liver were cultured. Independent of the timing of ceftriaxone treatment, all treated mice in the separate experiments were always culture negative from all tissues tested (Table 1: ceftriaxone treated mice).

Ceftriaxone treatment at two weeks of infection significantly reduced the mean levels of *B. burgdorferi* –specific IgG antibodies in both *B. garinii* and *B. burgdorferi* s.s. infected mice (p<0,005) (II). In *B. garinii* infected mice, ceftriaxone abolished antibody responses almost totally whereas in *B. burgdorferi* s.s. infected animals the effect was weaker. Ceftriaxone treatment at 8 weeks of infection significantly reduced the mean antibody levels of infected mice (p<0,005) (II). In contrast, the antibody levels of the mice treated at 18 weeks of infection were comparable to those of untreated mice. IgG antibody levels were at the same level in ceftriaxone treated SJL mice as in the corresponding C3H/He mice (Figure 7.).

Table 2. Culture and PCR findings of mice tissue samples. In Study 1, mice were treated with ceftriaxone for 5 days at 2 weeks and killed at 12 weeks of infection. In Study 2, mice were treated with ceftriaxone for 5 days at 18 weeks and killed at 39 weeks of infection. In Study 3, ceftriaxone for 18 days was started at 2 weeks of infection and mice were killed at 12 weeks of infection.

	Experimental design			**Culture[a]**			**PCR[a]**				**positive mice (total)**
							flaB		***ospA***		
	Infected	**n**	**CEF**	**Joint**	**Pinnae**	**Bladder**	**Joint**	**Bladder**	**Joint**	**Bladder**	
Study 1	+	30	2 wks	0/29[b]	0/29[b]	0/30	21/30	0/29[c]	12/30	0/30	22/30
	+	14	-	14/14	14/14	14/14	12/14	11/14	7/14	9/14	14/14
	-	7	-	0/7	0/7	0/7	0/7	0/7	0/7	0/7	0/7
Study 2	+	10	18 wks	0/10	0/10	0/10	6/9[c]	0/10	3/9	0/10	6/10
	+	9	-	8/9	9/9	9/9	7/8[c]	4/9	4/8	2/9	9/9
	-	5	-	0/5	0/5	0/5	0/5	0/5	0/5	0/5	0/5
Study 3	+	5	2 wks	0/5	0/5	0/5	5/5	0/5	0/5	0/5	5/5
	+	4	-	4/4	4/4	4/4	4/4	1/4	4/4	3/4	4/4

[a] Number of mice with positive samples/number of mice tested.

[b] One sample contaminated.

[c] One sample missed.

The early ceftriaxone treatment was started at two weeks of infection when the joint swelling had already developed. When ceftriaxone was administered at that time point, it weakened but did not completely eliminate the joint swelling of the mice. Their joints remained persistently less swollen than those of the infected control mice; however, the joint swelling did not disappear totally. The tibiotarsal joints were slightly swollen until the

end of the follow-up period of 12 weeks; neither recovery nor exacerbation was seen. In contrast, when the mice were treated with ceftriaxone at the late stage of infection, no improvement of joint swelling was observed between the untreated mice and those treated with ceftriaxone during the 19 week follow-up period.

5.3. The effects of anti-TNF-α treatment in *B. burgdorferi* infected mice after ceftriaxone (II)

The mice treated with anti-TNF-α alone (n=20) and infected control mice (n=10) were all culture positive. The mice treated with ceftriaxone only (n=19) were culture negative from all tissues tested. However, a number of mice treated with both ceftriaxone and anti-TNF-α were culture positive in one to three of the tissues studied (Table 3.). In *B. garinii* infected mice, this phenomenon occurred in 3 of 10 mice treated simultaneously with ceftriaxone and anti-TNF-α at two weeks of infection (group cef+aT2) and in 3 of 10 mice treated with anti-TNF-α four weeks after ceftriaxone treatment (group cef+aT6). The results of *B. burgdorferi* s.s. N40 infected mice were similar, with 2 of 9 (group cef+aT2) and 3 of 10 (group cef+aT6) mice being culture positive.

B. burgdorferi s.s. N40 spirochetes that had been recovered from a ceftriaxone and anti-TNF-α treated mouse were used to infect three C3H/He mice as described earlier. All mice developed tibiotarsal joint swelling at two weeks of infection. Ear punch biopsy samples were taken for culture at two weeks of infection, and two of the mice were found positive. At seven weeks of infection, all mice were culture positive from at least two of the three tissue samples taken (tibiotarsal joint, urinary bladder and pinna). These results showed that the virulence of the recovered spirochetes was similar to that of the *B. burgdorferi* s.s. N40 spirochetes used in the main experiment.

The plasmid profile of *B. burgdorferi* s.s. N40 spirochetes recovered from a ceftriaxone and anti-TNF-α treated mouse was identical to that of spirochetes used in the main experiment. This shows that there were no major changes in the plasmids of the recovered spirochetes.

The minimal inhibitory concentration of ceftriaxone for *B. burgdorferi* s.s. N40 spirochetes recovered from a ceftriaxone and anti-TNF-α treated mouse was 0.08 µg/ml. The minimal inhibitory concentration of the spirochetes used in the main experiment was 0.16 µg/ml. This shows that no selection of bacterial clones resistant to ceftriaxone occurred in the mice treated with ceftriaxone.

Anti-TNF-α had no influence on joint swelling whether it was given with or without ceftriaxone. Further, in *B. burgdorferi* s.s. infected mice, the anti-TNF-α treatment given concurrently with or four weeks after ceftriaxone had no effect on IgG antibody levels compared to the ceftriaxone treatment only. In contrast, in the sera of *B. garinii*

infected mice antibodies were higher in the mice that received anti-TNF-α in addition to ceftriaxone compared to mice treated with antibiotic only. Treatment with anti-TNF-α alone had no effect on the mean IgG antibody levels of the infected mice and IgG levels were comparable with those of the infected control mice.

Table 3. Dissemination of *B. garinii* Å218 and *B.burgdorferi* s.s. N40 in C3H/He mice

Borrelia strain	Treatment	Culture[a]			PCR	Number of
		Joint	Bladder	Pinna	Bladder	positive mice (culture or PCR)
B. garinii	none	3/5	5/5	4/4	3/5	5/5
	cef[b]	0/10	0/10	0/10	0/10	0/10
	cef[b]+aT2[c]	3/10	2/10	1/9	2/10[e]	4/10
	cef[b]+aT6[d]	3/10	2/10	2/10	1/10	3/10
B.burgdorferi	none	5/5	5/5	5/5	3/5	5/5
	cef[b]	0/10	0/10	0/9	0/10	0/10
	cef[b]+aT2[c]	2/9	1/9	0/9	0/9	2/9
	cef[b]+aT6[d]	1/10	2/10	1/10	1/10	3/10

[a] Number of mice with positive cultures/numbers of mice tested

[b] Ceftriaxone treatment was started at two weeks of infection

[c] anti-TNF-α treatment was started at the same time with ceftriaxone

[d] anti-TNF-α treatment was started four weeks after the ceftriaxone treatment

[e] One bladder sample only PCR positive

5.4. Detection of *B. burgdorferi* DNA in the tissues of infected mice after ceftriaxone treatment (II,III)

The above results suggested that *B. burgdorferi* is able to evade the effect of a five-day course of ceftriaxone. The possible niches for persisting spirochetes were searched for by using culture and two different PCR methods.

Infected mice were treated with ceftriaxone either at early (at two weeks of infection) or late (at 18 weeks of infection) stage of infection. At the early stage mice were treated for 5 (n=30) or 18 (n=5) days. After the treatment they were followed up for 10 to 12 weeks and finally killed to study the infection status. Tibiotarsal joint, bladder and pinna samples were collected from every mouse. A wider tissue sample panel of a subgroup was also studied including the heart, brain, kidney, mesenteric lymph node, spleen, eye ball and liver.

Mice treated for 5 or 18 days at two weeks of infection showed similar results. In both groups, all tissue samples were culture negative. However, 10 weeks after the treatment *B. burgdorferi* DNA was found in the joint samples in both treatment groups in altogether 26 of 35 mice (Table 2.). From 25 mice the heart and brain tissue samples were collected, and were positive in 4 and 1 mice, respectively (Table 1.). All other tissue specimens were negative.

Several tissues of all infected and non-treated mice (n=14; in subgroup n=9) were culture positive (Table 2. and Table 1). All of them were culture positive in the joint, bladder, pinna, heart and eye samples. Further, the kidney and spleen were positive in 8 of 9 and the mesenterial lymph node in 6 of 9 mice. Brain tissue samples were positive in only 2 of 9 mice. Several tissues of all infected and non-treated mice were also positive by PCR, but the PCR positivity rate was lower than that of culture. The joint, bladder, pinna and heart tissue samples were positive by PCR in 6 to 8 of 9 mice. Positive results were also obtained from the mesenterial lymph node and kidney specimens, 4 and 1 of 9, respectively. None of the non-treated mice was positive by PCR in the spleen, brain or liver tissue sample. All uninfected control mice were negative by PCR.

Mice treated at 18 weeks of infection were killed after a follow up period of 21 weeks. In this study, tibiotarsal joint, urinary bladder and pinna samples were collected for culture, and joint and bladder samples for PCR. All 10 ceftriaxone treated mice and five uninfected controls were culture negative (Table 2.). With the exception of the joint sample of one animal, all samples of nine untreated infected controls were culture positive. Importantly, 6 of 9 of the joint samples of the 10 ceftriaxone treated mice (one sample missed) were *flaB* PCR positive, whereas all 10 bladder samples of this group were negative. When the nine joint samples were studied by *ospA* PCR, three of them were positive, all being also *flaB* PCR positive. Of the infected control mice, 7 of 8 (two samples missed) joint samples and 4 of 9 (one sample missed) bladder samples were *flaB* PCR positive. In *ospA* PCR only 4 of 8 of joint and 2 of 9 of bladder samples were positive. All samples positive in *ospA* PCR were also positive in *flaB* PCR. All five uninfected control mice were negative in both PCR methods.

6. DISCUSSION

6.1. Treatment refractory *B. burgdorferi* infection in the mouse

An animal model was developed to investigate the pathogenesis and disease manifestations of *B. burgdorferi* infection. The focus was particularly on the late stage and long-term outcome of the infection after antibiotic treatment with special reference to potential joint findings.

The aim was to create a robust animal model in which every mouse is infected and has clear joint symptoms. Therefore, a relatively large inoculum of spirochetes (10^6) was used to infect the mice. The other reason for the use of a large inoculum was that *B. garinii* is thought to be less infectious than *B. burgdorferi*. However, both genospecies infected all animals and induced significant joint swelling in them in two weeks. Further, the infection proved persistent, since most tissue samples of the infected mice were culture positive at the end of the experiment regardless of the length of follow up period (the longest follow-up period was 52 weeks). Similarly, the levels of IgG antibodies to *B. burgdorferi* remained persistently elevated as has been described in similar models earlier (Barthold, de Souza et al. 1993).

Next, the effect of antibiotic treatment on the infection status, joint swelling and antibody levels of the mice was studied. Since at two weeks of infection mice had developed clear signs of infection (joint swelling, elevated IgG antibody levels and culture positive tissue samples), that time point was considered to be suitable for the onset of early treatment. Without treatment, joint swelling continues and IgG antibody level remains clearly elevated evidently for the lifetime of mice. Therefore, late antibiotic treatment was tested at two time points: at 8 and 18 weeks of infection.

In this model, ceftriaxone has different effects on disease manifestations and antibody responses when administered at the early or late stage of infection. When the treatment was administrated at 8 weeks of infection or earlier, it ameliorated the joint swelling and had a lowering effect on the levels of IgG antibodies to *B. burgdorferi*. These effects were not seen in the mice treated at 18 weeks of infection. However, all tissue samples of ceftriaxone treated mice were always culture negative independent of the timing of the treatment. Thus, both joint swelling and production of IgG antibodies to *B. burgdorferi* turned treatment-refractory when the infection continued for several months.

Our study results are partially in line with the results of earlier mouse studies. Elimination of tissue lesions has been described in *B. burgdorferi* infected mice treated at 7 to 14 days of infection (Moody, Adams et al. 1994; Bockenstedt, Mao et al. 2002). When treatment began at 30 or 90 days of infection, mild synovitis and vasculitis persisted in some mice.

In contrast to our results, ceftriaxone treatment even at 90 days of infection abrogated the development of antibodies against *B. burgdorferi*. We found only weakening of antibody response in the early treatment groups, and late treatment had no significant effect on antibody levels. These discrepancies may be due to the different *B. burgdorferi* or mouse strains used, or due to other differences in the experimental set-ups. In human patients, the long persistence of either IgM or IgG antibodies is a known phenomenon, though no clear correlation between the disease outcome and antibody levels has been reported (Kalish, McHugh et al. 2001; Nowakowski, Nadelman et al. 2003; Oksi, Nikoskelainen et al. 2007).

6.2. Relevance of the results obtained by the mouse model for understanding human Lyme borreliosis

Animal models have provided invaluable results for medicine. They have helped researchers and doctors to understand the pathogenesis of different diseases. Further, their use in drug development, toxicity testing and treatment trials, is irreplaceable. However, they are not necessarily useful in the study of extremely complex disorders (e.g. mental illness, some neurological conditions). LB is a complicated disease presenting symptoms from various organs. Results obtained from cell culture studies may be misleading because of the lack of complex regulatory networks, immune functions and interplay between tissues and organs operating in intact structured organisms. In human diseases, environmental effects naturally play a role that can not be simulated in experimental models.

Traditionally, animal models used to study a human disease have been spontaneous models in which the disease mimics the human disease. Rodents have been used very widely and they have been bred in numerous models for different diseases, e.g. NOD mice for type 1 diabetes mellitus. Another possibility is to use an induced model in which a condition resembling human disease is induced by different methods in a laboratory animal, e.g. collagen-induced arthritis in mice for a model of human RA. Today, gene technology has changed the field and it is even possible to generate a mutant animal harboring parts of the human genome.

Today, most animal studies of LB are performed using mice. Mouse models for LB have both similarities and dissimilarities with human LB. First, the infection is usually induced by needle injection. In a natural infection, various substances in tick saliva may have an effect on the primary reaction in the host skin. Further, tick saliva may even contain known or yet unknown pathogens that might help *B. burgdorferi* to disseminate. Second, the syringe inoculate may contain a much greater number of *B. burgdorferi* spirochetes than the number assumed to be transferred in tick saliva (Nardelli, Callister et al. 2008). The disease development may vary because of the number of infective spirochetes.

Third, mice used in these studies are susceptible to *B. burgdorferi* infection because of their genetic background. Therefore, their disease outcome may differ significantly from human disease depending on host immune reaction type. Further, antibiotic treatment is administered to mice intraperitoneally and under supervision. In disseminated human LB, intravenous treatment guarantees well controlled administration of the drug. However, courses of oral antibiotic courses are dependent on patients' compliance and defaulting may lead to treatment failures.

In Finland, ceftriaxone treatment is recommended in disseminated human LB and it is also widely used in mouse models (Mursic, Wilske et al. 1987; Moody, Adams et al. 1994; Pavia, Inchiosa et al. 2002; Hodzic, Feng et al. 2008). In vitro susceptibility studies indicate that it has high activity against *B. burgdorferi* (Mursic, Wilske et al. 1987). In human patients, ceftriaxone is administered intravenously but in our mouse model it is injected intraperitoneally. However, the serum concentration after even subcutaneous injection is at the same level with reported peak serum concentration in humans indicating that the systemic absorption occurrs in treated mice (Moody, Adams et al. 1994). Further, in our experimental model the mice were treated using a high dose of ceftriaxone (50 mg/kg) compared to the human dose (a recommended daily dose of 2 g correlates to 20 to 30 mg/kg).

We have focused on *B. burgdorferi* induced arthritis, though it is only one of the many manifestations of LB. However, for unknown reasons, the disseminated LB usually predominantly affects one organ system also in humans. Patients may suffer from neurologic, cutaneous, arthritic or cardiac manifestations of LB, but only infrequently do patients have prominent symptoms of more than one organ type after the primary EM lesion.

6.3. The persistence of *B. burgdorferi* and implications for treatment of Lyme borreliosis

The mechanisms underlying the chronic signs and symptoms of LB after antibiotic treatment remain to be discovered, and the explanatory theories presented range from persistent infection to autoimmune reaction. This special entity of LB has some similarities with RA that is a systemic autoimmune disorder of unknown etiology. Over time, persistent synovial tissue inflammation associated with RA causes irreversible damage in affected joints. Cytokines play an important role in the pathogenesis of RA and TNF-α is one of the most important pro-inflammatory cytokines in this respect. Over the last decade, anti-TNF-α treatment has changed the treatment of RA and other autoimmune disorders. Our aim was to evaluate the effect of anti-TNF-α treatment in our mouse model LB with persistent joint swelling and elevated antibody levels after ceftriaxone treatment.

Anti-TNF-α treatment proved to have no influence on the persisting joint symptoms of *B. burgdorferi* infected mice whether given with or without ceftriaxone. In contrast, we made an unexpected observation: when anti-TNF-α treatment was given to ceftriaxone treated mice, the spirochete could be recovered from several of them. Since this phenomenon could also be seen among mice treated with anti-TNF-α four weeks after ceftriaxone, it is clear that all spirochetes were not killed by the antimicrobial treatment. This study showed for the first time that immunomodulatory treatment of animals infected with *B. burgdorferi* and treated with a proper antibiotic can convert them from culture negative to culture positive. Previously, *B. burgdorferi* has been recovered by culture from antibiotic treated patients and animals. In the study of Bockenstedt et al., the spirochetes recovered were genetically different from the infecting spirochetes probably due to mutations within the gene itself correlated with infectivity or recombination effects (Bockenstedt, Mao et al. 2002). These recovered spirochetes were unable to further infect mice and, therefore, the authors conclude that these spirochetes constitute an attenuated population that is no longer infectious and will eventually die or be killed by the host defense system without causing significant pathology. Conversely, in our study spirochetes recovered from ceftriaxone and anti-TNF-α treated mice, and bacteria used primarily to infect the mice had similar plasmid profiles and virulence. This indicates that these spirochetes had successfully avoided the lethal effects of ceftriaxone without losing their infectivity. Since the recovered and infecting spirochetes had similar levels of ceftriaxone susceptibility, selection of bacterial clones resistant to ceftriaxone does not explain the survival of *B. burgdorferi* in the treated mice. Further, these results suggest that *B. burgdorferi* could be added to the list of pathogens potentially activated by anti-TNF-α treatment.

Based on our observations, we hypothesized that a small number of *B. burgdorferi* spirochetes are able to survive the combined action of ceftriaxone treatment and immune response through hiding in immune privileged tissue or in a tissue with poor vasculature. Therefore, an array of mouse tissue samples was analyzed using culture and two PCR methods targeting different genes of *B. burgdorferi*. We had earlier used a 5-day course of ceftriaxone in the treatment of mice. Because this can be considered too short, we further studied the effects of an 18-day course of ceftriaxone.

Our results show that various tissues of all untreated *B. burgdorferi* infected C3H control mice were culture positive while, after ceftriaxone treatment, *B. burgdorferi* could not be cultivated from any tissues of the infected mice. In contrast, *B. burgdorferi* DNA was detected with PCR methods in the joints of 21 of 30 animals treated at an early stage (2 weeks) of infection. The interval between treatment and sample collection was as long as 21 weeks. Thus, it can be concluded that residual *B. burgdorferi* DNA may persist in mouse joints or joint-related tissues several months after ceftriaxone has eradicated cultivable spirochetes from the animals. Mice treated for 5 or 18 days showed similar

results. Thus, borrelial DNA seems to reside in the joints of mice even after rather long treatment with ceftriaxone.

Though the precise histological location of the remaining spirochetes is unclear, it is probable that they are at least at some level reached by the host immune system. This is indicated by persistently elevated levels of IgG antibodies against *B. burgdorferi*. However, the amount of persisting *B. burgdorferi* remains low and under the detection level by culture, until the mice are immunosuppressed using anti-TNF-α. This phenomenon has a similarity with the latency that is a known mechanism occurring in some viral (e.g. *Herpes simplex* virus) and bacterial (*Mycobacterium tuberculosis*, *Treponema pallidum*) infections. In the human body, the microbe may persist in a form with low or partial infectivity (adenoviruses in the tonsils and adenoids) or in a completely non-infectious form without producing any antigens (*Herpes simplex* virus). It has been demonstrated in a murine model of cystitis that uropathogenic *Escherichia coli* that is considered a typical extracellular pathogen is able to form intracellular bacterial communities and seed recurrent infection (Mulvey, Schilling et al. 2001; Anderson, Palermo et al. 2003; Justice, Hung et al. 2004). A recently published study showed evidence of a similar mechanism in humans (Rosen, Hooton et al. 2007). Further, the causative agent of syphilis, spirochete *Treponema pallidum*, is able to evade immune response and cause a chronic infection. There is also evidence of treatment-refractory syphilis (Dunlop 1972), although penicillin resistant strains have not been found thus far. The pathogenesis of chronic syphilis infection is unknown and several possibilities have been suggested. *T. pallidum* penetrates into various tissues efficiently. By this means it could get into and stay in immune privileged tissues. It may also exploit its slow metabolism to avoid recognition by the host. Further, the number of persisting *T. pallidum* spirochetes may be very low and may be located in anatomical sites distant to one another and, thus, do not alert the immune response (Lafond and Lukehart 2006). Further, as in *B. burgdorferi* spirochetes, antigenic variation through gene conversion has been hypothesized to be one mechanism by which the organism evades host immune response (Radolf 1994; Peeling and Hook 2006).

Some of the above mentioned mechanisms could also operate in *B. burgdorferi* infection. However, at least in our mouse model, the persistence of *B. burgdorferi* spirochetes in immunologically privileged sites in general is unlikely. Fragments of *B. burgdorferi* genome were not found by PCR from the eye-balls of ceftriaxone treated mice and PCR was positive in the brain specimen of only one mouse. The brain specimens of the infected untreated controls were positive in 2 of 9 mice by culture and in 0 of 9 by PCR, suggesting that the *B. burgdorferi* strains used were probably not actively migrating into CNS. Since *B. burgdorferi* is thought to prefer solid rather than liquid tissues, it may exploit a joint related tissue, e.g. synovium, as a niche. A number of *B. burgdorferi* might be able to survive there, even if the host's immune system recognizes it to some extent, which is

evidenced by the persistently elevated levels of IgG antibodies. Results of staining using immunohistochemistry in murine samples also suggest that antigens are still produced after ceftriaxone treatment (Stephen W. Barthold, personal communication). Further, the persistence of *B. burgdorferi* has been suggested to be due to transformation of the spirochete into cystic forms. This phenomenon would give the spirochete a chance to overcome unfavorable environmental conditions (Brorson and Brorson 1997; Murgia and Cinco 2004). Cyst formation has been shown to occur in body fluids and in response to β-lactam antibiotics in vitro (Brorson and Brorson 1998; Murgia, Piazzetta et al. 2002), but its significance in vivo has yet to be demonstrated.

In human patients, *B. burgdorferi* has been cultured after treatment in rare cases (Preac-Mursic, Weber et al. 1989; Haupl, Hahn et al. 1993). The detection of *B. burgdorferi* DNA by PCR after treatment has been successful more often (Battafarano, Combs et al. 1993; Priem, Burmester et al. 1998; Hulinska, Votypka et al. 1999; Limbach, Jaulhac et al. 2001) but the clinical relevance of positive PCR results is a matter of debate.

Today, patients are treated symptomatically if the prolonged or repeated antibiotic treatment does not provide substantial help. This practice is based on studies in which a long antibiotic regimen did not alter the disease outcome after the initial treatment (Klempner, Hu et al. 2001; Krupp, Hyman et al. 2003; Oksi, Nikoskelainen et al. 2007; Fallon, Keilp et al. 2008). However, Fallon et al. found in their study that, although cognitive improvement was not sustained, there was a greater improvement on the secondary measures of pain and physical functioning among more impaired patients given ceftriaxone compared to placebo. This improvement was sustained over six months. This study suggests therefore that there might be a sub-population of patients with chronic symptoms who benefit from repeated treatment. If persistence of *B. burgdorferi* spirochetes is the reason for treatment refractory LB in some patients, treatment methods other than just a course of a single antibiotic should be tested. As in the treatment of tuberculosis, perhaps a combination of several antimicrobial substances would eradicate the bacteria more efficiently than one antibiotic agent alone.

7. SUMMARY

Treatment refractory LB is a recognized clinical entity. Its pathogenesis is unknown and theories of the mechanisms underlying the disease range from autoimmune reaction to persistent infection, but none of the theories has been proven undeniably valid.

In this study, *B. burgdorferi* infection-induced joint manifestations were examined in a mouse model. Without treatment, infection and joint swelling continued and IgG antibody levels against *B. burgdorferi* remained elevated through the lifetime of the C3H/He mice. This model provides excellent tools to study the natural pathogenesis of LB. Further, it can be used as a disease model for persistent bacterial infection for other purposes because of its constant repeatability.

Early administered ceftriaxone treatment diminished joint swelling and lowered IgG antibody levels. When mice were treated at 18 weeks of infection, ceftriaxone did not have an effect on joint swelling or IgG levels. Independent of the timing of ceftriaxone treatment, every mouse treated was always culture negative. However, using two different PCR methods, DNA of *B. burgdorferi* could be detected in a number of mice and almost exclusively in the joint tissue samples. When mice were treated with immunosuppressive anti-TNF-α treatment either simultaneously with ceftriaxone or four weeks apart, a number converted to culture positive. Anti-TNF-α treatment did not have an effect on joint swelling whether given with ceftriaxone or alone.

Based on the results presented here, it can be concluded that *B. burgdorferi* is able to avoid the effect of ceftriaxone in mice. It may seek its way to a niche, possibly with poor vasculature, to be able to survive and evade the effects of antibiotics and immune defense. It is also possible that the spirochete protects itself from the effects of antibiotics by reducing its metabolic activity or transforming to a less susceptible form (e.g. cystic). Thus far, we have succeeded in culturing *B. burgdorferi* from antibiotically-treated mice only after causing immunosuppression in them by anti-TNF-α treatment. Without immunosuppressive treatment, no viable spirochetes but only DNA of *B. burgdorferi* can be found. We tested a large panel of mouse tissues, and positive PCR results were almost exclusively obtained from the joint samples. This suggests that the joints or joint-related tissues of mice are the niche where *B. burgdorferi* hides and protects itself from the effects of antibiotics and host defense. Although we demonstrated the presence of segments of two different genes of *B. burgdorferi* in the joints of mice, this does not confirm that viable spirochetes existed in the specimens. However, it is hard to believe that lifeless fragments of the spirochete could maintain strong antibody production for months after the antibiotic treatment. Where and in what form the spirochetes reside in mice and humans is a challenging question warranting extensive further studies.

ACKNOWLEDGEMENTS

This study was carried out at the department of Medical Microbiology and Immunology, University of Turku, and at the National Public Health Institute (current National Institute for Health and Welfare).

I am most grateful to my supervisors Professor Matti Viljanen and Docent Jarmo Oksi. Your encouraging guidance and never-ending patience helped me during the moments when everything seemed somewhat hopeless. Matti, you have a special quality to inspire instead of using your authority. Your broad and deep knowledge of the field as well as your rational and flexible thinking make you a real role model in science. I also thank you for the instructive moments you spent with me to correct my manuscripts. After discussions and meetings with you, I always felt that this work is so interesting that I am privileged to do it. Jarmo, working under the guidance of the leading clinician of Lyme borreliosis in the country was important to me during these years. You have the most experience of complicated LB patients and your comments on my study designs and result interpretations have always been valuable. I am also thankful that you sometimes let me to come and meet your patients at borrelia-poli.

I warmly thank Professors emeriti Paavo Toivanen and Heikki Arvilommi for providing excellent facilities to carry out this research. I also thank Professor Paavo Toivanen for inviting me to join the diagnostic and teaching staff of the department.

Professors Hilpi Rautelin and Brian Fallon are warmly thanked for their constructive comments. Brian, your support in my study meant a lot to me. Finding my way to your office was such a happy coincidence!

My co-authors Kaija Hartiala, Jukka Hytönen, Carl-Ove Söderström and Xiao-yu Song are thanked for their contribution. Kaija, I missed your enthusiasm and female thinking in our group when you chose another career. Jukka, thank you for your precise and disciplined work.

All the past and present fellows of the Turku borrelia-group: Juha Suhonen, Pauliina Hartiala, Jemiina Neuvonen, Joonas Tynkkynen, Taina Kirjonen, Markus Penttinen, Jenni Pelkonen, Helena Tuominen-Gustafsson, Ulla Toivonen, Marju Niskala, Tuula Rantasalo, Aija Kaitaranta, Hanna Soini, Merja Marjamäki and Johanna Mäkinen, are thanked for creating such a nice atmosphere to work in! Pauliina, you have been great company to work and travel to congresses with. Our shared passion for dance and food (not to forget those spiral shaped creatures) has kept us going to many places together. Having you in the group has really been refreshing. Juha, it was always fun to work with you. Your special sense of humour brought a smile to my face many times during this

work. I especially want to thank all of you who have helped me with my mouse studies and even hunted run-away mice when required.

Ulla-Marjut Jaakkola, Seija Lindqvist and Tiina Kyrölä are thanked for all your assistance with the mice.

Seniors of our department Markku Viander, Olli Lassila, Erkki Eerola, Sirpa Jalkanen, Jaakko Uksila, Olli Vainio as well as Pentti Huovinen, Kaisa Granfors, Quishui He from the THL are acknowledged for their teaching and advice. Markku, your help has always been available – and every time it comes with smile. Olli, never give up! Your endless supply of energy is a motor for the entire department.

Fellow assistants and other colleagues at the department Milja Möttönen, Jussi Kantele, Kalle-Pekka Nera, Antti Hakanen, Arno Hänninen, Jukka Alinikula, Laura Mustonen, Perttu Terho, Catharina Alam, Suvi Valkonen, Laura Kopu, Pia Suonpää, Jenni Heikkinen, Janne Atosuo, Janne Komi, Pekka Kohonen, Laura Pirilä, Jussi Vaahtovuo, Elli Narvi, Jussi Liippo, Bas Hoffman, Jussi Salmi, Kaisa Katevuo, Egle Simelyte, Juha Tuokko, Kimmo Koskela, Kaisu Rantakokko-Jalava, Jari Jalava, Raija Manninen, Riitta Koskinen, Tong Chen, Xiang Zhang, are warmly thanked for company. Milja, thank you for good conversations concerning a wide range of topics; your support in this work has been important. Laura, thank you for being there and also sharing The Moods - which luckily are a minor part of life. I also want to thank the great Mikro staff, especially Paula Vahakoski, Mervi Salo, Raija Raulimo, Diina Ryynänen, Tuula Rikalainen and Matti Toivonen. Mike Nelson is thanked for language revision and excellent oral presentation skills classes.

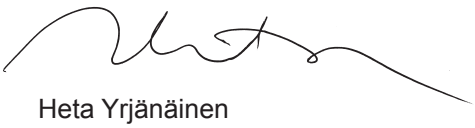
I want to thank my capoeira teachers and capoeira friends all over the world, especially Mestre João Grande and Cabello. Our group here in Finland means so much to me, without you Marika, Johanna, Elina, Lasse, Samuli and Pizzi, I would feel lonely. Thank you, Ritva, for your friendship and teaching, rest in peace.

My friends - thank you for every shared moment. Marja, our friendship is unique, your simultaneous conservativeness and radicalness is a rare combination. I call it wisdom. Laura and Joni, you are my extended family; Tapani, you know how to put the words to make me laugh; Leena, even when going through hard times yourself you always have time to ask me how I feel; Kari and Janne, those were the days, oh boys, I never forget the songs 1 and 11. Marjo, thank you for brightening my life with flowers and music, Elissa and Tuija, I cherish every endless conversation I have with you. Thanks to all of my friends, with your help life is like a bed of roses.

My parents, you are always there when needed and also when not needed at all and I appreciate everything you do for me and my family. Mum, your everlasting curiosity and intensity in everything you do is between science and art, you were the first in line when

energy was being given out. Dad, thank you for your positive support and witty phrases. I admire your natural optimism and the skill to look ahead. I thank my siblings Kaija and Antti and their families for their love and support.

Finally, my most important creation, Alma and Aaro, I am lucky to have you and I have always enjoyed our family life. The school of motherhood led by you has taught me so many things about life that high school and medical school failed to teach me. This work is important but you are my masterpiece.



Heta Yrjänäinen

REFERENCES

Aguero-Rosenfeld, M. E., G. Wang, et al. (2005). "Diagnosis of lyme borreliosis." Clin Microbiol Rev **18**(3): 484-509.

Anderson, G. G., J. J. Palermo, et al. (2003). "Intracellular bacterial biofilm-like pods in urinary tract infections." Science **301**(5629): 105-7.

Anderson, J. F. and L. A. Magnarelli (2008). "Biology of ticks." Infect Dis Clin North Am **22**(2): 195-215, v.

Appel, M. J., S. Allan, et al. (1993). "Experimental Lyme disease in dogs produces arthritis and persistent infection." J Infect Dis **167**(3): 651-64.

Asbrink, E. (1985). "Erythema chronicum migrans Afzelius and acrodermatitis chronica atrophicans. Early and late manifestations of Ixodes ricinus-borne Borrelia spirochetes." Acta Derm Venereol Suppl (Stockh) **118**: 1-63.

Asbrink, E. and A. Hovmark (1985). "Successful cultivation of spirochetes from skin lesions of patients with erythema chronicum migrans Afzelius and acrodermatitis chronica atrophicans." Acta Pathol Microbiol Immunol Scand [B] **93**(2): 161-3.

Asbrink, E. and A. Hovmark (1988). "Early and late cutaneous manifestations in Ixodes-borne borreliosis (erythema migrans borreliosis, Lyme borreliosis)." Ann N Y Acad Sci **539**: 4-15.

Baranton, G., D. Postic, et al. (1992). "Delineation of Borrelia burgdorferi sensu stricto, Borrelia garinii sp. nov., and group VS461 associated with Lyme borreliosis." Int J Syst Bacteriol **42**(3): 378-83.

Barbour, A. G. (1984). "Isolation and cultivation of Lyme disease spirochetes." Yale J Biol Med **57**(4): 521-5.

Barbour, A. G. (1988). "Plasmid analysis of Borrelia burgdorferi, the Lyme disease agent." J Clin Microbiol **26**(3): 475-8.

Barbour, A. G. and S. F. Hayes (1986). "Biology of Borrelia species." Microbiol Rev **50**(4): 381-400.

Barthold, S. W. (1991). "Infectivity of Borrelia burgdorferi relative to route of inoculation and genotype in laboratory mice." J Infect Dis **163**(2): 419-20.

Barthold, S. W., D. S. Beck, et al. (1990). "Lyme borreliosis in selected strains and ages of laboratory mice." J Infect Dis **162**(1): 133-8.

Barthold, S. W. and M. de Souza (1995). "Exacerbation of Lyme arthritis in beige mice." J Infect Dis **172**(3): 778-84.

Barthold, S. W., M. S. de Souza, et al. (1993). "Chronic Lyme borreliosis in the laboratory mouse." Am J Pathol **143**(3): 959-71.

Barthold, S. W., K. D. Moody, et al. (1988). "Experimental Lyme arthritis in rats infected with Borrelia burgdorferi." J Infect Dis **157**(4): 842-6.

Barthold, S. W., K. D. Moody, et al. (1988). "An animal model for Lyme arthritis." Ann N Y Acad Sci **539**: 264-73.

Battafarano, D. F., J. A. Combs, et al. (1993). "Chronic septic arthritis caused by Borrelia burgdorferi." Clin Orthop Relat Res(297): 238-41.

Beck, G., J. L. Benach, et al. (1989). "Isolation of interleukin 1 from joint fluids of patients with Lyme disease." J Rheumatol **16**(6): 800-6.

Benach, J. L., E. M. Bosler, et al. (1984). "Experimental transmission of the Lyme disease spirochete to rabbits." J Infect Dis **150**(5): 786-7.

Benach, J. L., E. M. Bosler, et al. (1983). "Spirochetes isolated from the blood of two patients with Lyme disease." N Engl J Med **308**(13): 740-2.

Berger, B. W. (1989). "Dermatologic manifestations of Lyme disease." Rev Infect Dis **11 Suppl 6**: S1475-81.

Berglund, J., R. Eitrem, et al. (1995). "An epidemiologic study of Lyme disease in southern Sweden." N Engl J Med **333**(20): 1319-27.

Binder, E., R. Doepfmer, et al. (1955). "[Experimental transmission of chronic erythema migrans from man to man.]." Hautarzt **6**(11): 494-6.

Bockenstedt, L. K., J. Mao, et al. (2002). "Detection of attenuated, noninfectious spirochetes in Borrelia burgdorferi-infected mice after antibiotic treatment." J Infect Dis **186**(10): 1430-7.

Bowie, V. L., K. A. Snella, et al. (2004). "Listeria meningitis associated with infliximab." Ann Pharmacother **38**(1): 58-61.

Brorson, O. and S. H. Brorson (1997). "Transformation of cystic forms of Borrelia burgdorferi to normal, mobile spirochetes." Infection **25**(4): 240-6.

Brorson, O. and S. H. Brorson (1998). "In vitro conversion of Borrelia burgdorferi to cystic forms in spinal fluid, and transformation to mobile spirochetes by incubation in BSK-H medium." Infection **26**(3): 144-50.

Burgdorfer, W. (1984). "The New Zealand white rabbit: an experimental host for infecting ticks with Lyme disease spirochetes." Yale J Biol Med **57**(4): 609-12.

Burgdorfer, W., A. G. Barbour, et al. (1982). "Lyme disease-a tick-borne spirochetosis?" Science **216**(4552): 1317-9.

Cadavid, D., T. O'Neill, et al. (2000). "Localization of Borrelia burgdorferi in the nervous system and other organs in a nonhuman primate model of lyme disease." Lab Invest **80**(7): 1043-54.

Canica, M. M., F. Nato, et al. (1993). "Monoclonal antibodies for identification of Borrelia afzelii sp. nov. associated with late cutaneous manifestations of Lyme borreliosis." Scand J Infect Dis **25**(4): 441-8.

Casjens, S., N. Palmer, et al. (2000). “A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete Borrelia burgdorferi.” Mol Microbiol **35**(3): 490-516.

Charon, N. W. and S. F. Goldstein (2002). “Genetics of motility and chemotaxis of a fascinating group of bacteria: the spirochetes.” Annu Rev Genet **36**: 47-73.

Christen, H. J. (1996). “Lyme neuroborreliosis in children.” Ann Med **28**(3): 235-40.

Coburn, J., L. Magoun, et al. (1998). ”Integrins alpha(v)beta3 and alpha5beta1 mediate attachment of lyme disease spirochetes to human cells.” Infect Immun **66**(5): 1946-52.

Comstock, L. E. and D. D. Thomas (1989). “Penetration of endothelial cell monolayers by Borrelia burgdorferi.” Infect Immun **57**(5): 1626-8.

Craft, J. E., R. L. Grodzicki, et al. (1984). “Antibody response in Lyme disease: evaluation of diagnostic tests.” J Infect Dis **149**(5): 789-95.

Dadamessi, I., F. Brazier, et al. (2001). “[Hepatic disorders related to Lyme disease. Study of two cases and a review of the literature].” Gastroenterol Clin Biol **25**(2): 193-6.

de Koning, J., J. A. Hoogkamp-Korstanje, et al. (1989). ”Demonstration of spirochetes in cardiac biopsies of patients with Lyme disease.” J Infect Dis **160**(1): 150-3.

De Rosa, F. G., D. Shaz, et al. (2003). ”Invasive pulmonary aspergillosis soon after therapy with infliximab, a tumor necrosis factor-alpha-neutralizing antibody: a possible healthcare-associated case?” Infect Control Hosp Epidemiol **24**(7): 477-82.

de Souza, M. S., A. L. Smith, et al. (1993). “Variant responses of mice to Borrelia burgdorferi depending on the site of intradermal inoculation.” Infect Immun **61**(10): 4493-7.

Dejmkova, H., D. Hulinska, et al. (2002). “Seronegative Lyme arthritis caused by Borrelia garinii.” Clin Rheumatol **21**(4): 330-4.

Donaldson, J. O. and R. A. Lewis (1983). “Lymphocytic meningoradiculitis in the United States.” Neurology **33**(11): 1476-9.

Dunlop, E. M. (1972). “Persistence of treponemes after treatment.” Br Med J **2**(5813): 577-80.

Duray, P. H. (1989). “Clinical pathologic correlations of Lyme disease.” Rev Infect Dis **11 Suppl 6**: S1487-93.

Duray, P. H. and A. C. Steere (1986). “The spectrum of organ and systems pathology in human Lyme disease.” Zentralbl Bakteriol Mikrobiol Hyg [A] **263**(1-2): 169-78.

Ecklund, K., S. Vargas, et al. (2005). ”MRI features of Lyme arthritis in children.” AJR Am J Roentgenol **184**(6): 1904-9.

Eisendle, K., T. Grabner, et al. (2007). “Focus floating microscopy: “gold standard” for cutaneous borreliosis?” Am J Clin Pathol **127**(2): 213-22.

Elias, A. F., P. E. Stewart, et al. (2002). “Clonal polymorphism of Borrelia burgdorferi strain B31 MI: implications for mutagenesis in an infectious strain background.” Infect Immun **70**(4): 2139-50.

Elliott, M. J., R. N. Maini, et al. (1994). “Repeated therapy with monoclonal antibody to tumour necrosis factor alpha (cA2) in patients with rheumatoid arthritis.” Lancet **344**(8930): 1125-7.

Elliott, M. J., R. N. Maini, et al. (1993). “Treatment of rheumatoid arthritis with chimeric monoclonal antibodies to tumor necrosis factor alpha.” Arthritis Rheum **36**(12): 1681-90.

Fallon, B. A., J. G. Keilp, et al. (2008). ”A randomized, placebo-controlled trial of repeated IV antibiotic therapy for Lyme encephalopathy.” Neurology **70**(13): 992-1003.

Feder, H. M., Jr., M. A. Gerber, et al. (1992). “Persistence of serum antibodies to Borrelia burgdorferi in patients treated for Lyme disease.” Clin Infect Dis **15**(5): 788-93.

Feldmann, M., F. M. Brennan, et al. (1996). “Role of cytokines in rheumatoid arthritis.” Annu Rev Immunol **14**: 397-440.

Fish, A. E., Y. B. Pride, et al. (2008). ”Lyme carditis.” Infect Dis Clin North Am **22**(2): 275-88, vi.

Franz, J. K. and A. Krause (2003). “Lyme disease (Lyme borreliosis).” Best Pract Res Clin Rheumatol **17**(2): 241-64.

Garcia-Monco, J. C., N. S. Miller, et al. (1997). “A mouse model of Borrelia meningitis after intradermal injection.” J Infect Dis **175**(5): 1243-5.

Gardam, M. A., E. C. Keystone, et al. (2003). “Anti-tumour necrosis factor agents and tuberculosis risk: mechanisms of action and clinical management.” Lancet Infect Dis **3**(3): 148-55.

Glatz, M., M. Golestani, et al. (2006). “Clinical relevance of different IgG and IgM serum antibody responses to Borrelia burgdorferi after antibiotic therapy for erythema migrans: long-term follow-up study of 113 patients.” Arch Dermatol **142**(7): 862-8.

Gnann, J. W., Jr., W. E. Goetter, et al. (1982). ”Ceftriaxone: in vitro studies and clinical evaluation.” Antimicrob Agents Chemother **22**(1): 1-9.

Goodman, J. L., J. F. Bradley, et al. (1995). “Bloodstream invasion in early Lyme disease: results from a prospective, controlled, blinded study using the polymerase chain reaction.” Am J Med **99**(1): 6-12.

Grab, D. J., G. Perides, et al. (2005). ”Borrelia burgdorferi, host-derived proteases, and the blood-brain barrier.” Infect Immun **73**(2): 1014-22.

Guo, B. P., S. J. Norris, et al. (1995). “Adherence of Borrelia burgdorferi to the proteoglycan decorin.” Infect Immun **63**(9): 3467-72.

Haerter, G., B. J. Manfras, et al. (2004). “Cytomegalovirus retinitis in a patient treated with anti-tumor necrosis factor alpha antibody therapy for rheumatoid arthritis.” Clin Infect Dis **39**(9): e88-94.

Halperin, J. J. (1997). “Neuroborreliosis: central nervous system involvement.” Semin Neurol **17**(1): 19-24.

Halperin, J. J. (1998). “Nervous system Lyme disease.” J Neurol Sci **153**(2): 182-91.

Halperin, J. J. (2008). “Nervous system Lyme disease.” Infect Dis Clin North Am **22**(2): 261-74, vi.

Hansen, K. and A. M. Lebech (1992). “The clinical and epidemiological profile of Lyme neuroborreliosis in Denmark 1985-1990. A prospective study of 187 patients with Borrelia burgdorferi specific intrathecal antibody production.” Brain **115 (Pt 2)**: 399-423.

Hansen, K., C. Rechnitzer, et al. (1987). ”Borrelia meningitis in Denmark.” Zentralbl Bakteriol Mikrobiol Hyg [A] **263**(3): 348-50.

Hardin, J. A., A. C. Steere, et al. (1979). “Immune complexes and the evolution of Lyme arthritis. Dissemination and localization of abnormal C1q binding activity.” N Engl J Med **301**(25): 1358-63.

Harrer, T., W. Geissdorfer, et al. (2007). ”Seronegative Lyme neuroborreliosis in a patient on treatment for chronic lymphatic leukemia.” Infection **35**(2): 110-3.

Haupl, T., G. Hahn, et al. (1993). “Persistence of Borrelia burgdorferi in ligamentous tissue from a patient with chronic Lyme borreliosis.” Arthritis Rheum **36**(11): 1621-6.

Hodzic, E., S. Feng, et al. (2008). “Persistence of Borrelia burgdorferi following antibiotic treatment in mice.” Antimicrob Agents Chemother **52**(5): 1728-36.

Holl-Wieden, A., S. Suerbaum, et al. (2007). “Seronegative Lyme arthritis.” Rheumatol Int **27**(11): 1091-3.

Hudson, B. J., M. Stewart, et al. (1998). ”Culture-positive Lyme borreliosis.” Med J Aust **168**(10): 500-2.

Hulinska, D., J. Votypka, et al. (1999). “Persistence of Borrelia garinii and Borrelia afzelii in patients with Lyme arthritis.” Zentralbl Bakteriol **289**(3): 301-18.

Humair, P. and L. Gern (2000). “The wild hidden face of Lyme borreliosis in Europe.” Microbes Infect **2**(8): 915-22.

Hunfeld, K. P., E. Ruzic-Sabljic, et al. (2006). “Risk of culture-confirmed borrelial persistence in patients treated for erythema migrans and possible mechanisms of resistance.” Int J Med Microbiol **296 Suppl 40**: 233-41.

Huppertz, H. I., M. Bohme, et al. (1999). “Incidence of Lyme borreliosis in the Wurzburg region of Germany.” Eur J Clin Microbiol Infect Dis **18**(10): 697-703.

Ilowite, N. T. (1995). “Muscle, reticuloendothelial, and late skin manifestations of Lyme disease.” Am J Med **98**(4A): 63S-68S.

Ivacic, L., K. D. Reed, et al. (2007). “A LightCycler TaqMan assay for detection of Borrelia burgdorferi sensu lato in clinical samples.” Diagn Microbiol Infect Dis **57**(2): 137-43.

Johnston, Y. E., P. H. Duray, et al. (1985). “Lyme arthritis. Spirochetes found in synovial microangiopathic lesions.” Am J Pathol **118**(1): 26-34.

Justice, S. S., C. Hung, et al. (2004). “Differentiation and developmental pathways of uropathogenic Escherichia coli in urinary tract pathogenesis.” Proc Natl Acad Sci U S A **101**(5): 1333-8.

Kahl, O., C. Janetzki-Mittmann, et al. (1998). “Risk of infection with Borrelia burgdorferi sensu lato for a host in relation to the duration of nymphal Ixodes ricinus feeding and the method of tick removal.” Zentralbl Bakteriol **287**(1-2): 41-52.

Kaiser, R. (1998). ”Neuroborreliosis.” J Neurol **245**(5): 247-55.

Kalish, R. A., R. F. Kaplan, et al. (2001). ”Evaluation of study patients with Lyme disease, 10-20-year follow-up.” J Infect Dis **183**(3): 453-60.

Kalish, R. A., G. McHugh, et al. (2001). “Persistence of immunoglobulin M or immunoglobulin G antibody responses to Borrelia burgdorferi 10-20 years after active Lyme disease.” Clin Infect Dis **33**(6): 780-5.

Kannian, P., G. McHugh, et al. (2007). “Antibody responses to Borrelia burgdorferi in patients with antibiotic-refractory, antibiotic-responsive, or non-antibiotic-treated Lyme arthritis.” Arthritis Rheum **56**(12): 4216-25.

Karma, A. and H. Mikkila (1996). “Ocular manifestations and treatment of Lyme disease.” Curr Opin Ophthalmol **7**(3): 7-12.

Kazragis, R. J., L. L. Dever, et al. (1996). “In vivo activities of ceftriaxone and vancomycin against Borrelia spp. in the mouse brain and other sites.” Antimicrob Agents Chemother **40**(11): 2632-6.

Keane-Myers, A. and S. P. Nickell (1995). “T cell subset-dependent modulation of immunity to Borrelia burgdorferi in mice.” J Immunol **154**(4): 1770-6.

Keane, J., S. Gershon, et al. (2001). “Tuberculosis associated with infliximab, a tumor necrosis factor alpha-neutralizing agent.” N Engl J Med **345**(15): 1098-104.

Klempner, M. S., L. T. Hu, et al. (2001). ”Two controlled trials of antibiotic treatment in patients with persistent symptoms and a history of Lyme disease.” N Engl J Med **345**(2): 85-92.

Klempner, M. S., R. Noring, et al. (1995). ”Binding of human plasminogen and urokinase-type plasminogen activator to the Lyme disease spirochete, Borrelia burgdorferi.” J Infect Dis **171**(5): 1258-65.

Klempner, M. S., G. H. Wormser, et al. (2005). “A case-control study to examine HLA haplotype associations in patients with posttreatment chronic Lyme disease.” J Infect Dis **192**(6): 1010-3.

Kornblatt, A. N., A. C. Steere, et al. (1984). ”Experimental Lyme disease in rabbits: spirochetes found in erythema migrans and blood.” Infect Immun **46**(1): 220-3.

Krinsky, W. L., S. J. Brown, et al. (1982). “Ixodes dammini: induced skin lesions in guinea pigs and rabbits compared to erythema chronicum migrans in patients with lyme arthritis.” Exp Parasitol **53**(3): 381-95.

Krupp, L. B., L. G. Hyman, et al. (2003). “Study and treatment of post Lyme disease (STOP-LD): a randomized double masked clinical trial.” Neurology **60**(12): 1923-30.

Kuiper, H., I. Cairo, et al. (1994). “Solitary erythema migrans: a clinical, laboratory and epidemiological study of 77 Dutch patients.” Br J Dermatol **130**(4): 466-72.

Lafond, R. E. and S. A. Lukehart (2006). “Biological basis for syphilis.” Clin Microbiol Rev **19**(1): 29-49.

Lawson, J. P. and A. C. Steere (1985). “Lyme arthritis: radiologic findings.” Radiology **154**(1): 37-43.

Limbach, F. X., B. Jaulhac, et al. (2001). “Treatment resistant Lyme arthritis caused by Borrelia garinii.” Ann Rheum Dis **60**(3): 284-6.

Lipsker, D., N. Antoni-Bach, et al. (2002). ”Long-term prognosis of patients treated for erythema migrans in France.” Br J Dermatol **146**(5): 872-6.

Littman, M. P., R. E. Goldstein, et al. (2006). ”ACVIM small animal consensus statement on Lyme disease in dogs: diagnosis, treatment, and prevention.” J Vet Intern Med **20**(2): 422-34.

Ma, Y., K. P. Seiler, et al. (1998). ”Distinct characteristics of resistance to Borrelia burgdorferi-induced arthritis in C57BL/6N mice.” Infect Immun **66**(1): 161-8.

Marconi, R. T. and C. F. Garon (1992). “Phylogenetic analysis of the genus Borrelia: a comparison of North American and European isolates of Borrelia burgdorferi.” J Bacteriol **174**(1): 241-4.

Marini, M., G. Bamias, et al. (2003). “TNF-alpha neutralization ameliorates the severity of murine Crohn’s-like ileitis by abrogation of intestinal epithelial cell apoptosis.” Proc Natl Acad Sci U S A **100**(14): 8366-71.

Marques, A. (2008). “Chronic Lyme disease: a review.” Infect Dis Clin North Am **22**(2): 341-60, vii-viii.

Masters, E. J., C. N. Grigery, et al. (2008). “STARI, or Masters disease: Lone Star tick-vectored Lyme-like illness.” Infect Dis Clin North Am **22**(2): 361-76, viii.

Matyniak, J. E. and S. L. Reiner (1995). “T helper phenotype and genetic susceptibility in experimental Lyme disease.” J Exp Med **181**(3): 1251-4.

Mikkilä, A. (1998). Ocular Lyme borreliosis, diagnosis and clinical characteristics.

Miller, L. C., E. A. Lynch, et al. (1993). ”Balance of synovial fluid IL-1 beta and IL-1 receptor antagonist and recovery from Lyme arthritis.” Lancet **341**(8838): 146-8.

Montgomery, R. R., D. Lusitani, et al. (2004). “Tick saliva reduces adherence and area of human neutrophils.” Infect Immun **72**(5): 2989-94.

Moody, K. D., R. L. Adams, et al. (1994). “Effectiveness of antimicrobial treatment against Borrelia burgdorferi infection in mice.” Antimicrob Agents Chemother **38**(7): 1567-72.

Moody, K. D., S. W. Barthold, et al. (1990). “Experimental chronic Lyme borreliosis in Lewis rats.” Am J Trop Med Hyg **42**(2): 165-74.

Morgan, J. R., A. Paull, et al. (1985). ”The penetration of ceftriaxone into synovial fluid of the inflamed joint.” J Antimicrob Chemother **16**(3): 367-71.

Mullegger, R. R. (2004). “Dermatological manifestations of Lyme borreliosis.” Eur J Dermatol **14**(5): 296-309.

Mullegger, R. R., G. McHugh, et al. (2000). ”Differential expression of cytokine mRNA in skin specimens from patients with erythema migrans or acrodermatitis chronica atrophicans.” J Invest Dermatol **115**(6): 1115-23.

Mulvey, M. A., J. D. Schilling, et al. (2001). “Establishment of a persistent Escherichia coli reservoir during the acute phase of a bladder infection.” Infect Immun **69**(7): 4572-9.

Murgia, R. and M. Cinco (2004). “Induction of cystic forms by different stress conditions in Borrelia burgdorferi.” Apmis **112**(1): 57-62.

Murgia, R., C. Piazzetta, et al. (2002). “Cystic forms of Borrelia burgdorferi sensu lato: induction, development, and the role of RpoS.” Wien Klin Wochenschr **114**(13-14): 574-9.

Mursic, V. P., B. Wilske, et al. (1987). ”In vitro and in vivo susceptibility of Borrelia burgdorferi.” Eur J Clin Microbiol **6**(4): 424-6.

Nadelman, R. B., J. Nowakowski, et al. (1996). “The clinical spectrum of early Lyme borreliosis in patients with culture-confirmed erythema migrans.” Am J Med **100**(5): 502-8.

Nadelman, R. B., C. S. Pavia, et al. (1990). ”Isolation of Borrelia burgdorferi from the blood of seven patients with Lyme disease.” Am J Med **88**(1): 21-6.

Nadelman, R. B. and G. P. Wormser (1995). “Erythema migrans and early Lyme disease.” Am J Med **98**(4A): 15S-23S; discussion 23S-24S.

Nardelli, D. T., S. M. Callister, et al. (2008). “Lyme arthritis: current concepts and a change in paradigm.” Clin Vaccine Immunol **15**(1): 21-34.

Nocton, J. J., F. Dressler, et al. (1994). “Detection of Borrelia burgdorferi DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis.” N Engl J Med **330**(4): 229-34.

Nowakowski, J., R. B. Nadelman, et al. (2003). "Long-term follow-up of patients with culture-confirmed Lyme disease." Am J Med **115**(2): 91-6.

Oksi, J., M. Marjamäki, et al. (1999). "Borrelia burgdorferi detected by culture and PCR in clinical relapse of disseminated Lyme borreliosis." Ann Med **31**(3): 225-32.

Oksi, J., H. Marttila, et al. (2001). "Early dissemination of Borrelia burgdorferi without generalized symptoms in patients with erythema migrans." Apmis **109**(9): 581-8.

Oksi, J., J. Nikoskelainen, et al. (2007). "Duration of antibiotic treatment in disseminated Lyme borreliosis: a double-blind, randomized, placebo-controlled, multicenter clinical study." Eur J Clin Microbiol Infect Dis **26**(8): 571-81.

Oksi, J., J. Savolainen, et al. (1996). "Decreased interleukin-4 and increased gamma interferon production by peripheral blood mononuclear cells of patients with Lyme borreliosis." Infect Immun **64**(9): 3620-3.

Pachner, A. R. (1995). "Early disseminated Lyme disease: Lyme meningitis." Am J Med **98**(4A): 30S-37S; discussion 37S-43S.

Pachner, A. R., K. Amemiya, et al. (2001). "Lyme borreliosis in rhesus macaques: effects of corticosteroids on spirochetal load and isotype switching of anti-borrelia burgdorferi antibody." Clin Diagn Lab Immunol **8**(2): 225-32.

Pachner, A. R., E. Delaney, et al. (1995). "Inoculation of nonhuman primates with the N40 strain of Borrelia burgdorferi leads to a model of Lyme neuroborreliosis faithful to the human disease." Neurology **45**(1): 165-72.

Pachner, A. R., H. Gelderblom, et al. (2001). "The rhesus model of Lyme neuroborreliosis." Immunol Rev **183**: 186-204.

Paster, B. J., F. E. Dewhirst, et al. (1991). "Phylogenetic analysis of the spirochetes." J Bacteriol **173**(19): 6101-9.

Pavia, C., M. A. Inchiosa, Jr., et al. (2002). "Efficacy of short-course ceftriaxone therapy for Borrelia burgdorferi infection in C3H mice." Antimicrob Agents Chemother **46**(1): 132-4.

Pavia, C. S., G. P. Wormser, et al. (2001). "Efficacy of an evernimicin (SCH27899) in vitro and in an animal model of Lyme disease." Antimicrob Agents Chemother **45**(3): 936-7.

Peeling, R. W. and E. W. Hook, 3rd (2006). "The pathogenesis of syphilis: the Great Mimicker, revisited." J Pathol **208**(2): 224-32.

Pfister, H. W., K. Einhaupl, et al. (1984). "The spirochetal etiology of lymphocytic meningoradiculitis of Bannwarth (Bannwarth's syndrome)." J Neurol **231**(3): 141-4.

Pfister, H. W. and T. A. Rupprecht (2006). "Clinical aspects of neuroborreliosis and post-Lyme disease syndrome in adult patients." Int J Med Microbiol **296 Suppl 40**: 11-6.

Picha, D., L. Moravcova, et al. (2006). "Symptoms of post-Lyme syndrome in long-term outcome of patients with neuroborreliosis." Scand J Infect Dis **38**(8): 747-8.

Preac-Mursic, V., K. Weber, et al. (1989). "Survival of Borrelia burgdorferi in antibiotically treated patients with Lyme borreliosis." Infection **17**(6): 355-9.

Priem, S., G. R. Burmester, et al. (1998). "Detection of Borrelia burgdorferi by polymerase chain reaction in synovial membrane, but not in synovial fluid from patients with persisting Lyme arthritis after antibiotic therapy." Ann Rheum Dis **57**(2): 118-21.

Priem, S., K. Munkelt, et al. (2003). "[Epidemiology and therapy of Lyme arthritis and other manifestations of Lyme borreliosis in Germany: results of a nation-wide survey]." Z Rheumatol **62**(5): 450-8.

Probert, W. S. and B. J. Johnson (1998). "Identification of a 47 kDa fibronectin-binding protein expressed by Borrelia burgdorferi isolate B31." Mol Microbiol **30**(5): 1003-15.

Radolf, J. D. (1994). "Role of outer membrane architecture in immune evasion by Treponema pallidum and Borrelia burgdorferi." Trends Microbiol **2**(9): 307-11.

Reichardt, P., I. Dahnert, et al. (2002). "Possible activation of an intramyocardial inflammatory process (Staphylococcus aureus)after treatment with infliximab in a boy with Crohn disease." Eur J Pediatr **161**(5): 281-3.

Ribeiro, J. M. and I. M. Francischetti (2003). "Role of arthropod saliva in blood feeding: sialome and post-sialome perspectives." Annu Rev Entomol **48**: 73-88.

Ribeiro, J. M., J. J. Weis, et al. (1990). "Saliva of the tick Ixodes dammini inhibits neutrophil function." Exp Parasitol **70**(4): 382-8.

Rosa, P. A., K. Tilly, et al. (2005). "The burgeoning molecular genetics of the Lyme disease spirochaete." Nat Rev Microbiol **3**(2): 129-43.

Rosen, D. A., T. M. Hooton, et al. (2007). "Detection of intracellular bacterial communities in human urinary tract infection." PLoS Med **4**(12): e329.

Rupprecht, T. A., U. Koedel, et al. (2008). "The pathogenesis of lyme neuroborreliosis: from infection to inflammation." Mol Med **14**(3-4): 205-12.

Russell, H., J. S. Sampson, et al. (1984). "Enzyme-linked immunosorbent assay and indirect immunofluorescence assay for Lyme disease." J Infect Dis **149**(3): 465-70.

Sadziene, A., D. D. Thomas, et al. (1991). "A flagella-less mutant of Borrelia burgdorferi. Structural, molecular, and in vitro functional characterization." J Clin Invest **88**(1): 82-92.

Sadziene, A., P. A. Thompson, et al. (1996). "A flagella-less mutant of Borrelia burgdorferi as a

live attenuated vaccine in the murine model of Lyme disease." J Infect Dis **173**(5): 1184-93.

Schaible, U. E., S. Gay, et al. (1990). "Lyme borreliosis in the severe combined immunodeficiency (scid) mouse manifests predominantly in the joints, heart, and liver." Am J Pathol **137**(4): 811-20.

Schaible, U. E., M. D. Kramer, et al. (1989). "The severe combined immunodeficiency (scid) mouse. A laboratory model for the analysis of Lyme arthritis and carditis." J Exp Med **170**(4): 1427-32.

Schmidt, B., R. R. Muellegger, et al. (1996). "Detection of Borrelia burgdorferi-specific DNA in urine specimens from patients with erythema migrans before and after antibiotic therapy." J Clin Microbiol **34**(6): 1359-63.

Schmitz, J. L., R. F. Schell, et al. (1988). "Induction of lyme arthritis in LSH hamsters." Infect Immun **56**(9): 2336-42.

Scrimenti, R. J. (1970). "Erythema chronicum migrans." Arch Dermatol **102**(1): 104-5.

Seidel, M. F., A. B. Domene, et al. (2007). "Differential diagnoses of suspected Lyme borreliosis or post-Lyme-disease syndrome." Eur J Clin Microbiol Infect Dis **26**(9): 611-7.

Shin, J. J., L. J. Glickstein, et al. (2007). "High levels of inflammatory chemokines and cytokines in joint fluid and synovial tissue throughout the course of antibiotic-refractory lyme arthritis." Arthritis Rheum **56**(4): 1325-35.

Shrestha, M., R. L. Grodzicki, et al. (1985). "Diagnosing early Lyme disease." Am J Med **78**(2): 235-40.

Sicklinger, M., R. Wienecke, et al. (2003). "In vitro susceptibility testing of four antibiotics against Borrelia burgdorferi: a comparison of results for the three genospecies Borrelia afzelii, Borrelia garinii, and Borrelia burgdorferi sensu stricto." J Clin Microbiol **41**(4): 1791-3.

Sigal, L. H. (1995). "Early disseminated Lyme disease: cardiac manifestations." Am J Med **98**(4A): 25S-28S; discussion 28S-29S.

Skotarczak, B. (2002). "Canine borreliosis--epidemiology and diagnostics." Ann Agric Environ Med **9**(2): 137-40.

Sonck, C. E. (1965). "Erythema chronicum migrans with multiple lesions." Acta Derm Venereol **45**(1): 34-6.

Sonnesyn, S. W., J. C. Manivel, et al. (1993). "A guinea pig model for Lyme disease." Infect Immun **61**(11): 4777-84.

Sood, S. K., M. B. Salzman, et al. (1997). "Duration of tick attachment as a predictor of the risk of Lyme disease in an area in which Lyme disease is endemic." J Infect Dis **175**(4): 996-9.

Stanek, G., S. O'Connell, et al. (1996). "European Union Concerted Action on Risk Assessment in Lyme Borreliosis: clinical case definitions for Lyme borreliosis." Wien Klin Wochenschr **108**(23): 741-7.

Stanek, G. and F. Strle (2003). "Lyme borreliosis." Lancet **362**(9396): 1639-47.

Stanek, G., G. Wewalka, et al. (1985). "Isolation of spirochetes from the skin of patients with erythema chronicum migrans in Austria." Zentralbl Bakteriol Mikrobiol Hyg [A] **260**(1): 88-90.

Steere, A. C., P. H. Duray, et al. (1988). "Spirochetal antigens and lymphoid cell surface markers in Lyme synovitis. Comparison with rheumatoid synovium and tonsillar lymphoid tissue." Arthritis Rheum **31**(4): 487-95.

Steere, A. C., R. L. Grodzicki, et al. (1983). "The spirochetal etiology of Lyme disease." N Engl J Med **308**(13): 733-40.

Steere, A. C., D. Gross, et al. (2001). "Autoimmune mechanisms in antibiotic treatment-resistant lyme arthritis." J Autoimmun **16**(3): 263-8.

Steere, A. C., G. J. Hutchinson, et al. (1983). "Treatment of the early manifestations of Lyme disease." Ann Intern Med **99**(1): 22-6.

Steere, A. C., R. E. Levin, et al. (1994). "Treatment of Lyme arthritis." Arthritis Rheum **37**(6): 878-88.

Steere, A. C., S. E. Malawista, et al. (1977). "Lyme arthritis: an epidemic of oligoarticular arthritis in children and adults in three connecticut communities." Arthritis Rheum **20**(1): 7-17.

Steere, A. C., R. T. Schoen, et al. (1987). "The clinical evolution of Lyme arthritis." Ann Intern Med **107**(5): 725-31.

Steere, A. C., V. K. Sikand, et al. (1998). "Vaccination against Lyme disease with recombinant Borrelia burgdorferi outer-surface lipoprotein A with adjuvant. Lyme Disease Vaccine Study Group." N Engl J Med **339**(4): 209-15.

Steere, A. C., V. K. Sikand, et al. (2003). "Asymptomatic infection with Borrelia burgdorferi." Clin Infect Dis **37**(4): 528-32.

Straubinger, R. K. (2000). "PCR-Based quantification of Borrelia burgdorferi organisms in canine tissues over a 500-Day postinfection period." J Clin Microbiol **38**(6): 2191-9.

Straubinger, R. K., A. F. Straubinger, et al. (2000). "Status of Borrelia burgdorferi infection after antibiotic treatment and the effects of corticosteroids: An experimental study." J Infect Dis **181**(3): 1069-81.

Straubinger, R. K., B. A. Summers, et al. (1997). "Persistence of Borrelia burgdorferi in experimentally infected dogs after antibiotic treatment." J Clin Microbiol **35**(1): 111-6.

Strle, F., R. B. Nadelman, et al. (1999). "Comparison of culture-confirmed erythema migrans caused by Borrelia burgdorferi sensu stricto in New York State and by Borrelia afzelii in Slovenia." Ann Intern Med **130**(1): 32-6.

Strle, F., J. A. Nelson, et al. (1996). "European Lyme borreliosis: 231 culture-confirmed cases involving patients with erythema migrans." Clin Infect Dis **23**(1): 61-5.

Szczepanski, A., M. B. Furie, et al. (1990). “Interaction between Borrelia burgdorferi and endothelium in vitro.” J Clin Invest **85**(5): 1637-47.

Terekhova, D., R. Iyer, et al. (2006). “Comparative genome hybridization reveals substantial variation among clinical isolates of Borrelia burgdorferi sensu stricto with different pathogenic properties.” J Bacteriol **188**(17): 6124-34.

van der Linde, M. R. (1991). “Lyme carditis: clinical characteristics of 105 cases.” Scand J Infect Dis Suppl **77**: 81-4.

Wang, G., C. Ojaimi, et al. (2002). “Disease severity in a murine model of lyme borreliosis is associated with the genotype of the infecting Borrelia burgdorferi sensu stricto strain.” J Infect Dis **186**(6): 782-91.

Weber, K., G. Schierz, et al. (1984). “European erythema migrans disease and related disorders.” Yale J Biol Med **57**(4): 463-71.

Weis, J. J., B. A. McCracken, et al. (1999). “Identification of quantitative trait loci governing arthritis severity and humoral responses in the murine model of Lyme disease.” J Immunol **162**(2): 948-56.

Weis, J. J., L. Yang, et al. (1997). “Pathological manifestations in murine Lyme disease: association with tissue invasion and spirochete persistence.” Clin Infect Dis **25 Suppl 1**: S18-24.

Viljanen, M. K., J. Oksi, et al. (1992). “Cultivation of Borrelia burgdorferi from the blood and a subcutaneous lesion of a patient with relapsing febrile nodular nonsuppurative panniculitis.” J Infect Dis **165**(3): 596-7.

Viljanen, M. K. and J. Punnonen (1989). “The effect of storage of antigen-coated polystyrene microwells on the detection of antibodies against Borrelia burgdorferi by enzyme immunoassay (EIA).” J Immunol Methods **124**(1): 137-41.

Williams, R. O., M. Feldmann, et al. (1992). “Anti-tumor necrosis factor ameliorates joint disease in murine collagen-induced arthritis.” Proc Natl Acad Sci U S A **89**(20): 9784-8.

Wilske, B. (2005). “Epidemiology and diagnosis of Lyme borreliosis.” Ann Med **37**(8): 568-79.

Wolgemuth, C. W., N. W. Charon, et al. (2006). ”The flagellar cytoskeleton of the spirochetes.” J Mol Microbiol Biotechnol **11**(3-5): 221-7.

Wormser, G. P., R. Ramanathan, et al. (2003). “Duration of antibiotic therapy for early Lyme disease. A randomized, double-blind, placebo-controlled trial.” Ann Intern Med **138**(9): 697-704.

Yang, L., J. H. Weis, et al. (1994). ”Heritable susceptibility to severe Borrelia burgdorferi-induced arthritis is dominant and is associated with persistence of large numbers of spirochetes in tissues.” Infect Immun **62**(2): 492-500.

Yin, Z., J. Braun, et al. (1997). “T cell cytokine pattern in the joints of patients with Lyme arthritis and its regulation by cytokines and anticytokines.” Arthritis Rheum **40**(1): 69-79.