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Clinical, Epidemiological and Immunological Aspects of Lyme Borreliosis

with Special Focus on the Role of the
Complement System

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*Croyez ceux qui cherchent la vérité,
doutez de ceux qui la trouvent;
doutez de tout, mais ne doutez pas
de vous même.*

(André Gide)

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ABSTRACT

Lyme borreliosis (LB) is the most common vector-borne disease in the Northern Hemisphere. The infection is caused by spirochetes belonging to the *Borrelia burgdorferi* sensu lato complex, and it is transmitted to humans by ticks. LB is associated with several clinical manifestations, of which erythema migrans (EM) and neuroborreliosis (NB) are the most common in Europe. The course of the disease is usually benign, but can vary between individuals. The underlying pathogenic mechanisms are not fully understood, but the prognosis is probably determined by a complex interplay between the bacteria and the host's immune response. Previous studies have indicated that a strong initial T helper (Th) 1-response followed by a Th2 response is beneficial for the clinical outcome in LB.

The aims of this thesis were to follow the incidence of NB in Jönköping County, Sweden, over an extended period of time, to search for clinical and laboratory markers associated with the risk of developing long-lasting post-treatment symptoms, and to explore the role of the complement system as well as the relative balance between Th-associated cytokine/chemokine responses in LB.

The number of NB cases, diagnosed by cerebrospinal fluid (CSF) analysis, increased from 5 to 10/100,000 inhabitants/year in Jönköping County during 2000-2005. Post-treatment symptoms persisting more than 6 months occurred in 13 %, and were associated with higher age, longer-lasting symptoms prior to treatment, higher levels of *Borrelia*-specific IgG in CSF, and reported symptoms of radiculitis. Facial palsy, headache and fever were frequent manifestations in children, whereas unspecific muscle and joint pain were the most commonly reported symptoms in older patients.

Complement activation occurred both locally in the skin in EM and in CSF of NB patients. However, no activation could be detected in blood in the NB patients. Elevated levels of C1q, C4 and C3a in CSF, along with correlation between C1q and C3a levels, suggest complement activation via the classical pathway locally in the central nervous system in NB. *In vitro* experiments with two clinical *Borrelia* isolates revealed that *B. garinii* LU59 induced higher complement activation in human plasma compared to *B. afzelii* K78, which recruited more of complement regulator factor H. To elucidate the role of complement in the phagocytosis process, experiments were performed using whole blood from healthy donors incubated with fluorescence-labelled spirochetes and different complement inhibitors. The results illustrated the central role of complement for phagocytosis of *Borrelia* spirochetes.

We also studied the relative contribution of different Th-associated cytokine/chemokine responses in NB. The results support the notion that early NB is dominated by a Th1 response, eventually accompanied by a Th2 response. IL-17A was increased in CSF in half of the patients with confirmed NB, suggesting a hitherto unknown role of Th17 in NB.

In conclusion, in NB the risk of developing long-lasting post-treatment symptoms tend to increase mainly with age and duration of symptoms prior to treatment. The complement system seems to play an important role in host defence by recognizing and killing *Borrelia* spirochetes. However, complement activation in inappropriate sites or to an excessive degree may cause tissue damage, and therefore, the role of complement in relation to the disease course needs to be studied further. Likewise, the role of Th17 in LB pathogenesis and host defence should be further evaluated in prospective studies.

SAMMANFATTNING PÅ SVENSKA

Lyme borrelios (LB) är den vanligaste vektorburna infektionen på norra halvklotet. Infektionen orsakas av spiroketer tillhörande *Borrelia burgdorferi* sensu lato-komplexet, och överförs till människor via fästingar. LB är associerat med flera kliniska manifestationer, av vilka erythema migrans (EM) och neuroborrelios (NB) är vanligast i Europa. Sjukdomsförloppet är i allmänhet godartat, men kan variera mellan individer. De bakomliggande sjukdomsmekanismerna är ofullständigt kända, men prognosen styrs sannolikt av ett komplext samspel mellan bakterien och värdens immunförsvar. Resultat från tidigare studier tyder på att vissa immunceller, T-hjälparceller typ 1 (Th1) är viktiga tidigt i förloppet, medan Th2-celler behövs senare under infektionen för att skapa ett effektivt försvar mot *Borrelia*.

De huvudsakliga syftena med avhandlingsarbetet var att följa incidensen av NB i Jönköpings län över tid, söka efter kliniska och laborativt mätbara markörer associerade med risken att utveckla ett långdraget sjukdomsförlopp, inklusive att studera betydelsen av immunförsvaret vid LB. Speciellt undersöktes betydelsen av komplementsystemet som är en viktig del i det tidiga immunförsvaret, samt betydelsen av olika typer av Th-celler och de signalsubstanser (cytokiner och kemokiner) som de är associerade med.

Antalet NB-fall diagnostiserade med cerebrospinalvätske (CSV)-analys ökade från 5 till 10 av 100 000 invånare/år i Jönköpings län under 2000-2005. Symtom som kvarstod mer än 6 månader efter behandling förekom hos 13 %, och dessa patienter var signifikant äldre, hade haft sina symtom längre innan de fick behandling, hade högre nivåer av *Borrelia*-specifikt IgG i CSV, och rapporterade oftare radikulitsymtom. Facialispares (ansiktsförlamning), huvudvärk och feber var vanliga symtom hos barn, medan specifik värk i muskler och leder var de vanligaste symtomen hos äldre patienter.

Komplementaktivering förekom framförallt lokalt, både i huden vid EM och i CSV vid NB. Däremot noterades ingen systemisk aktivering av komplementsystemet i blodet hos NB-patienterna, vilket visar att *Borrelia*-infektionen lokaliseras och bekämpas i olika organ. Förhöjda nivåer av komplementfaktorerna C1q, C4 och C3a i CSV, tillsammans med att C1q- och C3a-nivåerna korrelerade med varandra, antyder att komplementaktivering sker lokalt i centrala nervsystemet via klassisk väg vid NB. *In vitro*-försök med två kliniska *Borrelia*-isolat visade att *B. garinii* LU59 inducerade mer komplementaktivering i human plasma jämfört med *B. afzelii* K78 som rekryterade mer av det komplementreglerande proteinet faktor H. För att belysa komplementsystemets roll för eliminering av *Borrelia* via en mekanism benämnd fagocytos gjordes experiment med helblod som inkuberades med fluorescensmärkta spiroketer och olika komplementhämmare. Resultaten visar att komplement har en central roll för fagocytos av *Borrelia*-spiroketer.

Vi studerade även den relativa balansen mellan olika Th-celler och deras associerade cytokiner och kemokiner vid NB. Resultaten stödjer uppfattningen att tidig NB domineras av ett Th1-svar som sedermera åtföljs av ett Th2-svar. IL-17A var förhöjt i CSV hos hälften av NB-patienterna, vilket antyder en hittills okänd roll för den nyligen upptäckta celltypen Th17 vid NB.

Sammanfattningsvis förefaller risken att få långdragna symtom trots behandling att öka huvudsakligen med ålder och symtomduration före behandling vid NB. Komplementsystemet verkar spela en viktig roll i värdens immunförsvar för att känna igen och avdöda *Borrelia*-spiroketer. Dock kan komplementaktivering på fel ställe eller på ett ohämmat sätt leda till vävnadsskada, och därför bör komplementaktiveringens betydelse i relation till sjukdomsförloppet studeras ytterligare. På samma vis bör Th17-svarets roll vid LB och i immunförsvaret mot *Borrelia* undersökas i fler prospektiva studier.

LIST OF PAPERS

I. **AJ Henningsson**, BE Malmvall, J Ernerudh, A Matussek, P Forsberg: Neuroborreliosis – an epidemiological, clinical and healthcare cost study from an endemic area in the south-east of Sweden. *Clin Microbiol Infect.* 2010 Aug;16(8):1245-51. Epub 2009 Sep 29.

II. **AJ Henningsson**, J Ernerudh, K Sandholm, SA Carlsson, H Granlund, C Jansson, D Nyman, P Forsberg, K Nilsson Ekdahl: Complement activation in Lyme neuroborreliosis – increased levels of C1q and C3a in cerebrospinal fluid indicate complement activation in the CNS. *J Neuroimmunol.* 2007 Feb;183(1-2):200-7. Epub 2006 Dec 8.

III. K Sandholm*, **AJ Henningsson***, S Säve, M Nordberg, U Garpmo, C Jansson, SA Carlsson, D Nyman, S Bergström, P Forsberg, J Ernerudh, K Nilsson Ekdahl: Early immune responses to *Borrelia garinii* and *Borrelia afzelii* in Lyme borreliosis: Local complement activation in erythema migrans and *in vitro* studies of complement activation, phagocytosis and cytokine profile. *Manuscript.*

*KS and AJH contributed equally.

IV. **AJ Henningsson**, I Tjernberg, BE Malmvall, P Forsberg, J Ernerudh: Indications of Th1 and Th17 responses in cerebrospinal fluid from patients with Lyme neuroborreliosis: a large retrospective study. *J Neuroinflamm.* 2011; 8(1), 36. Epub 2011 Apr 20.

ABBREVIATIONS

ACA	acrodermatitis chronica atrophicans
AI	antibody index
AP	alternative pathway (of complement)
APC	antigen-presenting cell
<i>B.</i>	<i>Borrelia</i>
BBB	blood-brain-barrier
BL	borrelial lymphocytoma
BLC	B-lymphocyte chemoattractant (CXCL13)
BSA	bovine serum albumin
BSK II	Barbour-Stoenner-Kelly II
°C	degrees Celsius
C1INH	C1-inhibitor
C3aR	C3a-receptor
C4BP	C4-binding protein
C5aR	C5a-receptor
C5aRa	C5a-receptor antagonist
Ca ²⁺	calcium
CCL	C-C motif ligand
CXCL	C-X-C motif ligand
CD	cluster of differentiation
CFS	chronic fatigue syndrome
CLM	clinical laboratory of microbiology
CNS	central nervous system
CO ₂	carbon dioxide
CP	classical pathway (of complement)
CR1	complement receptor 1
CRASP	complement regulator-acquiring surface protein
CSF	cerebrospinal fluid
DAF	decay-accelerating factor
DbpA	decorin-binding protein
DC	dendritic cell
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid

ELISA	enzyme-linked immunosorbent assay
ELISPOT	enzyme-linked immunospot
EM	erythema migrans
Erp	OspE/F-related proteins
FHL-1	factor H-like protein-1
FHR	factor H-related protein
FITC	fluorescein isothiocyanate
GM-CSF	granulocyte-macrophage colony-stimulating factor
<i>I.</i>	<i>Ixodes</i>
Ig	immunoglobulin
IFA	immunofluorescence assay
IHC	immunohistochemistry
IL	interleukin
IP-10	IFN- γ inducible protein 10
HRP	horseradish peroxidase
IHC	immunohistochemistry
kbp	kilobase pairs
kDa	kilodalton
LA	Lyme arthritis
LB	Lyme borreliosis
LC	Lyme carditis
LP	lectin pathway (of complement)
LPS	lipopolysaccharide
MAC	membrane attack complex
MASP	MBL-associated serine protease
MBAA	multiple bead array assay
MBL	mannan-binding lectin
MCP	membrane cofactor of proteolysis
MDC	macrophage-derived chemokine
MFI	mean fluorescence intensity
Mg ²⁺	magnesium
MHC	major histocompatibility complex
NB	neuroborreliosis
NK cell	natural killer cell
NLR	NOD-like receptor
NOD	nucleotide binding and oligomerization domain
OD	optical density

Osp	outer surface protein
P	plasma
PAMPs	pathogen-associated molecular patterns
PCR	polymerase chain reaction
PBS	phosphate-buffered saline
PLDS	post-Lyme disease syndrome
PRRs	pathogen recognition receptors
PVDF	polyvinylidene fluoride
S	serum
sC5b-9	soluble C5b-9 (terminal complement complex)
SDS-PAGE	sodium dodecylsulphate-polyacrylamide gel electrophoresis
SPSS	statistical products and service solution
TBE	tick-borne encephalitis
Tc	cytotoxic T cell
TCC	terminal complement complex (C5b-9)
TCR	T cell receptor
Th	T helper cell
TLR	Toll-like receptor
TNF	tumour necrosis factor
TP	terminal pathway (of complement)
Treg	regulatory T cell
VlsE	variable major protein-like sequence, expressed
WB	Western blot
WCS	whole-cell sonicate

INTRODUCTION

Lyme borreliosis (LB) is the most common vector-borne disease in the Northern Hemisphere (Stanek and Strle, 2003; Wormser et al., 2006), showing great variation in prevalence between different regions. The infection is caused by spirochetes belonging to the *Borrelia burgdorferi* sensu lato (s.l.) complex, and transmitted to humans by ticks. Typically, the skin, joints, heart or nervous system can be affected. The symptoms can be classified as localized, disseminated or late persistent, with a disease course that is highly variable between individuals (Kaiser, 1994; Oschmann et al., 1998; Weber, 2001; Berglund et al., 2002; Vrethem et al., 2002). Although various clinical manifestations of LB have been described in the medical literature since the late 17th century as reviewed by Berglund (Berglund, 2004), it was not until 1982 that *B. burgdorferi* s.l. was identified as the causative agent of LB (Burgdorfer et al., 1982). The discovery of the pathogen had been preceded by the investigation of an outbreak of epidemic arthritis among citizens, mainly children, in the area of Lyme in Connecticut, USA in 1975; hence the name Lyme borreliosis (Steere et al., 1977). Since then, much research has been done in the field and an important insight into the genetics, physiology, pathogenesis and ecology of this bacterium and its tick/mammal life cycle has been gained. However, many questions still remain to be answered. The pathogenic mechanisms are not fully understood. Why is the course of the infection so variable between individuals? How can the disease be prevented? Better diagnostic laboratory methods are also needed. This thesis deals with the local epidemiology and clinical course of neuroborreliosis (NB) in Jönköping County, Sweden, and aspects of the immune response triggered in humans towards *B. burgdorferi* s.l. with special emphasis on the role of the complement system.

Borrelia burgdorferi sensu lato

The spirochete causing LB has been identified as a distinct species of the genus *Borrelia* (Johnson et al., 1984). Genetic analysis of the *Borrelia* strains associated with clinical LB in humans has resulted in the delineation of three different genospecies; *i.e.* *B. burgdorferi* sensu stricto (s.s.), *B. garinii* and *B. afzelii* (Baranton et al., 1992; Canica et al., 1993). It has become evident that the *Borrelia* spirochetes isolated from LB patients and ticks, as well as the clinical presentation of LB, differ between Europe and North America (Stanek et al., 1985; Wilske et al., 1985). While LB in Europe is caused by a greater variety of genospecies (*B. burgdorferi* s.s., *B. garinii* and *B. afzelii*), and occasionally other species as mentioned below, *B. burgdorferi* s.s. is the only pathogenic genospecies found in the USA so far (Stanek and Strle, 2003; Stanek

and Strle, 2008; Stanek and Reiter, 2011). Gradually, new genospecies have been identified in Europe, North America and Asia, and up to this point 18 genospecies have been recognized within the *B. burgdorferi* s.l. complex (Stanek and Reiter, 2011). Some of them (e.g. *B. spielmanii*, *B. bissettii*, *B. valaisiana*) have occasionally been found to cause human disease, but only three (*B. burgdorferi* s.s., *B. garinii* and *B. afzelii*) are widely accepted as human pathogens (Fingerle et al., 2008; Rudenko et al., 2008; Strle and Stanek, 2009; Stanek and Reiter, 2011). The clinical role of the more recently discovered genospecies remains to be further evaluated. A newly published study of ticks detached from humans in Sweden revealed the relative distribution of genospecies here, as presented in Table 1 (Wilhelmsson et al., 2010).

Table 1. Prevalence of *Borrelia* (*B.*) species in ticks detached from humans in Sweden, (Wilhelmsson et al., 2010).

<i>Borrelia</i> species	Prevalence (%)
<i>B. afzelii</i>	53
<i>B. garinii</i>	20
<i>B. valaisiana</i>	11
<i>B. burgdorferi</i> s.s.	1.3
<i>B. lusitaniae</i>	1.3
<i>B. miyamotoi</i> -like	1.3
Untypeable	12
Total prevalence	19 (75/399)

Various studies have suggested that each of the three pathogenic species exerts a certain organotropism and is responsible for a predominant clinical form of LB. Although all three genospecies can cause the entire spectrum of the clinical LB manifestations, there is evidence for *B. afzelii* being more often associated with skin manifestations, *B. garinii* with neurological symptoms, and *B. burgdorferi* s.s. preferentially associated with arthritis (van Dam et al., 1993; Balmelli and Piffaretti, 1995).

The *Borrelia* spirochetes are flat-wave shaped bacteria that measure 10-30 µm in length and 0.2-0.5 µm in width (Figure 1a-b) (Burgdorfer et al., 1982; Hovind-Hougen, 1984; Rosa, 1997). Their cell wall consists of two lipid bilayers that somewhat resemble, yet are distinctly different from those of Gram-negative bacteria. The inner membrane surrounds the protoplasmic cylinder containing a linear chromosome of about 900 kbp as well as multiple linear and circular plasmids (Fraser et al., 1997; Casjens et al., 2000). Genome sequences have been determined for *B. burgdorferi* s.s., *B. garinii* and *B. afzelii* (Fraser et al., 1997; Casjens et al., 2000; Samuels and Radolf, 2010). In the periplasmic space 7-11 flagella are attached to the inner membrane and twisted around the protoplasmic cylinder (Burgdorfer et al.,

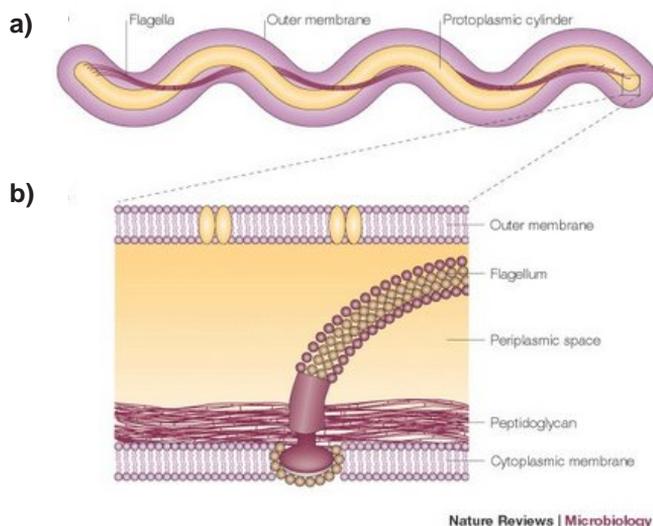


Figure 1. Schematic illustration of *Borrelia burgdorferi sensu lato* (a) and of its cell wall (b).

Adapted by permission from Macmillan Publishers Ltd: Nat Rev Microbiol (Rosa PA et al.), copyright 2005.

1982). The main structural component of the flagella is a 41-kDa protein called flagellin (Ge et al., 1998). The flagella give the spirochete rapid bidirectional motility and the ability to penetrate various tissues, and constitute an important virulence factor (Rosa, 1997; Sal et al., 2008).

The most important features that distinguish the cell wall of the *Borrelia* spirochetes from that of Gram-negative bacteria are the absence of phosphatidylethanolamine (Belisle et al., 1994) and lipopolysaccharide (LPS) (Takayama et al., 1987), and the presence of non-LPS glycolipid antigens (Belisle et al., 1994). The outer membrane contains an abundance of lipoproteins, including certain outer surface proteins (Osp) A-F, and variable major protein-like sequence, expressed (VlsE) (Rosa, 1997; Eicken et al., 2002). OspE, OspF and outer surface E/F-like leader peptide constitute what is referred to as OspE/F-related proteins (Erp) (Stevenson et al., 1996). An additional important virulence factor is another group of surface proteins called complement regulator-acquiring surface proteins (CRASP). CRASP and also Erp bind human complement regulators in order to protect the *Borrelia* spirochete from the effects of complement activation.

Ecology

Borrelia spirochetes are transmitted to humans by hard ticks; in Europe by *Ixodes ricinus* and in North America by *I. scapularis* and *I. pacificus* (Piesman and Gern, 2004; Gern, 2008). The preferential habitats for *I. ricinus* and *I. scapularis* are grassy woodlands, forests and pastures with high relative humidity, while *I. pacificus* favour shrub, desert and coniferous forest areas (Parola and Raoult, 2001).

The ticks have four life stages: egg, larva, nymph and adult, and a blood meal is required for the development from one stage to the next in the life cycle (Figure 2) (Parola and Raoult, 2001). It usually takes 2-3 years for the ticks to complete their life cycle. During winter the ticks enter a resting state, diapause, characterized by reduced metabolism. Ticks feed on their hosts for several days. Their mouthparts are specially adapted for firm attachment to the skin of the host, and their salivary secretion is a complex composition of vasodilator, anti-inflammatory, anti-hemostatic and anaesthetic substances (Parola and Raoult, 2001). Of interest for the context of this thesis, is that tick saliva contains several complement inhibitor proteins (Valenzuela et al., 2000; Schroeder et al., 2007; Schuijt et al., 2008; Gillet et al., 2009). These attributes facilitate the prolonged feeding time, since tick bites are generally painless and often go unnoticed for lengthy periods of time.

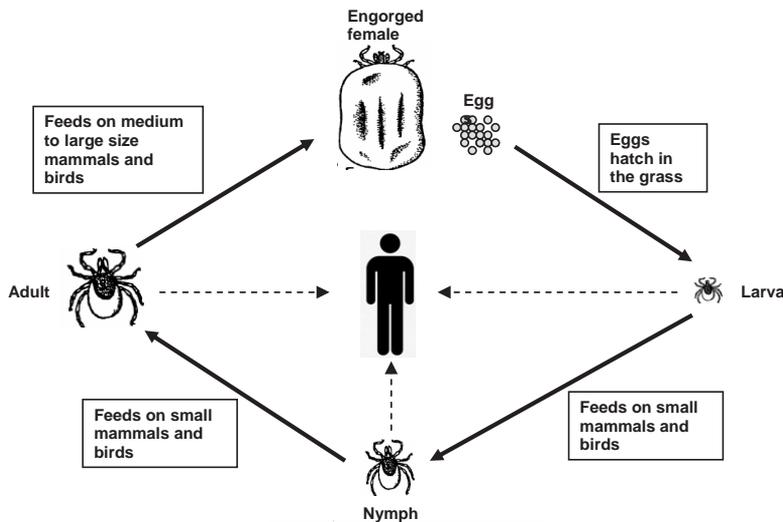


Figure 2. Life cycle of *Ixodes ricinus*. A blood meal is required to develop from one stage into the next, and for the adult female to mate and lay eggs. It normally takes 2-3 years to complete a life cycle. Humans are incidental hosts for the ticks.

The ticks are infected by *Borrelia* spirochetes when feeding on bacteremic reservoir hosts, *i.e.* smaller mammals and birds (Parola and Raoult, 2001). The transmission time of the spirochetes from ticks to humans has been estimated as 24-48 hours, since the spirochetes that reside in the tick mid-gut need to migrate to the salivary glands (Piesman et al., 1987a). Thus, the risk of disease transmission increases with the attachment time (Piesman et al., 1987b).

Epidemiology

LB is the most common vector-borne disease in North America and in Europe (Stanek and Strle, 2003; Wormser et al., 2006). In southern Sweden, the overall annual incidence of LB was found to be 69 cases per 100,000 inhabitants in 1992-93 with marked regional variability (Berglund et al., 1995). Most patients present from May through October, but cases are diagnosed all year round. The highest incidence was found in children 5-9 years of age, and in adults aged 60-74 years (Berglund et al., 1995). No significant difference in incidence between men and women was found, but re-infections have been reported to occur more frequently among women (Bennet and Berglund, 2002; Jarefors et al., 2006).

LB incidence in humans is determined by tick population distribution and density, the prevalence of human pathogenic *Borrelia* spirochetes in the ticks, as well as the nature and extent of human activity in tick areas (Mejlon and Jaenson, 1993; Vassalo et al., 2000; Hubalek, 2009). The distributional area of *I. ricinus* appears to be increasing in Sweden (Jaenson et al., 2009), along with the density of the tick populations. A combination of mild winters and extended spring and autumn seasons in recent years is thought to be one explanation for this increase (Lindgren et al., 2000; Randolph, 2004). The overall prevalence of *B. burgdorferi* s.l. in field-collected *I. ricinus* in Sweden 1988-91 was reported to be 10 % in nymphs and 15 % in adults, and the distribution of *Borrelia* spirochetes coincided with that of the ticks (Gustafson et al., 1995). The overall *Borrelia* prevalence in ticks that have bitten humans in the south-east of Sweden has recently been reported to be 19 % (Table 1) (Wilhelmsson et al., 2010). Even though humans are frequently exposed to ticks infected with *B. burgdorferi* s.l., the risk of contracting LB is low (Robertson et al., 2000b; Stjernberg and Berglund, 2002; Jacobs et al., 2008; Fryland et al., 2011).

Clinical disease

The clinical manifestations and their relative frequency differ between Europe and North America, depending on the geographical distribution and organotropism of various *B. burgdorferi* s.l. genospecies as described earlier (van Dam et al., 1993; Balmelli and Piffaretti, 1995; Piesman and Gern, 2004). Consequently, borreliar arthritis and carditis are more frequent in the USA, while neurologic and late skin manifestations are more common in Europe (Weber, 2001; Steere, 2006). The skin manifestation erythema migrans (EM) displays a faster expansion in the skin and is more frequently associated with systemic symptoms when caused by *B. burgdorferi* s.s. (USA). The relative frequency of the various LB manifestations in Sweden is presented in Table 2. The most common anatomical site of tick bites in children is the head and neck region, while adults are most commonly bitten on the lower extremities (Berglund et al., 1995). This may be related to the fact that children more often than adults present with lymphocytoma (which commonly affects an ear lobe or a nipple) and NB (Berglund et al., 1995; Huppertz et al., 1999).

Table 2. The relative frequency of Lyme borreliosis manifestations in Sweden, (Berglund et al, 1995). Patients can have more than one manifestation.

Manifestation	Frequency (%)
Erythema migrans	77
Neuroborreliosis	16
Arthritis	7
Acrodermatitis	3
Lymphocytoma	3
Carditis	<1

Traditionally, LB has been divided into different stages (Table 3)(Steere, 1989; Wormser et al., 2006). However, subdivision is becoming increasingly rare in the scientific literature, since it has become evident that most patients do not exhibit all stages or develop symptoms in a chronological order, and overlap between stages is not uncommon (Evans, 2000).

Borrelia spirochetes are inoculated into the skin during tick feeding. The spirochetes can then both spread locally within the skin and disseminate hematogenically to various tissues of the body (Oksi et al., 2001; Wormser, 2006b; Norman et al., 2008; Wormser et al., 2008).

Table 3. Stages of Lyme borreliosis.

Stage I Early localized	Stage II Early disseminated	Stage III Late persistent
Erythema migrans	Neuroborreliosis	Late neuroborreliosis
Borrelial lymphocytoma	Lyme arthritis	Chronic Lyme arthritis
	Lyme carditis	Acrodermatitis chronica atrophicans

Dermatoborreliosis

EM is the most frequent clinical sign of LB and occurs on both sides of the Atlantic (Stanek and Strle, 2003). It appears as a small maculopapular rash at the site of the tick bite several days to weeks after the bite. As the lesion slowly enlarges, it can be associated with local symptoms such as itching, burning or pain, or with systemic symptoms such as fatigue, headache and migrating arthralgia (Strle et al., 1996). Serology is positive in less than 50 % of patients with single EM (Strle et al., 1996), and the diagnosis is based on the clinical appearance. Typically, the EM adopts an annular shape with a central clearing, but can also be more homogenous, especially in women (Bennet et al., 2006) and when *B. garinii* is the causative genospecies (Carlsson et al., 2003; Bennet et al., 2006) (Figure 3a). Multiple EM lesions are an indication of systemic dissemination (Stanek and Strle, 2003).

Borrelial lymphocytoma (BL) is a solitary bluish-red swelling that appears in the vicinity of a tick bite after weeks to months (Stanek and Strle, 2003). BL is typically located at the ear lobe or the nipple, and is more common in children than in adults (Strle et al., 1992). The diagnosis can be supported by serology or histological examination (Figure 3b).

Acrodermatitis chronica atrophicans (ACA) is a late skin manifestation of LB that develops slowly over months to years. ACA has mainly been associated with *B. afzelii* infection (Ohlenbusch et al., 1996). The onset is subtle; a slight bluish-red discoloration and oedema that is most often located on the extensor sites of the hands, feet, elbows or knees (Stanek and Strle, 2003) (Figure 3c). Polyneuropathy, arthralgia and fatigue are some of the symptoms that may be associated with ACA. Unlike EM and BL, ACA does not resolve without antibiotic treatment (Asbrink and Hovmark, 1988). Gradually, the oedema vanishes and skin atrophy becomes more prominent. The condition is easily misinterpreted as a sign of vascular insufficiency. ACA is more often diagnosed in women than in men, and the patients are usually >40 years of age. Practically all patients with ACA are seropositive (Asbrink and Hovmark, 1988).

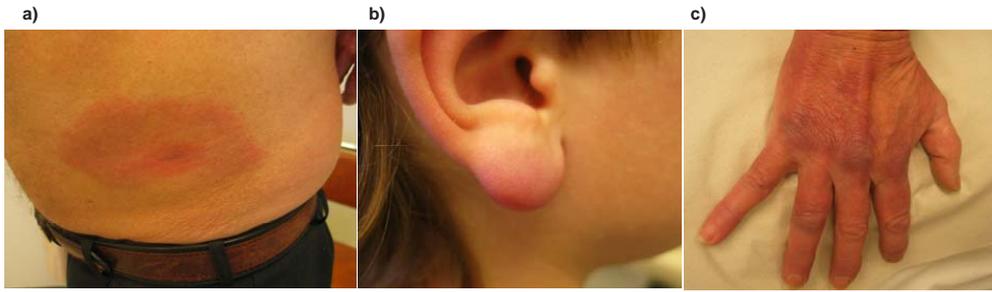


Figure 3. Skin manifestations of Lyme borreliosis. a) Erythema migrans. b) Borrelial lymphocytoma. c) Acrodermatitis chronica atrophicans. (Courtesy of the Department of dermatology, Ryhov County Hospital, Jönköping.)

Neuroborreliosis

Neurologic symptoms usually occur 1-12 (mostly 4-6) weeks after the tick bite (Mygland et al., 2010). NB is typically characterized by aseptic meningitis and involvement of cranial and peripheral nerves (Pachner and Steere, 1984). The most prominent clinical symptom is severe pain due to radiculoneuritis that is often located in the thoracic or abdominal regions or in a leg (Dotevall et al., 2003). The pain is neuralgic in its character and typically exacerbates at night (Stanek and Strle, 2003; Mygland et al., 2010). Motor nerves can be affected with paresis that is usually non-symmetrical (Kristoferitsch, 1991). Any cranial nerve can be involved, but the facial nerve is by far the most frequently affected, resulting in unilateral or bilateral peripheral facial palsy. Borrelial meningitis usually causes relatively mild or intermittent headache, and meningeal signs are only moderately expressed or absent. However, in some patients the head and neck pain can be more intense (Kristoferitsch, 1991). Other manifestations involving the central nervous system (CNS), such as myelitis and encephalitis, are rare. Sporadic cases of NB patients presenting with confusion, cerebellar ataxia, hemiparesis, acute stroke-like symptoms and cerebral vasculitis have been reported (Topakian et al., 2008; Mygland et al., 2010). Fever is usually absent. NB is typically an acute illness, with 95 % of cases classified as early NB (duration of neurologic symptoms <6 months at diagnosis). Less than 5 % present with a symptom duration exceeding 6 months (classified as late NB) (Mygland et al., 2010).

The diagnosis is based on the medical history, clinical signs and symptoms along with simultaneous laboratory analysis of serum (S) and cerebrospinal fluid (CSF) (Mygland et al., 2010). The inflammatory parameters in serum are usually normal, but may be slightly elevated. The CSF shows mononuclear pleocytosis up to several hundred cells $\times 10^6/L$. The CSF-albumin and CSF/S-albumin ratio are elevated in a substantial number of patients, but CSF-glucose is mostly normal. In cases with short

duration of symptoms, pleocytosis may be absent (Blanc et al., 2007) and intrathecal antibodies undetectable (Stanek and Strle, 2003; Mygland et al., 2010). Virtually all patients have detectable intrathecal *Borrelia*-specific antibody production 6-8 weeks after the onset of symptoms (Hansen and Lebech, 1991).

Arthritis

Lyme arthritis (LA) is the most common clinical sign of disseminated LB in the USA, but appears to be much less common in Europe (Berglund et al., 1995; Stanek and Strle, 2003). Onset of LA usually occurs 2 weeks to 6 months after the tick bite (Steere et al., 1977). The condition is characterized by acute monoarticular or oligoarticular inflammation of large joints, in most cases the knee. Sometimes the elbow, ankle, shoulder or hip may be affected. The joints become swollen and warm but are generally only mildly painful and not erythematous (Szer et al., 1991). Joint inflammation is usually intermittent and lasts for a few days to several weeks, or sometimes several months. The clinical course of LA is very variable, usually recurrent, and may continue for several years (Stanek and Strle, 2003; Wormser et al., 2006).

The erythrocyte sedimentation rate may be moderately raised, but the concentration of C-reactive protein is usually within the normal range (Szer et al., 1991; Stanek and Strle, 2003). White blood cell counts in synovial fluid range from 0.5 to $110 \times 10^9/L$ with a dominance of polymorphonuclear leukocytes (Nocton et al., 1994). Diagnosis of LA is based on the medical history, clinical features and serology. Detection of *Borrelia* deoxyribonucleic acid (DNA) in synovial tissue or synovial fluid by polymerase chain reaction (PCR) is a complementary method in ambiguous cases (Nocton et al., 1994; Stanek and Strle, 2003).

Rare manifestations

Lyme carditis (LC) has a reported relative frequency of 0.5 % in European LB patients (Berglund et al., 1995; Strle and Stanek, 2009), and 4-10 % in North American patients (Wormser, 2006b; Strle and Stanek, 2009). LC most often occurs within two months after the tick bite, and may be associated with EM or NB. Typically, LC presents with acute onset of changing atrioventricular blocks I-III as a result of conduction disturbances. Diagnosis is based on clinical signs and symptoms together with serology (Steere, 2001).

Eye manifestations are very rare and are either a result of inflammation in various eye tissues (conjunctivitis, keratitis, iridocyclitis, retinal vasculitis, choroiditis, optic neuritis), or of extraocular involvement (paresis of the cranial nerves, orbital myositis) (Mikkila et al., 2000).

Sporadic case reports of patients with, for example, myositis, osteomyelitis, fasciitis, scleroderma and symptoms from other organs such as the liver, urinary tract or respiratory tract have been interpreted as forms of LB, although the associations still remain to be firmly established (Stanek and Strle, 2003).

Asymptomatic seroconversion

Epidemiologic studies in areas with endemic LB have revealed that antibody reactivity to *B. burgdorferi* s.l. is not uncommon in healthy subjects that do not remember having any signs or symptoms of LB infection (Steere et al., 1986; Fahrler et al., 1991; Ekerfelt et al., 2001; Fryland et al., 2011). Also, these individuals display *Borrelia*-specific T-cell reactivity (Ekerfelt et al., 1999; Ekerfelt et al., 2001) and *Borrelia*-specific DNA has been detected in their urine (Karch et al., 1994). However, asymptomatic seroconversion seems to be less common in the USA than in Europe (Steere et al., 2003). The underlying mechanisms determining whether an individual develops asymptomatic seroconversion or clinical disease are mostly unknown. Variable invasive capacity of different *Borrelia* strains has been proposed as an explanation (Wormser et al., 2001), as well as interindividual differences in the immune response against *B. burgdorferi* s.l. (Sjowall et al., 2005; Jarefors et al., 2007).

Laboratory diagnosis

Direct detection methods

Microscopic detection:

Individual spirochetes may be visualized in various tissues after Giemsa, carbolfuchsin and silver staining (Aberer and Duray, 1991). However, immunological staining methods are more sensitive. *B. burgdorferi* s.l. may be detected either by direct immunofluorescence using, for example, fluorescein isothiocyanate (FITC)-labelled anti-*Borrelia*-antibodies, or by indirect immunofluorescence using a primary unlabelled anti-*Borrelia* antibody and a secondary FITC-labelled antibody. However, the diagnostic value of microscope-based assays in the clinical laboratory is limited since the spirochete density in clinical samples is generally very low. Microscopic detection is mainly suitable for specimens with a large number of spirochetes, such as suspensions of midgut tissue or salivary glands from infected ticks or spirochetes grown in culture media (Samuels and Radolf, 2010).

Antigen detection:

Detection of *B. burgdorferi* s.l. antigens with enzyme-linked immunosorbent assay (ELISA) has been used in CSF and in urine samples from patients with NB and EM, respectively (Dorward et al., 1991; Coyle et al., 1995). The limitations of antigen detection are the relatively low sensitivity, specificity and poor reproducibility (Klempner et al., 2001b), and therefore these assays are rarely used in clinical practice.

Culture:

B. burgdorferi s.l. is a slow-growing, fastidious bacteria that requires a special medium for laboratory culture since it is unable to synthesize amino acids, nucleotides, fatty acids, or most other cellular building blocks (Samuels and Radolf, 2010). The most frequently used culture medium, Barbour-Stoenner-Kelly II (BSK II), contains certain key components, particularly bovine serum albumin (BSA) and rabbit serum, the quality of which is critical for the growth-promoting capability (Barbour, 1984). Cultures are usually incubated at 30-34 °C under microaerophilic or anaerobic conditions. The generation time for *B. burgdorferi* s.l. during log phase growth is 7-20 hours and an incubation time of up to 12 weeks is usually needed before the culture is considered to be negative (Barbour, 1984; Aguero-Rosenfeld et al., 2005). *B. burgdorferi* s.l. can be recovered from various tissues and body fluids, including biopsy specimens of EM, BL, ACA, synovial tissue and cardiac tissue, as well as CSF, synovial fluid and blood samples (Aguero-Rosenfeld et al., 2005). However, the sensitivity of culture from clinical specimens has been reported to be low. The highest representative yield has been from EM biopsies from untreated patients (about 40 % in European studies) (Aguero-Rosenfeld et al., 2005). As a result of the low sensitivity and the cumbersome and time-consuming procedure, culture is not suitable for routine clinical diagnosis of LB.

Nucleic acid amplification:

The most sensitive direct detection method is amplification of *B. burgdorferi* s.l.-specific DNA sequences using PCR. Commonly used targets for amplification are the plasmid-borne *ospA* gene and the chromosomal gene for 16S rRNA (Schmidt, 1997; Aguero-Rosenfeld et al., 2005). The overall diagnostic sensitivity of PCR in clinical practice is comparable to that of culture (Schmidt, 1997; Wilske et al., 2007), with the exception of synovial fluid and synovial tissue where PCR has significantly higher sensitivity (~55-90 %) than culture methods (Nocton et al., 1994; Jaulhac et al., 1996). Detection by PCR amplification has so far not been widely applied in routine clinical diagnosis due to low sensitivity in blood and CSF, but may be a helpful complementary method to serology in investigation of LA. The low yield in blood (median 14 % from European studies) and CSF (median 38 %) could be a reflection of lack of spirochetemia or transient spirochetemia, very low numbers of spirochetes in blood and CSF, or the presence of PCR inhibitors in host blood and CSF (Aguero-Rosenfeld et al., 2005).

Indirect detection methods

Immunofluorescent antibody assay (IFA):

An early method of detection of *Borrelia*-specific antibodies in patient serum was IFA. Cultured *Borrelia* spirochetes were fixed onto glass slides and incubated with serum. After the addition of FITC-labelled anti-human immunoglobulin (Ig) G or IgM, the presence of antibodies bound to the spirochetes was detected by fluorescence microscopy (Aguero-Rosenfeld et al., 2005). The major limitations of this method are its cumbersome nature and the subjectivity involved in reading and interpreting the results. Hence IFA is not conducive to routine use in clinical laboratories, and has largely been replaced by ELISA and Western blot (WB) (Samuels and Radolf, 2010).

Enzyme-linked immunosorbent assay (ELISA):

The principles of ELISA methods are presented in the Materials and Methods section. The first commercially available ELISAs for detection of anti-*Borrelia*-antibodies used whole-cell sonicates (WCS) as test antigens (Aguero-Rosenfeld et al., 2005). The advantages of ELISA over IFA are that it allows the objective determination of antibodies using a numeric value (optical density, OD), and it has the potential for automation and large-scale testing (Samuels and Radolf, 2010). The main disadvantage of WCS-based ELISAs is that they include a number of antigens that are not unique to *B. burgdorferi* s.l. but cross-react with antigens in other bacteria. This is particularly pronounced when testing for the presence of IgM antibodies in serum samples. The second generation of ELISAs are based on purified native antigens, such as flagellin, to improve specificity. Third generation ELISAs may use recombinant antigens and synthetic peptides, such as the C6 peptide, in combination or as single-antigen tests (Aguero-Rosenfeld et al., 2005; Skogman et al., 2008a; Tjernberg, 2011). Currently, a plethora of different ELISAs are commercially available and applied in clinical practice, and this lack of standardization may cause difficulties when comparing diagnostic criteria and studies conducted at different centres (Ekerfelt et al., 2004).

Western blot (WB):

In order to improve the specificity of serological testing for LB, international recommendations are to use a two-step modality (Centers for Disease Control and Prevention, 1995). Samples are first tested using a sensitive method such as ELISA or IFA. Samples testing negative by these methods should be reported as negative, whereas samples with equivocal or positive results should be tested by separate IgG and IgM WB. The principles of WB are presented in the Materials and Methods section. However, there are several limitations to WB, such as the lack of standardization, the variations in preparation of the antigen source, and subjective interpretation of band intensity (Aguero-Rosenfeld et al., 2005). Criteria for WB interpretation have been established in the USA (Centers for Disease Control and

Prevention, 1995), but WB interpretation is more complicated in Europe, due to the diversity of *B. burgdorferi* s.l. genospecies, and consensus on criteria has not been reached (Hauser et al., 1997; Robertson et al., 2000a). This two-step approach is currently being re-evaluated and may in the future be superseded by the new ELISAs based on recombinant and synthetic antigens (ICLB, 2010; Samuels and Radolf, 2010; Tjernberg, 2011).

Enzyme-linked immunospot (ELISPOT):

The ELISPOT assay, which is based on an ELISA technique, is a highly sensitive method for visualization of *Borrelia*-specific cytokine secretion on a single cell level (Czerkinsky et al., 1988; Forsberg et al., 1995; Ekerfelt et al., 1997). Nitrocellulose-bottomed microtitre plates are coated with monoclonal anti-human antibodies directed against the cytokine to be studied. Peripheral blood mononuclear cells are separated, suspended in culture medium and added to the wells of the plate. The cells are then stimulated with *Borrelia* antigens and incubated for a specified period of time. Cytokines (or other cell products that may be of interest) secreted by activated cells are captured locally by the coating antibody. Cytokine secretion is then visualized by biotinylated antibodies in combination with, for example, streptavidin-horseradish peroxidase (HRP) and a precipitating substrate. Each spot that develops in the assay represents a single cytokine-secreting cell. The number of spots is counted either manually or by using an automated reader and computer software. This method is currently used exclusively for research purposes and not as a routine diagnostic test.

Detection of specific biomarkers:

The B-cell attractant chemokine C-X-C motif ligand 13 (CXCL13, previously called B lymphocyte chemoattractant, BLC) has in recent years aroused great interest as a sensitive and specific diagnostic biomarker in CSF in early NB (Rupprecht et al., 2005; Ljostad and Mygland, 2008; Senel et al., 2010; Tjernberg et al., 2011). CXCL13 appears to be a reliable marker of active NB infection and treatment response, since CSF levels decrease rapidly after initiation of antibiotic therapy (Schmidt et al., 2011). CXCL13 can be measured by ELISA or multiple bead array assays (described in the Materials and Methods section), but is not yet widely applied for routine diagnosis of NB.

Therapy

Therapy recommendations vary between different countries and parts of the world (Wormser et al., 2006; EUCALB, 2009). The current Swedish recommendations are presented in Table 4. Phenoxymethylpenicillin (PcV), amoxicillin, doxycycline and cefuroxime axetil for 10 days have been shown to be equally effective in the treatment of EM in adults (Nadelman et al., 1992; Bennet et al., 2003; Wormser et al., 2003; Wormser, 2006a). However, studies on azithromycin have been more ambiguous (Massarotti et al., 1992; Luft et al., 1996), and therefore this drug is only recommended in treatment of patients allergic to penicillin. Doxycycline, penicillin G (PcG), cefotaxime and ceftriaxone have been shown to be effective for treatment of NB with peripheral nerve involvement in children and in adults (Dotevall and Hagberg, 1999; Halperin et al., 2007; Ljostad et al., 2008). However, for NB patients with CNS symptoms or with treatment failure on oral doxycycline, intravenously administered ceftriaxone is recommended by the European Federation of Neurologic Societies (Mygland et al., 2010). The Swedish recommendations are antibiotic therapy for 10-21 days, depending on the manifestation of LB. Prolongation of the antibiotic treatment has not proven to be warranted (Oksi et al., 2007), but can instead cause serious adverse events (Ettestad et al., 1995; Patel et al., 2000; Krupp et al., 2003; Wormser et al., 2006).

In vitro susceptibility studies of *B. burgdorferi* s.l. have always been limited by the technical drawbacks of culture and a lack of standardized methodology, for example variations in incubation periods, density of the inoculum, and the criteria for correct determination of antibiotic-induced killing and growth inhibition *in vitro* (Hunfeld and Brade, 2006). To date, however, there is no scientific evidence for acquired anti-microbial resistance against drugs that are commonly used for treatment of *B. burgdorferi* s.l. (Hunfeld and Brade, 2006).

Table 4. Swedish treatment recommendations for Lyme borreliosis (Swedish Medical Products Agency, 2009).

Single EM	Adults: Pregnancy: Pc allergy: Children: Pc allergy:	PcV PcV Doxycycline or Azithromycin PcV Azithromycin	1 g x 3 for 10 days 2 g x 3 for 10 days 100 mg x 2 for 10 days* 500 mg x 1 day 1 ^a and 250 mg x 1 day 2-5 ^a 25 mg/kg x 3 for 10 days 10 mg/kg x 1 day 1 and 5 mg/kg x 1 day 2-5
EM + fever Multiple EM	Adults: Pregnancy: Children: < 8 y: Pc allergy, < 8 y:	Doxycycline Ceftriaxone Doxycycline Amoxicillin Azithromycin	100 mg x 2 for 10 days 2 g x 1 IV for 10 days 4 mg/kg x 1 for 14 days 15 mg/kg x 3 for 14 days 10 mg/kg x 1 day 1 and 5 mg/kg x 1 day 2-5
BL	Adults: Pregnancy: Pc allergy: Children: < 8 y: Pc allergy, < 8 y:	PcV PcV Doxycycline or Azithromycin Doxycycline Amoxicillin Azithromycin	1 g x 3 for 14 days 2 g x 3 for 14 days 100 mg x 2 for 14 days* 500 mg x 1 day 1 ^a and 250 mg x 1 day 2-5 ^a 4 mg/kg x 1 for 14 days 15 mg/kg x 3 for 14 days 10 mg/kg x 1 day 1 and 5 mg/kg x 1 day 2-5
ACA	Adults:	Doxycycline or PcV	100 mg x 2 for 21 days* 2 g x 3 for 21 days
NB	Adults: Pregnancy: Children: < 8 y:	Doxycycline or Ceftriaxone Ceftriaxone Doxycycline Ceftriaxone	200 mg x 1 for 14 days or 200 mg x 2 for 10 days 2 g x 1 IV for 14 days 2 g x 1 IV for 14 days 4 mg/kg x 1 for 10 days 50-100 mg/kg x 1 for 10 days
LA	Adults: Pregnancy: Children: < 8 y:	Doxycycline or Ceftriaxone Ceftriaxone Doxycycline Amoxicillin	100 mg x 2 for 14 days 2 g x 1 IV for 14 days 2 g x 1 IV for 14 days 4 mg/kg x 1 for 21 days 15 mg/kg x 3 for 21 days
LC	Adults: Pregnancy:	Doxycycline or Ceftriaxone Ceftriaxone	100 mg x 2 for 14 days 2 g x 1 IV for 14 days 2 g x 1 IV for 14 days

EM: erythema migrans.

BL: borreliolymphocytoma.

ACA: acrodermatitis chronica atrophicans.

NB: neuroborreliosis.

LA: Lyme arthritis.

LC: Lyme carditis.

y: years.

Pc: penicillin.

PcV: phenoxymethylpenicillin.

IV: intravenously.

* not in 2nd or 3rd trimester of pregnancy.^a not in 1st trimester of pregnancy.

Prognosis

Antibiotics are effective in all manifestations of LB and prognosis is usually excellent (Steere, 2001; Nowakowski et al., 2003). *Borrelia*-specific antibodies are generally detectable for an extended period of time after the infection, and do not mirror active infection (Kalish et al., 2001). Therefore, careful interpretation of *Borrelia* serology is crucial in endemic areas since re-infections are common (Berglund et al., 1996; Nowakowski et al., 1997; Bennet and Berglund, 2002; Nowakowski et al., 2003). However, a smaller proportion of patients with LA may develop antibiotic-refractory arthritis (Steere and Angelis, 2006; Tory et al., 2010), and require additional treatment with non-steroidal anti-inflammatory drugs, intra-articular steroids or disease-modifying antirheumatic drugs (Dressler et al., 2004). This condition is more commonly seen in North America where LA is more frequent than in Europe. The pathogenesis of refractory LA is not clear, but several hypotheses exist; a) persistent infection of the pathogen, b) retained spirochetal antigens but no living bacteria present, c) pathogen-induced autoimmunity due to molecular mimicry, d) dysregulation of the inflammatory response, or e) predefined rheumatological autoimmunity unmasked by borrelial infection (Girschick et al., 2009).

In the case of NB, the time to complete recovery after initiation of treatment varies greatly between individuals (Oschmann et al., 1998; Weber, 2001; Berglund et al., 2002; Oksi et al., 2007). Most NB patients improve rapidly with antibiotic therapy (Halperin et al., 2007; Ljostad et al., 2008), but the clinical outcome cannot be reliably evaluated at the completion of the antibiotic treatment, but rather 6-12 months afterwards (Oksi et al., 2007).

Various post-treatment symptoms have been reported in LB, such as fatigue, cognitive impairment, headache, arthralgia, myalgia and memory difficulties (Cimperman et al., 1999; Karkkonen et al., 2001; Berglund et al., 2002; Vrethem et al., 2002; Picha et al., 2006; Ljostad and Mygland, 2010; Eikeland et al., 2011). Persistent symptoms with objective sequelae (mainly consisting of neurologic deficits after NB) do occur but are not as common as subjective complaints (Wormser et al., 2006; Marques, 2008). Post-Lyme disease syndrome (PLDS) is characterized by continuous or relapsing non-specific, subjective symptoms lasting more than six months after treatment of LB (Wormser et al., 2006; Mygland et al., 2010; Stanek et al., 2011). The frequency of remaining subjective complaints is partly dependent on the follow-up time point, since a gradual decrease in symptoms has been observed (Berglund et al., 2002; Wormser et al., 2003; Picha et al., 2006). The same symptoms as in PLDS also occur in the general population, and have been shown to be as frequent in controls without a previous history of LB (Seltzer et al., 2000; Cerar et al., 2010). However, children appear to be less likely to develop PLDS (Wang et al., 1998; Skogman et al., 2008b). Patients with suspected PLDS should be thoroughly evaluated and differential

diagnoses, such as fibromyalgia, chronic fatigue syndrome (CFS), autoimmune diseases and psychiatric and neurological disorders excluded (Wormser et al., 2006).

The mechanisms underlying PLDS are still mainly unknown, but as in the case of LA, several explanations have been proposed. At present, there is fairly convincing evidence that PLDS is not caused by a persistent infection with *B. burgdorferi* s.l. (Wormser et al., 2006; Feder et al., 2007; Marques, 2008). Additional or prolonged antibiotic treatment of persistent symptoms has not been proven to be better than placebo (Klempner et al., 2001a; Kaplan et al., 2003; Krupp et al., 2003; Auwaerter, 2007; Fallon et al., 2008). Plausible hypotheses for PLDS instead include a) a low-grade inflammatory response sustained by spirochetal debris and triggering antigens (Fallon et al., 2010). Indeed, proteins of the complement system have been found to be elevated in patients with PLDS as compared to patients with CFS (Schutzer et al., 2011), b) an aberrant or dysregulated immune response that causes tissue damage (Widhe et al., 2002; Widhe et al., 2004), c) autoimmune inflammation initiated by the release of self-antigens as a result of tissue damage during spirochetal combating (Sigal, 1997), and finally, d) psychiatric co-morbidity and psychological factors may in some cases contribute to the illness of PLDS, since some studies have shown an association between PLDS and a previous history of psychological trauma, psychotropic medication, depression, anxiety disorders and other psychological factors (Solomon et al., 1998; Hassett et al., 2008; Hassett et al., 2009).

Immunity to infection

The human defence against microbes can be divided into three levels of increasing specificity (Janeway, 2005; Mölne and Wold, 2007). The first line of defence consists of various mechanical and chemical barriers, such as the skin, mucous membranes, antimicrobial peptides (for example defensins on the skin) and enzymes (for example lysozyme and phospholipase A in saliva). If a pathogen breaches these barriers, the second line of defence, the innate immune system, provides an immediate response triggered by stereotyped warning signals on the pathogen surface. If the pathogen successfully evades the innate response, the third line of defence, the adaptive immune system, is activated to improve the recognition of the specific pathogen. This improved response is retained after the pathogen has been eliminated, in the form of an immunological memory, which allows the adaptive immune system to mount faster and stronger responses if the pathogen is re-encountered in the future. Both innate and adaptive immunity depend on the ability to distinguish between components of the own body (self) and foreign substances (non-self). Once the pathogen is eliminated, the destructive effects of the powerful immune activation need to be counterbalanced by immune regulation in order to limit damage to self components. Down-regulation of the immune response normally occurs as a feedback mechanism, provided that the initial immune response is strong enough (Borish, 1998). An aberrant immune

response and inadequate immune regulation could lead to persistent inflammation and host tissue damage.

The cells of the immune system originate in the bone marrow, where most of them also mature. They then migrate to guard peripheral tissues, circulating in the blood and in the lymphatic system (Figure 4).

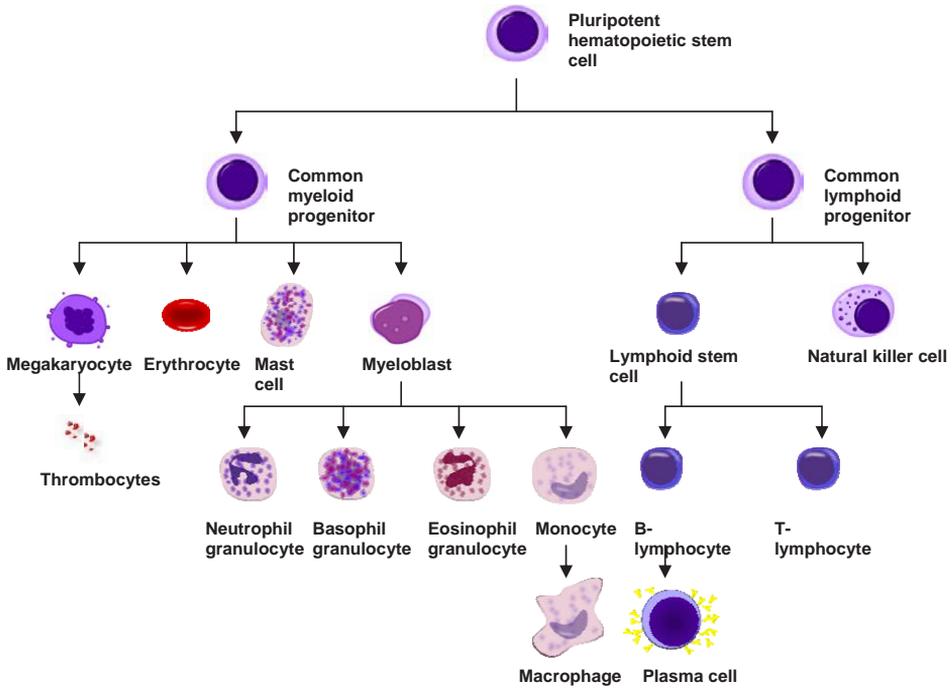


Figure 4. The development from a hematopoietic stem cell to mature and specialized blood cells. Cartoons used in the picture were obtained from Ciker.com – clip art – public domain royalty free clip art.

The innate immune response

The innate immune system is the dominant system of host defence in most organisms (Litman et al., 2005). It is triggered when microbes are identified by pattern recognition receptors (PRRs), which recognize repetitive pathogen-associated molecular patterns (PAMPs) that are conserved among broad groups of microorganisms (Janeway, 2005; Mölne and Wold, 2007). It can also be triggered by alarm signals sent out by injured or stressed cells. The PRRs include the collectin family of proteins (for example mannan-binding lectin, MBL), the macrophage mannose receptor, scavenger receptors as well as nucleotide binding and oligomerization domain (NOD)-like receptors (NLRs) and Toll-like receptors (TLRs) (Kaufmann et al., 2004). Thus, the innate immune system can discriminate between self and non-self, but responds to pathogens in a rather general way. Importantly, the innate immune system do not confer long-lasting immunity against a pathogen.

Inflammation:

Inflammation is one of the first responses of the immune system to infection (Janeway, 2005; Mölne and Wold, 2007). The symptoms of inflammation are redness, swelling, heat and pain, which are caused by increased blood flow into a tissue. Inflammation is produced by a broad set of molecules with overlapping effects; prostaglandins that produce fever and dilatation of blood vessels, leukotrienes that attract leukocytes, cytokines that are responsible for communication between cells, and chemokines that promote chemotaxis (Kaufmann et al., 2004).

The complement system:

The complement system is the major humoral component of the innate immune response (Kaufmann et al., 2004; Janeway, 2005; Mölne and Wold, 2007). The term “complement” was introduced by Paul Ehrlich in the late 1890s to represent the heat-labile component of normal plasma that augments the opsonization and killing of bacteria by antibodies. This activity was said to “complement” the antibacterial activity of antibodies; hence the name. The complement system consists of a complex network of more than 30 fluid-phase and cell membrane proteins. Several complement proteins are proteases that are themselves activated by proteolytic cleavage. The main functions of the complement system are:

- * Recognition of foreign substances, as well as altered host cells.
- * Opsonization of foreign or altered structures, thus facilitating phagocytosis.
- * Release of anaphylatoxins, which induce inflammation and recruit immune cells to the site of complement activation.
- * Destruction of pathogens via lysis.
- * Enhancing the adaptive immune response.

The complement system is activated through a triggered-enzyme cascade via three different pathways (Figure 5). The classical pathway (CP) is initiated by the binding of the C1-complex to different activating structures, such as antigen-bound IgG or IgM, C-reactive protein and intracellular debris from apoptotic cells. The large C1-complex (790 kDa) is Ca^{2+} -dependent and consists of the recognition molecule C1q and two of each of the smaller serine proteases C1r and C1s (Kishore and Reid, 2000). Activated C1s is able to cleave the complement protein C4 into C4a and C4b. Once C4b is attached, it can bind C2, which then will be cleaved by C1s to C2a and C2b. The classical C3-convertase (C4bC2a) is now formed and ready to cleave C3 into C3a and C3b. C3a is a potent anaphylatoxin (the name refers to the ability to induce overwhelming inflammation; anaphylaxis) involved in phagocyte recruitment, and C3b opsonises the pathogen and thereby enhances the phagocytosis process. A fraction of the generated C3b will become attached to the C4bC2a-complex and form the C5-convertase of the CP (C4bC2aC3b), which will have affinity for C5 instead of C3.

The lectin pathway (LP) is the most recently described complement activation pathway recognized today (Ikeda et al., 1987). It generates a classical C3-convertase (C4bC2a); however, the recognition molecules differ from the CP. The LP is initiated by the binding of MBL or ficolins to specific carbohydrate structures found on microbes. This triggers activation of MBL-associated serine proteases (MASP 1-2), leading to cleavage of C4 and eventually also C2.

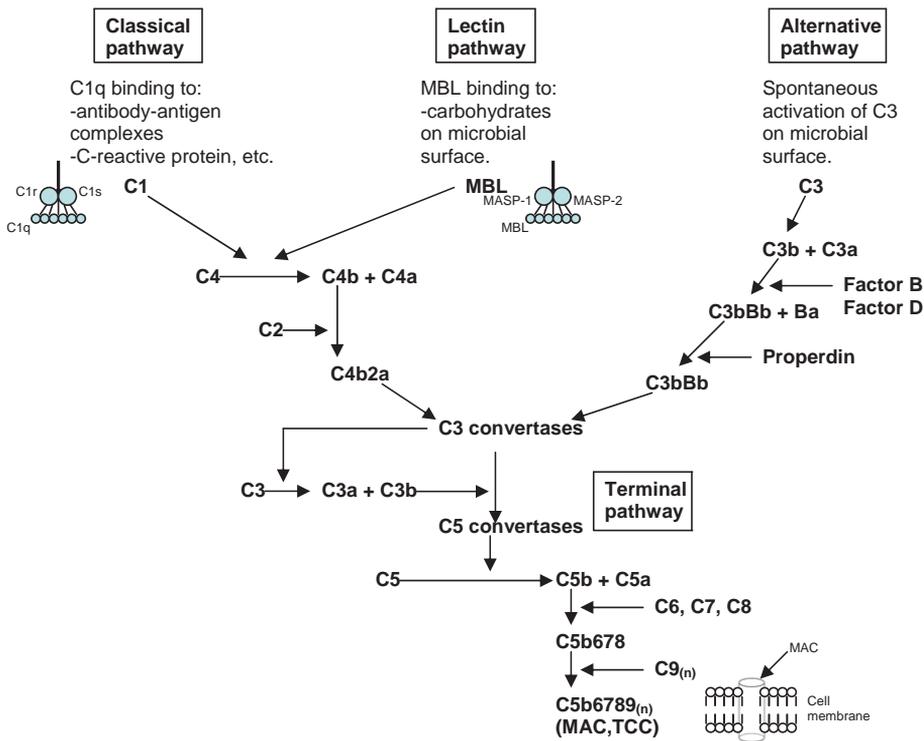


Figure 5. The complement system. MBL: mannan-binding lectin. MASP: MBL-associated serine protease. MAC: membrane attack complex. TCC: terminal complement complex.

The alternative pathway (AP) is activated by spontaneous hydrolysis of C3 or direct binding to specific carbohydrates on pathogen surfaces (Kaufmann et al., 2004; Janeway, 2005). The AP shows a remarkable ability to recognize foreign molecules and non-self substances, despite the inability to utilize specific antibody-antigen complexes for its activation. This capacity can be at least partially explained by its tight regulation on self surfaces by host regulatory proteins, such as factor H. C3b binds factor B in an Mg^{2+} -dependent manner. Factor D will cleave the bound factor B molecule, which results in the formation of the C3-convertase of the AP (C3bBb). The binding of properdin to C3bBb stabilizes the complex, but is not essential for its function. Recently, it has been shown that properdin is also a PRR that binds to certain microbial surfaces and apoptotic/necrotic cells. Once bound to a surface, properdin can direct convertase formation and target uptake (Kemper et al., 2010). Since C3b acts both as a building block and as a product of the C3-convertase, a powerful positive amplification loop is generated. By binding of an additional C3b molecule to the C3bBb-complex, the specificity of the convertase is changed from C3 to C5, and the C5 convertase of the AP is formed (C3bBbC3b).

The terminal pathway (TP) is initiated when C5 is cleaved by any of the two C5-convertases (C4bC2aC3b; C3bBbC3b) into C5a and C5b. C5a is a very potent anaphylatoxin with a broad spectrum of functions; for example activation of phagocytes via the C5a receptor. C5b has the ability to form a complex with C6. The sequential binding of C7, C8 and C9 leads to the formation of the terminal complement complex (TCC), also called the membrane attack complex (MAC; C5b-9), which is inserted in the cell membrane of the pathogen. After the binding of the first C9 molecule, a rapid polymerization with additional 10-16 C9 molecules will occur, leading to the formation of a pore in the cell membrane and osmotic lysis of the cell.

The complement system is a very powerful defence system with highly destructive effects. Uncontrolled complement activation can cause extensive damage to autologous cells and tissues. To prevent improper actions, the complement system is tightly regulated at several stages of the cascade (Table 5). The soluble regulators include C1-inhibitor (C1INH), factor I, C4b-binding protein (C4BP), factor H, factor H-like protein-1 (FHL-1), carboxypeptidase N, clusterin and vitronectin. Important surface-bound regulators are complement receptor 1 (CR1/CD35), membrane cofactor of proteolysis (MCP/CD46), decay-accelerating factor (DAF/CD55) and protectin (CD59).

The primary site of biosynthesis for the majority of the fluid-phase complement components is the hepatocyte, and more than 90 % of plasma complement is derived from the liver (Morgan and Gasque, 1996). All the hepatocyte-derived components behave as acute-phase reactants. Although the liver is the primary source of plasma complement, there are other cells and tissues that produce complement components, such as fibroblasts, endothelial cells, leukocytes, in particular monocytes and macrophages, gut epithelium, cells of the renal glomerulus and synovial lining cells (Volanakis, 1995). Locally produced complement might be particularly important in

Table 5. Regulators of the complement system.

Regulator	Soluble	Regulating function	Pathway inhibited
C1-inhibitor (C1INH)	Yes	Accelerator for decay of the C3-convertase and cofactor to factor I.	CP and LP
Factor I	Yes	Cleaves and inactivates C3b and C4b.	CP, LP and AP
C4-binding protein (C4BP)	Yes	Accelerator for decay of the C3-convertase and cofactor to factor I.	CP and LP
Factor H	Yes	Accelerator for decay of the C3-convertase and cofactor to factor I.	AP
Factor H-like protein-1 (FHL-1)	Yes	Accelerator for decay of the C3-convertase and cofactor to factor I.	AP
Carboxypeptidase N	Yes	Inactivation of C3a, C4a and C5a.	-
Clusterin	Yes	Decreases MAC-formation by interference with C5b-8 and C5b-9.	TP
Vitronectin	Yes	Decreases MAC-insertion by interference with C5b-7 and C5b-9.	TP
Complement receptor 1 (CR1)/CD35	No	Accelerator for decay of both C3-convertases and cofactor to factor I.	CP, LP and AP.
Membrane cofactor of proteolysis (MCP)/CD46	No	Cofactor to factor I.	CP, LP and AP.
Decay-accelerating factor (DAF)/CD55	No	Accelerator for decay of both C3-convertases	CP, LP and AP
Protectin/CD59	No	Decreases the MAC-insertion by interference with C5b-7 and C5b-9.	TP

CP: classical pathway; LP: lectin pathway; AP: alternative pathway; TP: terminal pathway; CD: cluster of differentiation.

early stages of inflammation, before recruitment of plasma complement and inflammatory cells occurs. The CNS tissue is separated from plasma by the blood-brain barrier (BBB) formed by the endothelial cells of brain microvessels and their underlying basement membrane. Little or no complement can reach the CNS in the presence of an intact BBB (Morgan and Gasque, 1996). It has been shown that human CNS cell lines, of which mainly the astrocytes have been studied so far, express and secrete all the components of the CP, the AP and the TP (Gasque et al., 1992; Gasque et al., 1993; Gasque et al., 1995) as well as complement regulators, for example C1INH, factor H, factor I and DAF. Synthesis of all components is enhanced, and C1q synthesis induced, by pro-inflammatory cytokines like interferon- γ (IFN- γ), interleukin-1 β (IL-1 β), and tumour necrosis factor (TNF) (Rus et al., 2006). Locally generated complement may play an important part in opsonizing and killing pathogens in the brain; however, overproduction or dysregulation of complement at an inflammatory site might lead to tissue damage and resultant pathology. It has been shown that complement activation occurs within the CNS in inflammatory and degenerative diseases such as multiple sclerosis and Alzheimer's disease, but also in stroke, cerebral trauma and infectious meningitis (Mavrikakis et al., 1980; Fryden et al., 1983; Francis et al., 2003).

Cellular defence:

The innate leukocytes include the phagocytes (macrophages, neutrophil granulocytes, dendritic cells), mast cells, eosinophil granulocytes, basophile granulocytes and natural killer (NK) cells (Janeway, 2005). Phagocytosis is an important feature of cellular innate immunity, and is performed by cells that are specialized in engulfing pathogens or particles. Phagocytes generally patrol the body searching for pathogens, but may also be called to specific locations by cytokines and chemokines secreted by other cells. Once a pathogen has been engulfed by a phagocyte, it becomes trapped in an intracellular vesicle called a phagolysosome. The pathogen is killed by the activity of digestive enzymes or following a so-called respiratory burst that releases free radicals into the phagolysosome.

Neutrophil granulocytes are normally found in the blood stream and are the most abundant type of phagocyte, normally representing 50-60 % of the total circulating leukocytes. During the acute phase of inflammation, neutrophils migrate towards the site of inflammation in a process known as chemotaxis. They are usually the first cells to arrive at the scene of infection.

Macrophages are versatile cells that reside within tissues and produce a wide variety of enzymes, complement proteins and cytokines. Macrophages also act as scavengers, removing pathogens, apoptotic cells and other debris, and as antigen-presenting cells (APC) that activate the adaptive immune response.

Dendritic cells (DC) are phagocytes mainly residing in tissues that are in contact with the external environment, such as the skin, as well as the respiratory and

gastrointestinal tracts. They serve as professional APCs, their main purpose being to phagocytose, process and present antigens to naïve T cells in lymphoid tissues.

Mast cells reside in connective tissues and mucous membranes. They regulate the inflammatory response, and are often associated with allergy and anaphylaxis by their release of histamine. Basophil and eosinophil granulocytes secrete mediators involved in the defence against parasites, and play a role in allergic reactions and asthma. NK cells are leukocytes that attack and destroy tumour cells or cells infected by viruses.

The adaptive immune response

The adaptive immune response is pathogen-specific and requires the recognition of specific non-self antigens in a process called antigen presentation (Janeway, 2005; Mölne and Wold, 2007). Antigen specificity allows for immune responses that are tailored for specific pathogens or pathogen-infected cells, and the generation of an immunological memory in which each pathogen is “remembered” by a signature antigen. The cells of the adaptive immune system are B lymphocytes (B cells) that are involved in the humoral immune response, and T lymphocytes (T cells) that are involved in the cell-mediated immune response. Both B cells and T cells carry receptor molecules that recognize specific targets. T cells recognize antigens only after they have been processed and presented by other cells in combination with a self receptor - a major histocompatibility complex (MHC) molecule. In contrast, the B cell antigen-specific receptor is an antibody molecule anchored in the B cell membrane, which recognizes whole pathogens without any need for processing or antigen presentation. When B cells and T cells are activated and begin to proliferate, some of the offspring will become long-lived memory cells.

T cells:

T cells originate from the bone marrow, mature in the thymus and are then present in the blood circulation and the secondary lymphoid organs, such as the spleen and the lymph nodes. There are two major types of T cells; T helper (Th) cells and cytotoxic T cells (Tc). Th cells regulate both the innate and the adaptive immune responses and help to determine which type of immune response is mounted against a particular pathogen (Janeway, 2005; Mölne and Wold, 2007). Th cells have no cytotoxic activity and do not kill infected cells or clear pathogens directly, but direct other cells to perform these tasks. Th cells express T cell receptors (TCR) that recognize antigen bound to MHC class II molecules on the surface of APCs. The ensuing activation of the Th cell causes it to release cytokines that influence the activity of many other cell types; for example enhancing the microbicidal function of macrophages and antibody production in B cells, as well as stimulating Tc cells.

Tc cells express TCRs that recognize antigen bound to MHC class I molecules that are present on all host cells of the body. Activation of Tc cells generally requires

additional signals from Th cells. When activated, the Tc cell releases cytotoxins, such as perforin and granulysin that cause destruction of the cells expressing foreign or abnormal antigens on their surface.

B cells:

B cells are produced and mature in the bone marrow before entering the blood circulation. A B cell that has taken up a specific foreign antigen via the B cell receptor, processes the antigen and present it on MHC class II molecules on its surface. This MHC-antigen complex attracts a matching Th cell, which releases cytokines and activates the B cell. Activated B cells proliferate and differentiate to antibody-producing plasma cells. The antibodies circulate in the blood and lymph, bind to pathogens and mark them for destruction by complement activation, and for uptake and destruction by phagocytes. Antibodies can also neutralize pathogens directly by binding to bacterial toxins or by interfering with receptors used by the pathogen to infect cells.

Cytokines and chemokines

Cytokines are small soluble proteins, peptides or glycoproteins secreted by a cell that can alter the behaviour or properties of the cell itself or of another cell (Janeway, 2005; Mölne and Wold, 2007). They are released by various cell types in response to activating stimuli, and they are involved in the determination of the type of immune response to be mounted against a pathogen (Borish and Steinke, 2003). Each cytokine has a matching cell surface receptor, and subsequent cascades of intracellular signalling lead to altered cell functions that may include up-regulation or down-regulation of several genes, resulting in production of other cytokines, increased number of cell surface receptors, or feedback inhibition or stimulation of the cytokine's own effects. Cytokines exert their effects in autocrine, paracrine or endocrine manners, and may be involved in cell proliferation and differentiation, as well as in activation and inhibition of immune responses. The effect of a particular cytokine on a given cell depends on the cytokine, its extra-cellular concentration, the presence and abundance of receptors on the target cell surface, the downstream signals activated by receptor binding, and also the specific phase of the immune response during which the cytokine is secreted (Borish and Steinke, 2003). Some cytokines are pleiotropic, *i.e.* they have versatile roles, such as both pro-inflammatory and anti-inflammatory effects. Cytokines are also characterized by their considerable redundancy, in that many cytokines appear to share similar functions. Cytokines can be divided into different groups according to their main effector functions. Thus, there are pro-inflammatory and anti-inflammatory cytokines. Important pro-inflammatory cytokines in humans include IL-1 β , TNF, IFN- γ and IL-12, whereas anti-inflammatory cytokines include transforming growth factor beta (TGF- β) and IL-10.

Chemokines are small chemotactic cytokines that are induced during an immune response to promote migration of immune cells to the site of infection (Borish and Steinke, 2003).

T helper cell subsets

The Th cells can be further divided into different subsets depending on the cytokines they secrete (Figure 6) (Wan, 2010). Th1 cells secrete mainly IFN- γ , TNF and lymphotoxin (previously called TNF- β). They stimulate macrophages, Tc cells and NK cells and are important for defence against intracellular pathogens. They also stimulate B cells to produce opsonizing and complement activating antibodies, in particular IgG₁ and IgG₃ in humans. The chemokine CXCL10 (previously named IFN- γ inducible protein 10, IP-10) is secreted by several cell types, such as monocytes, endothelial cells and fibroblasts, in response to IFN- γ , and plays an important role in attracting more T cells into the sites of Th1-inflammation (Luster and Ravetch, 1987). If unregulated, an excessive Th1 response may cause tissue damage and chronic inflammatory diseases (Wan, 2010).

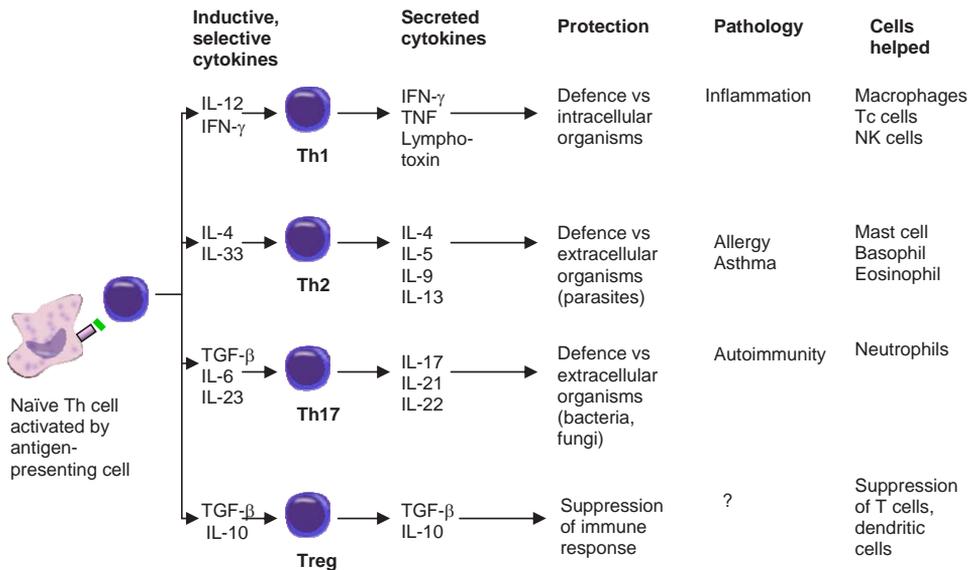


Figure 6. T helper cell subsets, differentiation and function.
 Th: T helper cell; Tc: cytotoxic T cell; NK: natural killer cell; vs: versus.

Th2 cells are characterized by production of IL-4, IL-5, IL-9 and IL-13. They affect mast cells and eosinophil granulocytes, and are involved in protection against extra-cellular pathogens, particularly parasites. Th2 cells stimulate B cells to produce IgE and IgG₄ in humans. The Th2-associated chemokine C-C motif ligand 22 (CCL22, previously named macrophage-derived chemokine, MDC) is secreted by DC and macrophages, and is a chemoattractant for monocytes, immature DC and NK cells (Godiska et al., 1997). Aberrant Th2 responses are involved in asthma and allergy.

Th17 cells secrete mainly IL-17A, IL-17F, IL-21 and IL-22, and are involved in the immune defence against extra-cellular bacteria and fungi (Sallusto and Lanzavecchia, 2009). They are involved in the recruitment, activation and migration of neutrophil granulocytes. Th17 cells are thought to play a key role in the induction and development of tissue injury in some autoimmune diseases, although so far this has mainly been shown in experimental models.

Regulatory T cells (Treg) are yet another Th population, mainly producing IL-10 and TGF- β (Janeway, 2005; Wan, 2010). They are a group of heterogenous, immunosuppressive cells that maintain self-tolerance and regulate the immune response, thus acting to prevent infection-induced immunopathology.

Immunology of Lyme borreliosis

The infection by *B. burgdorferi* s.l. is a complex process beginning with the translocation from the gut to the salivary glands of the tick during feeding on the host. Tick saliva contains a large number of immunosuppressive proteins that interfere with the host's innate and adaptive immune responses (Hovius, 2009), for example Salp15 that interacts with OspC and protects the spirochete from the complement attack by inhibiting the formation of MAC/TCC on its surface (Ramamoorthi et al., 2005; Schuijt et al., 2008). Inhibition of host immune responses could be beneficial for both tick attachment and *B. burgdorferi* s.l. infection.

Even before entering the host, the spirochete has to protect itself from the hostile immune response, as it is exposed to host blood already in the tick gut. One way this is achieved is by antigenic variation (Rupprecht et al., 2008). OspA, which is abundantly expressed in the tick and important for spirochetal adhesion to tick gut epithelium, is rapidly down-regulated during the feeding process on the host (Pal et al., 2000; Schwan and Piesman, 2000). In contrast, OspC is rapidly up-regulated during tick feeding, constituting an important initial survival factor by its ability to bind the complement-inhibiting protein Salp15 (Schwan et al., 1995; Tilly et al., 2007). However, OspC is down-regulated later during host infection, facilitating spirochetal persistence, despite anti-OspC antibody production (Xu et al., 2006). Taken together, the evidence mentioned above suggest that the *Borrelia* spirochete seems to suppress or hide several surface markers during the course of infection to remain unrecognized

by the immune system of the host, but transient expression can be used to utilize various protective mechanisms (Rupprecht et al., 2008).

In addition to the complement-blocking agents supplied by tick saliva, the *Borrelia* spirochetes display their own complement evasion strategies. All pathogenic *Borrelia* genospecies have been shown to activate the CP and/or the AP of complement *in vitro* (van Dam et al., 1997), and complement-resistant strains can inactivate human complement *in vitro* (Brade et al., 1992; Breitner-Ruddock et al., 1997; Kraiczy et al., 2000; Alitalo et al., 2001). Complement-resistant *Borrelia* isolates express outer surface proteins called CRASP and Erp, as described previously in this thesis. Currently, five different CRASP proteins have been identified (Skotarczak, 2009). The most important, CRASP-1, is up-regulated during transmission from tick to host, and down-regulated during persistent infection (von Lackum et al., 2005; Bykowski et al., 2007; Bykowski et al., 2008). Erp proteins are expressed on the spirochete surface during dissemination in various tissues (Stevenson et al., 1996; Hellwage et al., 2001; Stevenson et al., 2002). CRASP-1 and -2 bind factor H and factor H-like protein-1 (FHL-1), and CRASP-3, -4 and -5 and Erp bind factor H (Alitalo et al., 2002; Stevenson et al., 2002; Kraiczy et al., 2004). However, the *Borrelia* spirochetes differ in their susceptibility to complement activation. Most isolates of *B. afzelii* are complement-resistant, due to their efficient binding of factor H, FHL-1 as well as a number of proteins within the factor H-related protein (FHR) family (Breitner-Ruddock et al., 1997; van Dam et al., 1997; Alitalo et al., 2001; Kraiczy et al., 2001a; Siegel et al., 2010). *B. garinii* isolates are generally classified as being complement-sensitive; they bind factor H and FHL-1 only weakly or not at all (Kraiczy et al., 2001a; Wallich et al., 2005). One exception is *B. garinii* OspA serotype 4 (ST4) that binds FHL-1 well and is quite resistant to complement (van Burgel et al., 2010). *B. garinii* OspA ST4 is known to be frequently associated with NB in Europe (Wilske et al., 1993; Wilske et al., 1996; Marconi et al., 1999), and is genetically distinct from other *B. garinii* strains (Margos et al., 2009). It has been proposed that this distinction deserves species status under the name *B. bavariensis*. *B. burgdorferi* s.s. isolates are intermediate in terms of their complement resistance (Kraiczy et al., 2001b).

It has recently been shown in *in vitro* experiments that, apart from factor H and FHL-1, *B. burgdorferi* s.l. is also capable of binding C4BP, the complement inhibitor of the CP (Pietikainen et al., 2010). Binding was strongest to *B. garinii*, the weakest factor H-binder. This protection against antibody-induced complement attack is thought to play an important role in late persistent *Borrelia* infections. A 43 kDa protein (P43) on the spirochetal surface has been suggested as a potential ligand for C4BP.

When encountering the innate cellular immune defence, spirochetal lipoproteins are recognized by TLR-2 (Lien et al., 1999). This ligation leads to activation of monocytes and neutrophils, with subsequent secretion of inflammatory cytokines such as IL-1 β , IL-6, TNF and IL-12 (Radolf et al., 1995; Hirschfeld et al., 1999). However, anti-inflammatory cytokine responses to *B. burgdorferi* s.l. have also been observed.

Spirochetes and their lipoproteins have been shown to elicit IL-10 secretion in human mononuclear cells (Giambartolomei et al., 1998). For the host, there is a delicate balance between eradication of the microbe and protection against immune-mediated tissue damage. Mice deficient in IL-10 have a tenfold lower spirochete burden, but develop more severe Lyme arthritis compared to wild-type mice (Brown et al., 1999).

Studies on *Borrelia*-specific T cell responses in patients with borreliosis have unambiguously shown predominating Th1-type responses. These are most pronounced within the target organ, and mirrored by high levels of IFN- γ , IL-12 and IL-18 (Forsberg et al., 1995; Oksi et al., 1996; Ekerfelt et al., 1997; Yin et al., 1997; Ekerfelt et al., 1998; Gross et al., 1998; Widhe et al., 1998; Grusell et al., 2002). These findings suggest an important role for Th1-mediated effector responses in the eradication of *B. burgdorferi* s.l., i.e. phagocytosis and cytotoxicity, which is supported by the discovery of clonal accumulation of Tc cells in CSF in patients with early NB (Jacobsen et al., 2003) and the discovery of cytotoxic phenotypes (Ekerfelt et al., 2003) as well as cytokines (Nordberg et al., 2011) in NB.

The strong initial Th1-response needs to be counterbalanced by a subsequent Th2-response in order to limit tissue injury. In human borreliosis, an initial IFN- γ -response (Th1) followed by an up-regulation of IL-4 (Th2) seems to be associated with successful resolution of the symptoms (Widhe et al., 2004), whereas a persistent up-regulation of IFN- γ is associated with persistent symptoms after treatment in patients with NB and ACA (Widhe et al., 2002; Jarefors et al., 2007).

Interestingly, the Th17-associated cytokine IL-17 has also been shown to be increased in CSF in NB patients (Nordberg et al., 2011), and it has been suggested that Th17 cells and their associated cytokines are involved in the pathogenesis of LA (Burchill et al., 2003; Codolo et al., 2008; Kotloski et al., 2008). However, the possible role of Th17 responses in borreliosis needs to be further investigated.

The activation of B cells and the production of specific antibodies is essential for clearance of the *Borrelia* spirochetes (Seiler and Weis, 1996). The B cell attractant chemokine CXCL13 has been shown to be up-regulated in the CNS in NB patients (Rupprecht et al., 2005), and TLR-2 has been suggested as a major mediator of *Borrelia*-induced secretion of CXCL13 from human monocytes (Rupprecht et al., 2007). Following activation by Th cells, B cells start producing *B. burgdorferi* s.l.-specific IgM and IgG antibodies (Samuels and Radolf, 2010). The antibodies are directed against an increasingly diverse array of antigens as the infection progresses, which illustrates the large antigenic repertoire displayed by the *Borrelia* spirochete during the course of infection (Nowalk et al., 2006). The earliest antibody responses develop to flagellin and p66, followed by OspC, VlsE, and decorin-binding protein A (DbpA), whereas antibodies against OspA and OspB develop later during the course of the infection.

Borrelia spirochetes have been shown to be killed extracellularly by antibodies plus complement via the CP, as well as by phagocytes (Montgomery and Malawista, 1994). The importance of antibodies acting in concert with the complement system for

the clearance of *B. burgdorferi* s.l. is also supported by the discovery of a predominance of the complement-activating isotypes IgG₁ and IgG₃ in sera from borreliosis patients (Widhe et al., 1998). This can also be considered as a read-out of the *Borrelia*-induced Th1-response, since switching to production of opsonizing and complement-activating Ig isotypes in B cells is part of the Th1-mediated effector response. The significance of the CP of complement activation in the control of *B. burgdorferi* s.l. infection is further underlined by studies on immune evasion of the spirochete, where down-regulation of several antigens leads to insufficient killing by complement and antibodies (Kraiczy et al., 2001b; Kraiczy et al., 2002; Liang et al., 2002). Further, complement activation has been shown to be essential for T cell priming as reviewed by Sacks (Sacks, 2010). In the absence of C3a-receptor (C3aR) signalling, DC lose their capacity to induce potent Th1-responses, and this also favours the emergence of Treg. Disruption of C5aR signalling in TLR-2-stimulated DC causes naïve Th cells to undergo differentiation mainly to Th17 cells, but also to Treg.

Finally, it could be speculated that invasion of a protected niche can be another way for *B. burgdorferi* s.l. to hide from the hostile immune system as shown so far mainly *in vitro* (Liang et al., 2004). The extracellular matrix of joints and skin, as well as the CNS, are considered to be such immunologically privileged sites (Rupprecht et al., 2008).

AIMS

The general aims of this thesis were to increase the knowledge about the epidemiology of NB in Jönköping County, to elucidate possible disease mechanisms related to the innate and adaptive immune responses in LB, and to find out whether immunological biomarkers were related to clinical outcome.

The specific aims for each paper were:

- I. To assess the incidence of NB in Jönköping County, Sweden, and to determine if the incidence changed during 2000-2005; to find out if NB symptoms relate to age; to investigate if NB patients can present with specific anti-*Borrelia* antibodies in CSF but not in serum; to search for possible clinical and laboratory markers associated with the risk of developing long-lasting post-treatment symptoms; and to address some economic aspects of NB in terms of costs for health care and social benefits.
- II. To determine whether the complement system is activated in patients with well-defined NB; to monitor whether any such activation occurs systemically and/or intrathecally; and to characterize the pattern of complement activation.
- III. To investigate whether the complement system is activated locally in the skin in EM; to relate the findings to genospecies, clinical presentation and disease course; to compare clinical isolates of *B. afzelii* and *B. garinii* *in vitro* regarding the degree of complement activation and its impact on phagocytosis; to monitor early cytokine/chemokine release *in vitro* in response to live spirochetes.
- IV. To assess the relative contribution of different Th-associated cytokine/chemokine responses in serum and in CSF in well-characterized NB patients; and to relate the findings to age, sex, clinical presentation and course of the disease.

MATERIALS AND METHODS

Subjects

Case definitions

The following criteria were used in this thesis:

Erythema migrans (Paper III): cases of EM were clinically diagnosed based on the patient's history and physical examination, *i.e.* an expansible rash >5 cm in diameter.

Neuroborreliosis (Papers I, II, IV): cases of confirmed NB were defined by the presence of both CSF pleocytosis and elevated levels of *Borrelia*-specific antibodies in CSF or a positive *Borrelia*-specific antibody index (AI) as described below in the *Borrelia* serology section. Probable NB (Paper II) was defined as signs in serum of ongoing *Borrelia* infection (seroconversion or increasing levels of anti-*Borrelia*-antibodies) and signs in CSF of CNS involvement, *i.e.* pleocytosis, impairment of the BBB or positive *Borrelia*-specific AI. Possible NB (Paper IV) was defined as either the presence of *Borrelia*-specific antibodies or positive *Borrelia*-specific AI in CSF, or CSF pleocytosis in combination with clinical symptoms suggestive of NB. These criteria are in line with current European guidelines (Mygland et al., 2010), although we used the term “confirmed NB” instead of “definite LNB”, and in Paper II the term “probable NB” instead of “possible LNB”. Our criteria for “probable NB” were stricter than the criteria for “possible LNB” used in the European guidelines, since we required seroconversion or increasing levels of anti-*Borrelia*-antibodies in serum. These criteria are used by the clinicians and co-authors on Åland, since the area is highly endemic for *Borrelia* infections, the seroprevalence being nearly 20 % (Carlsson et al., 1998).

Patients and controls

In total, 655 patients with signs and symptoms suggestive of LB were included and investigated in the studies of this thesis (Paper I-IV). Eighty of the patients in Paper I were also included in Paper IV (but only counted once here). An additional group of patients (n=17) without clinical symptoms or laboratory findings suggestive of LB infection were included as controls in Paper IV (Table 6).

In Paper I, 150 patients with NB from Jönköping County were included. All of them had elevated *Borrelia*-specific antibodies (IgG and/or IgM) in CSF and pleocytosis, except for four cases with a long history of illness and high *Borrelia*-specific IgG indices where pleocytosis was not present. They were regarded as possible late NB cases and were therefore included in this clinical study.

Table 6. Subjects included in the thesis.

Diagnosis	Paper I Clinical, epidemiological study	Paper II C activation study in NB	Paper III C activation in EM and <i>in vitro</i> study	Paper IV Cytokine/chemokines in NB
Confirmed NB	146*	15	-	133*
Possible/probable NB	4	8	-	34
Non-CNS <i>Borrelia</i> infection	-	47	24	-
Other CNS-involvement	-	20	-	-
Other non-CNS infection	-	16	-	-
Controls with symptoms	-	192	24**	96
Asymptomatic controls	-	-	-	17
Total***	150	298	24	280

C: complement; NB: neuroborreliosis; EM: erythema migrans; CNS: central nervous system.

* Eighty of the patients in paper I were also included in paper IV.

** Control biopsies were taken from healthy skin on the opposite part of the body of the same patients.

*** In total, 655 patients with symptoms suggestive of LB were included and investigated in the studies of this thesis (Paper I-IV). From the total number indicated in the table above, 97 patients should be subtracted: 80 patients because they were included both in Paper I and IV; 17 patients in the asymptomatic control group because they did not have any signs or symptoms suggestive of NB.

In Paper II, 298 patients investigated in Åland Central Hospital for symptoms suggestive of NB were included. They were classified according to laboratory findings in serum and in CSF as shown later in Table 14 in the Results and Discussion section. Fifteen patients were diagnosed as having confirmed NB: CSF pleocytosis and intrathecal production of specific anti-*Borrelia* antibodies. Eight patients had probable NB: signs of ongoing *Borrelia* infection (seroconversion or increasing levels of serum anti-*Borrelia*-antibodies) and CNS involvement (pleocytosis or *Borrelia*-specific antibodies in CSF or significant damage to the BBB). In the subsequent analyses, the results of the probable NB group did not differ from the confirmed NB group, supporting the theory that the patients with probable NB were in fact true NB cases. Therefore, in all calculations the NB patients were treated as a single group (NB, $n=23$). Another 47 patients had signs of ongoing *Borrelia* infection (seroconversion or increasing levels of serum anti-*Borrelia*-antibodies) but no CNS involvement. They

were classified as other *Borrelia* infection (called group B in Paper II). Twenty patients had other, non-borreliosis-related CNS inflammation (called group CNS): CSF pleocytosis but no serologic evidence of ongoing *Borrelia* infection, e.g. tick-borne encephalitis (TBE), other CNS infection, or suspected multiple sclerosis. Sixteen patients were diagnosed with another ongoing infection not involving the CNS (called group I), e.g. pneumonia, bronchitis, anaplasmosis, viral myocarditis. The remaining 192 patients served as a control group (C): no CSF pleocytosis, ongoing *Borrelia* infection or other infection. Their symptoms could be related to various other conditions, e.g. dementia, status post TBE, folic acid or iron deficiency, hypercalcaemia, orthopaedic diagnoses or depression.

In Paper III, skin biopsies were obtained on Åland from 24 patients >18 years of age presenting with typical EM lesions. Control biopsies were taken from healthy skin on the contralateral body part. The biopsies were selected from a larger sample collection (n=102) obtained in another prospective follow-up study conducted from 2002 to 2004. Biopsies from 19 patients with *B. afzelii* and five patients with *B. garinii* detected by PCR analysis in the EM were chosen. For the *in vitro* analyses, blood was collected from healthy volunteers with (n=8) or without (n=1) specific anti-*Borrelia*-antibodies in serum.

In Paper IV, 263 patients investigated for suspected NB at the hospitals of Jönköping and Kalmar were included and stratified into four groups based on the CSF findings (shown later in Table 16 in the Results and Discussion section). Group 1 consisted of 133 patients with confirmed NB: pleocytosis and detectable *Borrelia*-specific antibodies in CSF or elevated *Borrelia*-specific AI. Group 2, possible late NB, involved 19 patients with long duration of symptoms, *Borrelia*-specific antibodies in CSF or elevated AI, but no pleocytosis. Group 3 comprised 15 patients with pleocytosis, but no detectable *Borrelia*-specific antibodies in CSF. They were all children with short duration of symptoms that were strongly indicative of NB (in all cases meningitis and/or peripheral facial palsy), and they were regarded as possible early NB cases. The remaining 96 patients had no pleocytosis and no detectable *Borrelia*-specific antibodies in CSF, and they were classified in the non-NB group (group 4). In addition, we included a control group (group 5), consisting of 17 patients undergoing elective orthopaedic surgery. They had not experienced symptoms suggestive of NB and were negative for anti-*Borrelia*-antibodies in serum and in CSF. Thus, the total number of included subjects was 280.

Excluded subjects

In Paper I, 157 patients in Jönköping County were found to have elevated levels of *Borrelia*-specific antibodies in CSF during the years 2000 through 2005. Seven of these patients were excluded from the study due to other obvious diagnoses (for example normal-pressure hydrocephalus, cerebellar infarction or *Varicella*

encephalitis) and/or absence of pleocytosis (Table 7). Their elevated anti-*Borrelia* antibody levels in CSF were considered to be an indication of a previous NB episode or IgM cross-reactivity to another pathogen (for example *Varicella*).

In Paper II, 314 paired serum and CSF samples were obtained from patients enrolled in this prospective study from July 2002 to February 2005 at Åland Central Hospital. Sixteen of these were excluded from the study. In the case of four patients, double samples had been collected: for these patients, the first sample obtained was used in the study and the second sample was omitted. Another 12 patients were excluded from the study: nine whose sample volumes were too small, one who had received 2½ months of antibiotic treatment immediately prior to our study, and two who could not be evaluated because of extensive co-morbidity (one with cardiovascular disease, chronic glomerulonephritis and stroke, one with immunosuppression and recently treated biliary disease).

In Paper III, no subjects were excluded.

In Paper IV, serum and CSF samples were initially available from 271 patients investigated for suspected NB in Kalmar and Jönköping Counties during 2003-2005. Eight patients were excluded because the sample volumes were insufficient for the analyses.

Our opinion is that the exclusion of the subjects as described above is unlikely to have caused a skewness of the results presented in this thesis. First of all, the number of excluded subjects was very small in relation to the size of the study population (Table 7). Most patients were excluded because of insufficient sample volumes. Some were excluded due to other obvious diagnoses, *i.e.* they should not have been included. Further, the excluded subjects represented all age groups and an equal number of men and women, except in Paper IV where all the excluded subjects happened to be men. However, they were all excluded solely because of insufficient sample volumes.

Table 7. Excluded subjects.

	Paper I	Paper II	Paper III	Paper IV
Excluded subjects (%)	4.5 (7/157)	5.1 (16/314)	0	3.0 (8/271)
Years of age (mean, range)	54 (8-83)	39 (6-83)	-	43 (6-62)
Sex (%)	M: 57 F: 43	M: 50 F: 50	-	M: 100 F: 0

M: males.
F: females.

Methods

Data collection

The patients' medical records were scrutinized by the author (Paper I), together with Dag Nyman and Pia Forsberg (Paper II), and with Ivar Tjernberg (Paper IV) according to standardized protocols. Data on health care cost were obtained from the Departments of Economy and Information Technology, Ryhov County Hospital. Monetary values were assigned to each hospitalization using the classification systems according to Health Care Financing Administration-Diagnosis Related Groups (DRG) during 2000-2003, and NordDRG during 2003-2005 (Fernström, 2007; Nordclass, 2007). Data on sickness and temporary parental benefits were provided by the Swedish Social Insurance Agency (Paper I). The EM patients in Paper III answered a health questionnaire at inclusion in the study and at follow-up after 3, 6, 12 and 24 months.

Borrelia serology

The Clinical Laboratory of Microbiology (CLM), Ryhov County Hospital, Jönköping, performed all *Borrelia* antibody tests in Paper I and those for the Jönköping patients in Paper IV ($n=98$), using the commercially available Lyme Borreliosis ELISA kit, 2nd Generation (Dako Cytomation A/S, Glostrup, Denmark). From January 2000 to August 2004, the *Borrelia*-specific AI was calculated as described by Peter (Peter, 1990), with the modification that total IgG was substituted for *Rubella*-specific IgG. The formula used was: [*Borrelia*-specific IgG in CSF (OD)/*Borrelia*-specific IgG in serum(OD)]/[*Rubella*-specific IgG in CSF (OD)/*Rubella*-specific IgG in serum (OD)]. From September 2004, the CLM used total IgG as a reference molecule: [*Borrelia*-specific IgG in CSF (OD)/*Borrelia*-specific IgG in serum(OD)]/[total IgG in CSF (mg/L)/total IgG in serum (g/L)]. A *Borrelia*-specific AI >2 was considered to indicate intrathecal anti-*Borrelia* antibody production with both methods.

The CLM in Åland Central Hospital performed all the *Borrelia* antibody tests in Paper II, and the CLM in Kalmar County Hospital carried out the *Borrelia* serology for the Kalmar patients in Paper IV ($n=165$). Both laboratories used Immunetics Quick ELISA C6 Assay kit, Immunetics, (Boston/Cambridge MA, USA) for the detection of *Borrelia*-specific antibodies in serum, and IDEIA Lyme Neuroborreliosis kit K6028 (Dako Cytomation, UK) for measuring intrathecal production of *Borrelia*-specific antibodies.

The CLM in Ryhov County Hospital, Jönköping, performed the serum *Borrelia*-antibody screening tests of the healthy subjects in Paper III, using Enzygnost Lyme link VlsE/IgG and Enzygnost Borreliosis/IgM (DADE Behring, Marburg, Germany) (Table 8).

Table 8. Overview of the different *Borrelia*-antibody test kits used in this thesis.

	Lyme Borreliosis ELISA Kit 2 nd Generation, Dako	IDEIA Lyme Neuroborreliosis, Dako	Quick ELISA C6 Assay, Immunetics	Enzygnost Lyme link VlsE/IgG, DADE Behring
Test antigen	Purified native flagellin from <i>B. burgdorferi</i> .	Purified native flagellin from <i>B. afzelii</i> DK1.	Synthetic peptide based on IR6 of the VlsE protein from <i>B. garinii</i> .	Deactivated <i>B. afzelii</i> PKo antigens and recombinant VlsE from <i>B. afzelii</i> , <i>B. garinii</i> and <i>B. burgdorferi</i> s.s.
Used for serum samples	Yes	Yes*	Yes	Yes
Used for CSF samples	Yes	Yes*	No	No
Used in paper	I, IV	II, IV	II, IV	III

CSF: cerebrospinal fluid.

IR6: invariable region 6.

VlsE: variable major protein-like sequence, expressed.

* simultaneous analysis of serum and CSF for direct measurement of intrathecal production of *Borrelia*-specific antibodies.

Principles of the ELISA method:

ELISA is used for detection and quantification either of an antibody or an antigen in a solution (Figure 7) (Leng et al., 2008). The wells of a microtitre plate are coated with antigen or a capture antibody specific for the antibody/antigen to be detected. The sample is added and the antibody/antigen binds to the antigen/capture antibody at the bottom of the wells. A detection-antibody specific for the measured antibody/antigen is used in combination with a substrate to generate a quantifiable signal proportional to the concentration of the antibody/antigen in the sample. A traditional ELISA typically involves chromogenic reporters and substrates that produce a colour change, the intensity of which can be measured spectrophotometrically.

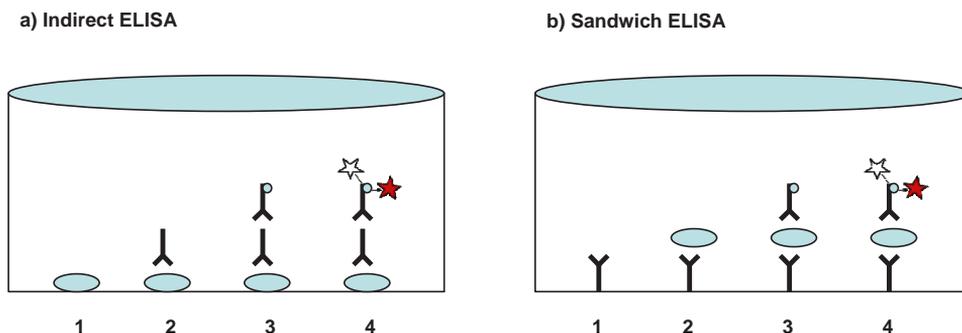


Figure 7. a) Indirect ELISA. 1: microtiter wells are coated with antigen. 2: patient serum is added and specific antibodies bind to the antigen. 3: biotinylated secondary antibody is added. 4: streptavidin-HRP and substrate are added, resulting in enzymatic colour change, and absorbancy is measured spectrophotometrically.

b) Sandwich ELISA. 1: microtiter wells are coated with a capture antibody. 2: sample is added, and if antigen is present, it binds to the capture antibody. 3: biotinylated detection antibody is added. 4: streptavidin-HRP and substrate are added.

Measurement of complement components and activation products

The selection of the complement components analysed in Paper II was based on a previous pilot study on C3, C4, MBL, C1q, C3a and TCC conducted as a student examination project at the Linneaus University, Kalmar, supervised by Kristina Nilsson Ekdahl.

For the quantification of complement components C1q, C3 and C4, and the activation products C3a and soluble (s) C5b-9 (Papers II and III), sandwich ELISAs were used (Figure 7b).

In each determination of C1q, C3 and C4, a control of pooled plasma (P) from five blood donors was included, and a sample with known concentrations of C1q, C3 and C4, respectively, was used as a standard.

For the C1q determination, microtitre plates were coated with anti-C1q and detection of bound C1q was performed using biotinylated anti-C1q followed by streptavidin-HRP.

In the C3 assay, the microtitre plates were coated with anti-C3c, which recognizes C3, C3b, iC3b and C3c, and detection of C3 and C3 fragments was carried out using biotinylated anti-C3c antibodies.

In the C4 assay, plates were coated with anti-C4c, which recognizes C4, C4b, iC4b and C4c. Biotinylated anti-C4 was used for detection of total C4, including activated forms.

For the C3a assay (Nilsson Ekdahl et al., 1992), zymosan-activated serum calibrated against a known C3a concentration was used as a standard, and pooled zymosan-activated serum from blood donors was used as a control. The microtitre plates were coated with a monoclonal antibody (MoAb 4SD17.3) against a neopeptide in C3a, and detection was performed using a biotinylated polyclonal antibody against C3a.

The sC5b-9 assay (Paper III) used a monoclonal antibody (anti-human C5b-9, Diatec, Oslo, Norway) against a neopeptide exposed in C9 when incorporated into the TCC, *i.e.* C5b-9. A biotinylated polyclonal anti-C5 antibody was used for detection (Mollnes et al., 1985).

For quantification of factor H and C4BP on the surface of the spirochetes after incubation with human plasma we used a variant of ELISA. The tubes with spirochetes and plasma were incubated for 30 minutes and then centrifuged at 2,500 g for 5 minutes. The pellets with spirochetes were washed, resuspended in phosphate-buffered saline (PBS) + Ca²⁺ and transferred to new tubes. Biotinylated antibodies against human factor H and C4BP, respectively, were added to the samples and incubated for 45 minutes. After centrifugation, washing and resuspension, streptavidin-HRP was added and absorbance was measured at 450 nm.

Complement components were also detected by WB analysis. In Paper II, CSF proteins of all patients were separated under reducing conditions by sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE). The proteins were transferred to a polyvinylidene fluoride (PVDF) membrane, and the presence of C3/C3-fragments was detected using biotinylated antibodies against human C3c and streptavidin-HRP. Purified human C3, C3b and iC3b served as controls (Nilsson et al., 1990).

In Paper III, WB was used for visualization of C3/C3-fragments and factor H on the spirochetes after incubation with human plasma. Detection was performed using biotinylated antibodies against human factor H and C3c, respectively, followed by streptavidin-HRP. Purified C3, C3b and factor H served as controls.

Principles of the Western blot technique

WB (or protein immunoblot) is an analytical technique used to detect specific proteins in a sample (Chandler and Colitz, 2006). Gel electrophoresis is used to separate the proteins by length of the polypeptide chain. The proteins are then transferred to a membrane, typically nitrocellulose or PVDF. This is followed by detection using labelled antibodies specific to the target protein (Figure 8).

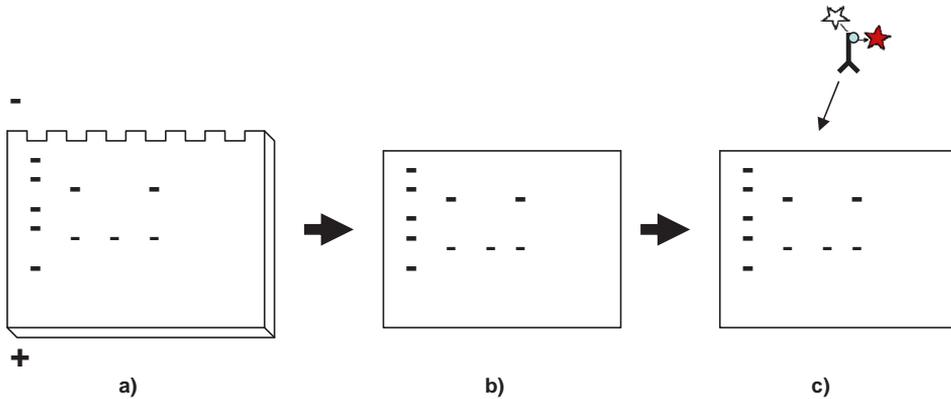


Figure 8. Western blot. a) Proteins are separated by molecular weight using gel electrophoresis. b) The proteins are transferred to a polyvinylidene fluoride membrane. c) Detection of specific protein bands by addition of biotinylated antibodies, streptavidin-horseradish peroxidase and a chromogenic substrate.

Calculation of intrathecal complement and chemokine production indices

By analogy to the AI used for calculations of intrathecal antibody production (Tibbling et al., 1977; Peter, 1990), an intrathecal production index was calculated for each complement component analyzed in Paper II, and for the chemokines analyzed in Paper IV. The formulas used were as follows: $[\text{CSF-complement}/\text{P-complement}]/[\text{CSF-albumin}/\text{S-albumin}]$, and $[\text{CSF-chemokine}/\text{S-chemokine}]/[\text{CSF-albumin}/\text{S-albumin}]$, respectively, thus relating the CSF/blood gradient of the substance analyzed to the albumin ratio. The albumin ratio was used as a marker of BBB damage and passive transudation of proteins.

Skin biopsies

For the analyses in Paper III, punch biopsies were taken from the outer edge of the red EM zone, and control biopsies were taken from healthy skin on the opposite part of the body from the same patient. The causative *Borrelia* species had previously been determined for each EM by research colleagues in Umeå (Sven Bergström's group) using PCR targeting the 16S rRNA gene. Biopsies from 19 patients with *B. afzelii* and five with *B. garinii* were selected from a large sample collection ($n=102$). All EM lesions were photographed and measured.

Immunohistochemistry

The skin biopsies from the EM patients (Paper III) were instantly snap-frozen in a mixture of isopentane and liquid CO₂ and stored at -70 °C. Serial sections of 5 µm were obtained using a cryostat. Antibodies were fluorescent labelled and diluted according to Table 9. The tissue sections were incubated with antibody dilutions for 30 minutes, washed, and then analyzed in a fluorescence microscope by an experienced clinical immunologist.

Table 9. Antibodies used for immunohistochemistry (paper IV).

Antibody	Manufacturer	Clonality	Labelling	Concentration	Dilution
Anti-hu-C3c	A0062 Dako A/S, Denmark	polyclonal	Alexa Fluor 488	1.98 mg/mL	1:10
Anti-hu-C3c	A0062 Dako A/S, Denmark	polyclonal	Alexa Fluor 555	2.46 mg/mL	1:20
Anti-hu-IgG	A0424 Dako A/S, Denmark	polyclonal	Alexa Fluor 488	1.98 mg/mL	1:10
Anti-hu-IgM	A0425 Dako A/S, Denmark	polyclonal	Alexa Fluor 488	2.08 mg/mL	1:10
Anti-hu-FH	PC030 Binding Site, UK	polyclonal	Alexa Fluor 488	2.5 mg/mL	1:10
Anti-hu-iC3b	in house 26.1	monoclonal	Alexa Fluor 488	1.98 mg/mL	1:10
Anti-hu-CD3	555332 BD, USA	monoclonal	FITC	0.53 mg/mL	1:10
Anti-hu-CD45	345808 BD, USA	monoclonal	FITC	50 µg/mL	1:20
Anti-hu-CD11b	77987 Abcam, UK	monoclonal	Alexa Fluor 488	1 mg/mL	1:10
Anti- <i>Borrelia</i>	BP1002 Acris, Germany	polyclonal	Alexa Fluor 555	2.86 mg/mL	1:10
Anti- <i>Borrelia</i>	01-97-92 KPL, USA	polyclonal	FITC	0.5 mg/mL	1:10

hu: human; FH: factor H; CD: cluster of differentiation (CD3: T lymphocytes, CD45: all leukocytes, CD11b: activated granulocytes and macrophages); FITC: fluorescein isothiocyanate.

Principles of immunohistochemistry:

Immunohistochemistry (IHC) is a method of localizing specific antigens within a tissue section by the use of labelled specific antibodies (Chandler and Colitz, 2006). The antigen-antibody interactions are visualized with a marker such as fluorescent dye, an enzyme, a radioactive element or colloidal gold. There are numerous IHC methods available. The selection of a suitable method should be based on parameters such as the type of specimen under investigation and the degree of sensitivity required. The basic principles of IHC are illustrated in Figure 9a-b.

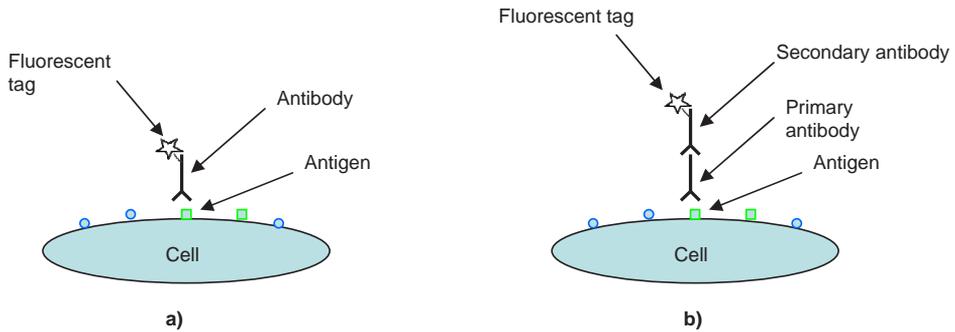


Figure 9. a) Direct immunohistochemistry. b) Indirect immunohistochemistry.

Bacterial strains and culture

The *Borrelia* strains *B. garinii* LU59 (complement sensitive), isolated from human CSF, and *B. afzelii* K78 (complement resistant), isolated from a human skin biopsy in Sven Bergström's laboratory, Umeå University, Sweden, were grown in BSK II medium (Barbour, 1984) supplemented with 7 % rabbit serum (Sigma, St Louis, USA) at 37 °C until cell density reached 10^8 - 10^9 cells/mL (Paper III).

Complement activation assay

Different concentrations of spirochetes (10^6 , 10^7 , 10^8 , 10^9 /mL plasma) were incubated for 60 minutes with hirudin plasma from blood donors with or without *Borrelia*-specific antibodies (Paper III). Complement activation was terminated by addition of ethylenediaminetetraacetic acid (EDTA), and the tubes were centrifuged at 2,500 *g* for 5 minutes. Complement activation was measured in the supernatants as the generation of activation products C3a and sC5b-9 using a sandwich ELISA as described above. Quantification of factor H bound to the surface of the spirochetes after plasma incubation was performed using the ELISA method described above. Hirudin plasma incubated without spirochetes served as controls.

Labelling of spirochetes

For the phagocytosis assay (Paper III), spirochetes were labelled with FITC using a modification of a previously described procedure (Hazebos et al., 1994). The labelled spirochetes were visualized by fluorescence microscopy and found to be viable.

Phagocytosis assay

FITC-labelled *B. garinii* LU59 and *B. afzelii* K78 were incubated for 30 minutes with hirudin blood from donors with or without *Borrelia*-specific antibodies (Paper III). Incubations were performed without complement inhibition, or with inhibition by addition of: a) EDTA, which binds the Ca^{2+} and Mg^{2+} ions necessary for the proteolytic reactions of the complement cascade, b) compstatin, a synthetic cyclic peptide that binds and inhibits activation of C3, and c) a C5a receptor antagonist (C5aRa) that blocks C5a mediated up-regulation of granulocytes and monocytes. Incubations without spirochetes or with a scrambled peptide of C5aRa, respectively, were used as negative controls. After lysis and removal of the red blood cells, the leukocytes were resuspended in PBS with 1 % paraformaldehyde and 0.075 % trypan blue, the latter being added to quench the FITC-signals from unphagocytosed spirochetes (Klippel and Bilitewski, 2007). Mean fluorescence intensity (MFI) was analyzed by flow cytometry.

Principles of flow cytometry:

Flow cytometry is a method for counting and examining microscopic particles, such as cells, and can be used for detection of cell surface markers, cytokine production, cell viability, as well as functional cell studies and cell sorting (Chandler and Colitz, 2006). The cells are detected by the use of specific fluorophore-labelled antibodies and a laser beam (Figure 10a-b). The labelled cells are lined up in a single-cell stream and passed through the laser beam, resulting in excitation and light emission of the fluorophore. The light hitting the cells is scattered and reveals information about the size (forward scatter) and granularity (side scatter) of each of the cells. The combination of scattered and fluorescent light is detected by the flow cytometer, recorded on a computer and analyzed with the aid of computer software. It is then possible to derive various types of information about the physical and chemical structure of each individual cell.

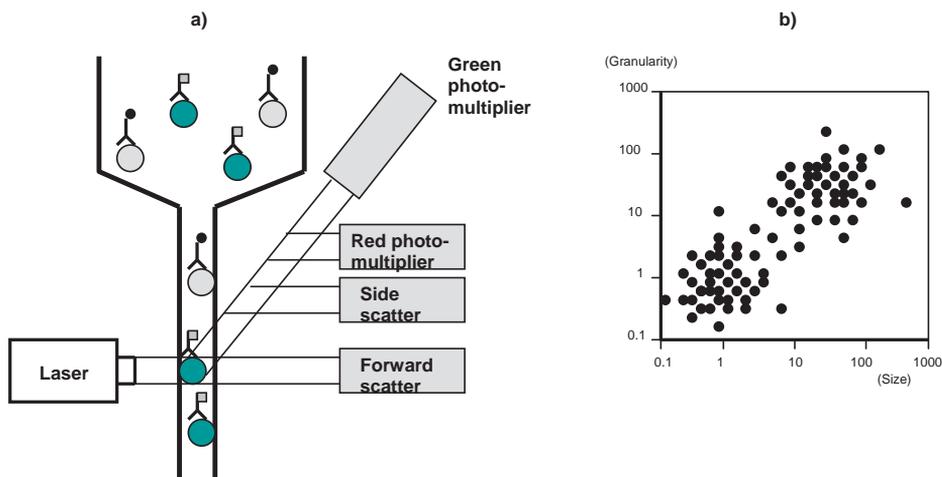


Figure 10. Flow cytometry. a) The cells are passed through the laser beam one by one, thus exciting the fluorescent dye. The light hitting the cells is also scattered and reveals information about cell size (forward scatter) and granularity (side scatter). b) Example of data presentation in a scatter dot plot.

Measurement of cytokines and chemokines

Concentrations of cytokines and chemokines in hirudin blood incubated with *Borrelia* spirochetes (Paper III) and in serum and CSF from patients (Paper IV) were measured by Luminex multiple bead array using Milliplex Human Cytokine/Chemokine kit from Millipore Corporation according to the instructions provided by the manufacturer. Values under the detection limit were given half the value of the lowest point on the standard curve. The various cytokines and chemokines analyzed in this thesis are presented in Table 10.

Principles of Luminex multiplex bead array:

This method is based on a combination of existing and established technology (microspheres, detection by specific antibody-antigen interactions or chemical reactions, flow cytometry and digital signal managing), and permits the simultaneous analysis of a large number of different biomolecules (*i.e.* proteins, peptides or nucleic acids) (Elshal and McCoy, 2006; Leng et al., 2008). Tiny polystyrene beads (*i.e.* microspheres with a diameter of 5.6 μm) are filled with red and infrared fluorescent dyes (Figure 11). Using different intensities of the two dyes, up to 100 different microsphere batches can be created, each with a unique spectral signature. Each bead set can then be coated with a reagent specific to a particular bioassay, *e.g.* a monoclonal antibody, allowing the capture of a specific analyte in a sample. The analyte is then detected by a reporter molecule, typically a secondary antibody labelled

Table 10. Analyzed cytokines and chemokines (Papers III, IV).

Cytokine/ Chemokine	Source	Function	Type of response	Lower detect- ion limit (pg/mL)
IL-1 β	macrophages epithelial cells	Fever, activation of T cells and macrophages	Inflammatory	3.2
IL-6	macrophages T cells, endo- thelial cells	Fever, stimulates growth and differentiation of T and B cells	Inflammatory Th17	1.6
IL-10	T cells macrophages	Suppresses macrophage functions	Anti- inflammatory	3.2
IL-12p70	macrophages DC	Activates NK cells, induces Th1 differentiation	Th1	1.6
IL-17A	Th17 cells	Induces cytokine production by epithelia, endothelia and fibroblasts	Th17	1.6
IL-23	DC	Increases IFN- γ production, induces Th17 differentiation	Th17	24.4
TNF	macrophages NK cells T cells	Induces inflammation, activates endothelial cells	Inflammatory	1.6
GM-CSF	macrophages T cells	Stimulates growth and differentiation of myeloid cells	Inflammatory	3.2
CXCL1	macrophages neutrophils epithelial cells	Attracts neutrophils	Th17	3.2
CXCL8	macrophages DC endothelia	Attracts neutrophils, basophils and T cells	Inflammatory Th17	1.6
CXCL10	monocytes endothelia fibroblasts	Attracts activated T cells	Th1	16.0
CCL2	monocytes macrophages DC	Attracts monocytes, DC and T memory cells	Inflammatory	3.2
CCL20	macrophages DC	Attracts DC, T and B cells and neutrophils	Th17	9.8
CCL22	macrophages DC	Attracts DC, NK cells and T cells	Th2	16.0

TNF: tumour necrosis factor; GM-CSF: granulocyte-macrophage colony-stimulating factor; DC: dendritic cells; IL: interleukin; NK: natural killer cells; Th: T helper cells; CXCL: C-X-C motif ligand; CCL: C-C motif ligand.

with a fluorochrome. When the beads pass through the flow cytometer, the red/infrared fluorescent dyes are excited by a red laser beam (for identification of specific bead sets), and the fluorochrome of the reporter molecule is excited by a green laser beam (allowing detection and quantification of the bound analyte).

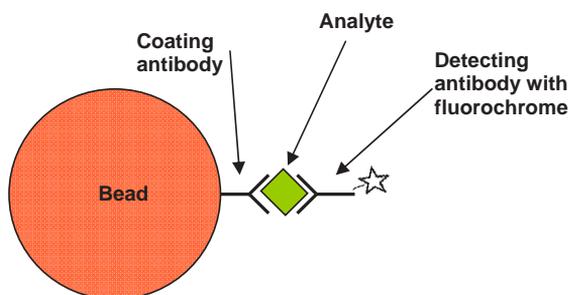


Figure 11. Luminex multiplex bead array. Polystyrene beads are filled with a mixture of red and infrared dyes and coated with antibodies that capture the analyte to be measured. A detecting antibody labelled with a fluorochrome is then added and binds to the analyte. By the use of a red and a green laser beam, the emitted light from the bead and from the fluorochrome can be detected.

Methodological considerations

In this thesis, several different commercial ELISA kits were used for the assessment of specific anti-*Borrelia* antibodies in serum and in CSF. This lack of standardization between laboratories is a potential limitation in clinical practice as well as in the thesis. However, serology can never be a stand-alone tool for LB diagnosis; it must be interpreted with caution and in relation to clinical presentation, medical history, duration of symptoms, other laboratory findings and the local epidemiology. Therefore, all the patients included in our studies were not only serologically, but also clinically, evaluated.

In cases of suspected NB, simultaneous analysis of specific anti-*Borrelia* antibodies in paired serum and CSF samples provides the possibility of determining the presence of intrathecally produced *Borrelia* antibodies by calculation of a specific *Borrelia* AI (Tibbling et al., 1977; Stanek et al., 1996; Wilske et al., 1986). AI has a high specificity, although the sensitivity has been reported to be only 55-80 %, with the lower sensitivity in early stages of the infection (Stiernstedt et al., 1985 ; Steere et al., 1990; Tumani et al., 1995; Picha et al., 2000). Furthermore, a positive AI may persist for several months or even years after the infection, despite adequate treatment (Hammers-Berggren et al., 1993; Stanek and Strle, 2003; Ljostad et al., 2007). Again, it is worth noting that *Borrelia* serology as well as AI must be interpreted in relation to

the patient's medical history, clinical symptoms and other laboratory findings (Blanc et al., 2007).

By analogy to the AI described by Tibbling et al. 1977, we used the CSF/serum-albumin ratio as a marker of BBB impairment when calculating intrathecal production of complement components and chemokines (Papers II and IV). However, these formulas must be evaluated further since a linear relationship between the CSF/serum ratio of the analytes and the CSF/serum-albumin ratio cannot be automatically assumed, especially in patients with severe BBB damage (Ohman et al., 1989). Also, the different sizes and electric charges of the analyzed molecules could influence their ability to cross the BBB.

In Paper II and III, we used plasma for the complement assays (except for the phagocytosis assay where whole blood was used). Serum can also be used for complement studies; however, plasma is considered to be of higher biological relevance due to the presence of coagulation factors which allows the important crosstalk between the two cascade systems (Amara et al., 2008).

For the *in vitro* complement assays we used recombinant hirudin (lepirudin) as anticoagulant. Hirudin is a direct thrombin inhibitor and does not interfere with the complement system (Johnson, 1994). This is advantageous in comparison with heparin since the latter, apart from binding to antithrombin, also interacts with factor H and thus reduces complement activation.

For the measurement of complement components (Papers II, III), we used well established ELISA methods because of their high sensitivity. Other advantages of ELISA methods are that they are relatively easy to perform and do not require expensive laboratory equipment. Possible limitations of the method could be intra- and inter-assay variation. The use of positive and negative controls is important to ensure that the analysis is robust and reproducible over time. If several analytes are to be measured, ELISA is a rather time-consuming method and quite large sample volumes are needed.

Certain parallels could be drawn between ELISAs and the multiplex bead array assays (MBAAAs) that we used in Paper III and IV. However, there are some major differences between the methods (Elshal and McCoy, 2006; Leng et al., 2008). For instance, MBAAAs use fluorescence as the reporter system, whereas ELISAs use enzyme amplification of a colorimetric substrate. Luminex uses capture antibodies coated on spherical beads, while ELISAs generally rely upon solid surfaces in microtitre plates. Most importantly, ELISA methodologies generally study one analyte at a time, whereas MBAAAs offer the possibility of analyzing multiple analytes simultaneously in small sample volumes which makes the method cost- and time-effective. MBAAAs also allow collection of data from numerous beads for each analyte, thus providing statistical rigor. Another advantage of MBAAAs is that the dynamic range (range over which there is a linear relationship between the analyte concentration and the level of the fluorescence reading) is wider than the range for ELISA-based assays. Although good correlations have been found between ELISAs

and MBAs using identical capture and reporter antibodies and similar diluents and serum blockers, side-by-side comparisons should be made with caution and are not recommended. Any study involving sequential or comparative monitoring of patients should be performed using only a single technique, one laboratory platform, and one commercial vendor for all samples.

In Paper II, we analyzed C3/C3-fragments using ELISA followed by WB. The advantage of this two-step procedure was that ELISA gives sensitive quantification of the total quantity of C3/C3-fragments, while WB gives a relative quantification of different C3-fragments that can be identified by their molecular weights. In Paper III, we used WB for visualization of C3/C3-fragments and factor H bound to the surface of the spirochetes. However, with this method we were not able to demonstrate any differences in the quantity of C3/C3-fragments or factor H bound by *B. afzelii* K78 and *B. garinii* LU59, respectively, and therefore we used ELISA for more precise quantification.

In Paper III, we chose IHC for the analysis of complement components, antibodies and inflammatory cells in the skin biopsies from EM patients, since this method gives direct information on the localization of the cells and proteins within the tissue section in relation to the spirochetes and tissue architecture. By analyzing biopsies from EM caused by *B. afzelii* and *B. garinii* along with control biopsies from unaffected skin, we were able to get an estimate of the relative quantity of the analytes in the tissue sections. We used a one-step staining method on frozen tissue sections, since it is fast, relatively gentle on the antigen structure, and requires small amounts of reagents. However, further optimization of the method, if required, is certainly possible. For better preservation of the tissue structure and cell morphology, the frozen sections could be fixed in cold acetone or alcohol. In our study, fixation in paraformaldehyde was not an option, since this probably would have caused inhibition problems in the PCR analysis used for detection and genotyping of the spirochetes. Further, fixation may also cause conformational changes to antigen structures which may lead to decreased antibody binding. In order to increase the sensitivity of our method, we could use a two-step staining method with a secondary antibody directed against several antigenic sites of the primary antibody (Figure 9b), resulting in amplification of the signal from the reporter molecule (Chandler and Colitz, 2006).

For the phagocytosis assay in Paper III, we used flow cytometry for detection of FITC-labelled spirochetes since the method allows rapid and simultaneous multiparametric analysis of the cells. By adding trypan blue to the samples after incubation with human blood, we quenched the FITC signal from unphagocytosed spirochetes (Klippel and Bilitewski, 2007). Trypan blue absorbs light in the range from 475 to 675 nm, which covers the wavelength of FITC fluorescence emission (519 nm). Since trypan blue is not able to permeate cell membranes, it cannot interact with fluorescent particles that are internalized by phagocytes. Thus, the amount of internalized fluorescent material correlates to the phagocytic activity and was determined by the fluorescence intensity of phagocytes. The flow cytometer is a delicate instrument and thus there may be variations in detection level if serial

measurements are made at different time points. By using standardized settings and making adjustments on each analysis occasion the variations of the instrument were corrected for (Chandler and Colitz, 2006).

Statistics

SPSS software for Windows, version 15.0 was used for the statistical analyses in this thesis. The non-parametric Kruskal-Wallis test was used when comparing continuous data between several diagnostic groups, and the Mann-Whitney U test was applied as a *post hoc* analysis (Paper I-IV). The Chi² test was used for non-continuous data (Papers I, III), and Fishers' exact test was applied when expected counts were <5. Correlations between parameters were calculated using the non-parametric Spearman correlation analysis (Papers I-IV). Trend analysis of NB incidence (Paper I) was carried out using linear regression. Two-tailed tests were used, and p-values of <0.05 were considered to be significant, except in Paper IV where p-values of <0.01 were considered as significant in order to avoid mass-significance problems.

However, it is important for the interpretation of the results of the statistical analysis to consider the following: although differences between compared groups might be statistically significant at some level, this does not automatically imply that they are of clinical importance. In some cases the calculated statistical significance results are due to a large population (mass significance) but have no clinical importance. On the other hand, a result might not be statistically significant but clinically important, as a result of a small (sub-) population. Therefore, the researcher has a responsibility to judge the size of the difference, since size matters. Consequently, one must judge the differences according to the size, their clinical relevance for the patients and the number of objects in the (sub-) analysis.

Ethics

Approvals were obtained from the Regional Ethical Review Board in Linköping, according to the Swedish act concerning the Ethical Review of Research Involving Humans (2003:460), and from the Ethical Committee of Åland, Finland. Informed consent was obtained from the patients or their parents (Paper II, III). The patients in paper IV had given a general consent to their samples being stored in a biobank and used for research purposes. Permission to read patients' medical records was given by the medical director of each department after approval from the Regional Ethical Review Board of Linköping (Paper I).

RESULTS AND DISCUSSION

Neuroborreliosis epidemiology and clinical disease (Paper I)

During the study period 2000-2005, the annual number of NB cases, verified by CSF analysis, increased from 16 to 32 cases, *i.e.* from 5 to 10 cases/100,000 inhabitants/year (Figure 12). The NB incidence previously reported from Jönköping County in 1992-1993 was also 10/100,000 inhabitants/year (Berglund et al., 1995). However, these two studies are not really comparable, since Berglund et al. included patients diagnosed with NB on the basis of either CSF findings or clinical symptoms and anti-*Borrelia* antibodies in serum, while we used the presence of anti-*Borrelia* antibodies in CSF as an inclusion criterion. This inclusion criterion was chosen in order to ensure that only true NB cases were studied. However, this approach means that very early NB cases, where anti-*Borrelia* antibodies were not yet measurable in CSF, were not included. Furthermore, we may have missed a number of patients who, during the study period, were treated with antibiotics for suspected NB infection, and for whom the diagnosis was based on symptoms and the presence of anti-*Borrelia* antibodies in serum. The true number of NB cases in the county can thus be assumed to be even higher than 10/100,000 inhabitants/year. Consequently, it cannot be claimed that our study gives a complete epidemiologic overview of NB in Jönköping County 2000-2005, but rather a reflection of the situation. The strength of the study is that the NB diagnoses were confirmed by CSF analyses.

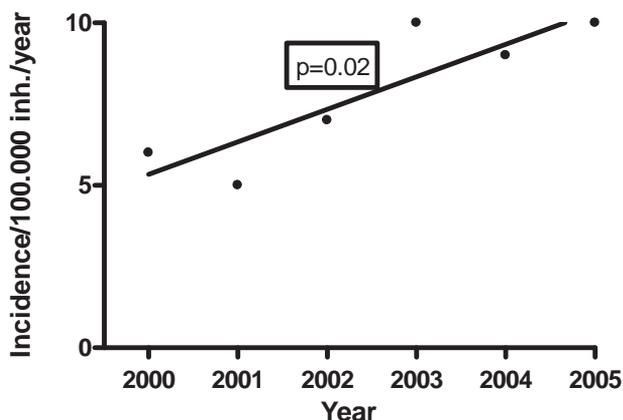


Figure 12. Incidence of neuroborreliosis cases, verified by cerebrospinal fluid analysis, in Jönköping County, Sweden, 2000-2005. Trend analysis: $p=0.02$ (Paper I).

The increase of NB cases during the study period may possibly be explained by greater awareness of *Borrelia* infections, both in the general population and among physicians. However, there might well be a real increase in NB cases, and there are several plausible explanations for this; for example a milder and more humid climate in the region (Alexandersson, 2006; Westermark et al. 2007) that may be favourable to the ticks (Kovats et al., 2001; Randolph, 2001), and increasing populations of fallow deer and wild boars in the county during the study period (Svenska Jägareförbundet, 2006). Indeed, there are indications that the tick population is increasing in Sweden (Jaenson et al., 2009; Jaenson and Lindgren, 2011), and that this is associated both with climate conditions and with the occurrence of hosts (Jaenson, 1991).

Of the included patients, 62 % were men and 38 % were women. Most cases were either children <19 years of age or adults aged 50-79 years (Figure 13). These findings are mainly consistent with earlier observations (Berglund et al., 1995; Hubalek, 2009).

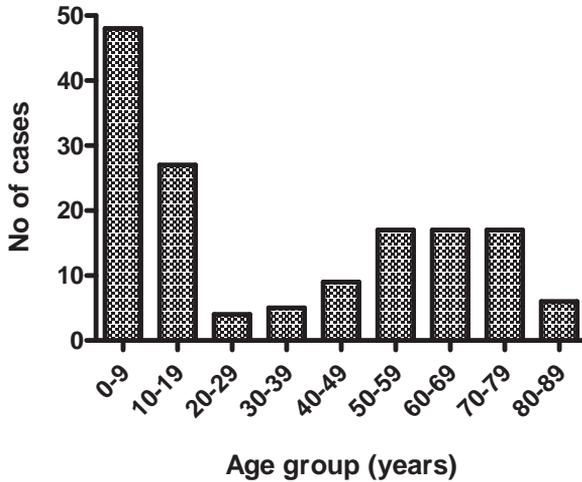


Figure 13. Number of cases of neuroborreliosis in each age group (Paper I).

Patients under 40 years of age tended to have distinct symptoms, such as facial palsy, neck pain and fever (but also fatigue) more frequently than patients over 40 years ($p=0.0001-0.031$), who more often reported unspecific symptoms, such as muscle and joint pain, radiating pain, paresthesias, vertigo and concentration difficulties ($p=0.0001-0.003$). Patients under 40 also had shorter duration of symptoms prior to diagnosis ($p<0.0001$), and they displayed more pronounced signs of CNS inflammation, as monitored by CSF mononuclear pleocytosis ($p<0.0001$), CSF-albumin ($p<0.0001$), CSF-albumin/S-albumin ratio ($p<0.0001$), CSF-lactate ($p<0.001$) and CSF-glucose ($p<0.001$), compared to patients over 40 years of age.

At follow-up, 106 patients had completely recovered, but 44 still had symptoms at the last visit (*i.e.* they were not followed until complete recovery). The 106 patients for whom the clinical course until recovery was clearly documented were classified according to the duration of post-treatment symptoms (Table 11). Group A recovered within 2 weeks, group B within 2-4 weeks, group C within 1-6 months, and group D had symptoms lasting >6 months. Significant differences in age distribution were found among all groups ($p=0.0001-0.05$), except when group B was compared to group C. Higher age was associated with longer duration of post-treatment symptoms. There were also significant differences between the groups regarding duration of symptoms prior to treatment, except when comparing group B with groups A and C ($p=0.0001-0.025$). Longer duration of symptoms before treatment was associated with longer duration of post-treatment symptoms. There were no significant differences between the groups regarding sex distribution, type of antibiotic treatment or duration of treatment. The only laboratory parameter in the acute phase that differed significantly among the groups was *Borrelia*-specific CSF IgG, which was highest in group D, but this probably just reflects the longer duration of symptoms at diagnosis in this group.

Table 11. Duration of symptoms (A-D) in relation to sex, age and duration of symptoms before treatment (Paper I).

		Patient groups, n=106*			
		A n=26	B n=33	C n=33	D n=14
Males	number (%)	16 (61.5)	25 (75.8)	21 (63.6)	6 (42.9)
Females	number (%)	10 (38.5)	8 (24.2)	12 (36.4)	8 (57.1)
Age (years)**	median (range)	8 (4-66)	12 (3-79)	22 (3-85)	56,5 (10-64)
Duration of symptoms before treatment (weeks)**	median (range)	2 (0.1-8)	2,5 (0.5-6)	3 (0.1-36)	5,5 (2-77)

* 106/150 patients where data were available.

** significant differences between groups, except between group B and C regarding age.

Groups according to duration of symptoms post-treatment: A: 0-2 weeks; B: 2-4 weeks; C: 1-6 months; D: >6 months.

The acute phase symptoms reported in groups A-D are presented in Table 12. Post-treatment symptoms lasting more than 6 months were reported by 13 % of the patients (14/106). The clinical parameters in the acute phase characterizing this patient group were advanced age, long duration of symptoms prior to treatment, symptoms of radiculitis, especially radiculitis involving the lower extremities, and unspecific symptoms, such as muscle and joint pain, paresthesia, vertigo and concentration difficulties.

Table 12. Patients categorized according to time to recovery after start of antibiotic treatment, in relation to symptoms with which they presented at the time of lumbar puncture (%) (Paper I).

Symptom	Patient groups, n=106*			
	A n=26	B n=33	C n=33	D n=14
Headache	58	42	36	43
Fatigue	58	49	49	36
Fever	31	30	15	14
Neck pain	39	46	30	43
Vertigo	0	6	9	29
Concentration difficulties	0	0	6	14
Radiculitis				
Leg	15	21	21	57
Arm	4	12	9	29
Trunk	8	3	9	36
Muscle/ joint pain	35	42	61	86
Paresthesia	4	15	33	57
Facial palsy	46	61	55	7
Other cranial nerve palsy	0	6	6	7

* 106/150 patients where data were available.

Group A: 0-2 weeks. Group B: 2-4 weeks. Group C: 1-6 months. Group D: > 6 months. Patients could have one or several of the symptoms.

Advanced age was the most prominent factor associated with long-lasting post-treatment symptoms. Based on our findings, one could speculate that young individuals can mobilize a strong and rapid inflammatory response to the *Borrelia* infection, causing more distinct clinical symptoms, which in turn lead to early diagnosis and treatment as well as effective elimination of bacteria. Accordingly, a strong initial inflammatory response has previously been demonstrated to be associated with good prognosis in borreliosis (Forsberg et al., 1995; Zeidner et al., 1996; Kang et al., 1997; Widhe et al., 2002; Sjowall et al., 2005).

Increased levels of anti-*Borrelia* antibodies were found in CSF but not in serum in 25 (17 %) of the patients at the time of diagnosis. These patients had symptoms with a median duration of 3 weeks (range 1 day to 8 weeks). This finding emphasizes the importance of CSF analysis for the diagnosis of NB, especially in patients whose symptoms have lasted less than 8 weeks when they seek medical care.

Possible limitations of this study are that very early NB cases may have been overlooked, as discussed above, and the retrospective study design presumably entailed a bias in both reported and documented symptoms. In addition, there were difficulties obtaining data on time to recovery for all patients. However, the data were available in a substantial number of cases and, furthermore, our data are in line with those of previous prospective studies (Berglund et al., 1995), supporting the accuracy of the results.

Since previous data on economic aspects of NB in a European perspective were scarce (Joss et al., 2003), we wanted to estimate the cost for health care and social benefits associated with this diagnosis (Table 13). The number of out-patient visits per individual varied between 0 and 10 (median 3), and 79 patients (53 %) were hospitalized (median 5 days, range 1-60 days). The estimated total cost of hospital care was 4,200 EUR per patient. (For comparison, the estimated costs of hospital care were 2,800 EUR per patient for pneumonia, 2,300 EUR per patient for viral meningitis, and 5,200 EUR per patient for sepsis). Of the adult patients of working age (16-64 years), 26 (51 %) received sickness benefits from the Social Insurance Agency (median 32 days, range 4-528 days). The mean cost of sickness benefits was 4,600 EUR per patient. Temporary parental benefits were granted to the parents of 39 (51 %) of the children (median 5 days, range 0.25-19 days). The estimated total cost of all social benefits taken together was 2,000 EUR per patient. We conclude that NB leads to important expenditure on health care as well as on social benefits, and reduces the working capacity of individuals for extended periods of time.

Table 13. Health care cost and social benefits, n=150 (Paper I).

	Study population	Per patient	Estimated cost (EUR)
No. of visits to out-patient department	490	range 0-10 median 5	130,000
No. of hospitalizations	79 (=603 d.) (53 % of patients)	range 1-60 d. median 5 d.	330,000
Ceftriaxone doses administered in out-patient department	509 doses	-	38,000
No. of cases with temporary parental benefit	39 (=211 d.) (51 % of children)	range 0.25-19 d. median 5 d.	14,000
No. of cases with sickness benefit	26 (=1653 d.) (51 % of adults aged 16-64 y.)	range 4-528 d. median 32 d.	120,000
Total			632,000

d: days; y:years; EUR: Euro.

Complement activation and evasion (Paper II-III)

Human studies

Since previous data from *in vitro* experiments and animal studies have shown that *B. burgdorferi* s.l. activates complement and that some *Borrelia* species also have the ability to circumvent the effects of complement (Brade et al., 1992; Breitner-Ruddock et al., 1997; van Dam et al., 1997; Kraiczy et al., 2000; Alitalo et al., 2001), it is reasonable to hypothesize that complement activation is important for protective immunity against *Borreliae* also in humans. However, published data on complement activation patterns from human *in vivo* studies have been lacking. Therefore, we monitored the concentration and activation of complement in plasma and in CSF from 298 patients, of whom 23 were diagnosed with NB (Paper II). The different patient groups are recapitulated in Table 14.

No significant differences were found in the plasma levels of C1q, C4, C3 or C3a between the control patients and those in groups NB, B, CNS and I, with the exception of C4, which was slightly lower in group B than in the control group ($p=0.003$).

Table 14. Patients and diagnostic groups (Paper II).

Diagnostic group, subgroup	<i>n</i>	Mean age (range)	Male:female ratio	CSF pleocytosis	CSF <i>Borrelia</i> antibodies	* Seroconversion or increasing titers of serum antibodies
NB	23	55 (6-87)	10:13	+/-	+/-	+
Confirmed	15	55 (6-87)	5:10	+	+	+
Probable	8	54 (30-79)	5:3	+/-	+/-	+
Other <i>Borrelia</i> infection (B)	47	51 (3-93)	25:22	-	-	+
CNS involvement, non- <i>Borrelia</i> (CNS)	20	56 (22-85)	12:8	+	-	-
Other non-CNS infection (I)	16	52 (29-83)	9:7	-	-	-
Controls (C)	192	57 (5-91)	90:102	-	-	-

* Since Åland is an endemic area for Lyme borreliosis, some patients in the CNS, I and C groups could have positive *Borrelia*-specific IgG titers, but these titers were stationary and not increasing.

However, in contrast to serum levels, C1q, C4, C3 and C3a in the CSF were significantly elevated in patients with NB when compared to controls and to the groups B and I (Figure 14a-b), see also Appendixes, Paper II, Table 3 for C4 and C3 data). In the case of the CNS group, the C1q level was significantly higher than that of the control group ($p < 0.0001$). The other complement components also showed a tendency toward being elevated in the CNS group ($p = 0.03-0.1$), but this increase was not always significant (see Appendixes, Paper II, Table 3). For the NB and CNS groups, correlations were found between the C1q and the C3a levels in CSF (for the NB group: $r = 0.498$, $p = 0.018$; for the CNS group: $r = 0.697$, $p < 0.0001$).

Regarding the NB group, the elevated levels of C3a in CSF indicate that activation of the complement system does occur within the CNS. The elevated levels of C1q and C4, together with the correlation between C1q and C3a, suggests activation via the CP, and points to the possible involvement of complement-fixing antibodies, *i.e.* a Th1-type of immune response. This is in concordance with previous studies showing that LB is a Th1-dominated disease (Forsberg et al., 1995; Oksi et al., 1996; Ekerfelt et al., 1997; Yin et al., 1997; Ekerfelt et al., 1998; Gross et al., 1998; Grusell et al., 2002), and that the predominant IgG subclasses in both serum and in CSF in LB are IgG₁ and IgG₃, the subclasses that are complement-activating as well as opsonizing in humans (Olsson et al., 1987; Hechemy et al., 1988; Widhe et al., 1998). Thus, our complement data suggest an important role for complement activation in NB and support the notion of a pro-inflammatory Th1-biased immune response.

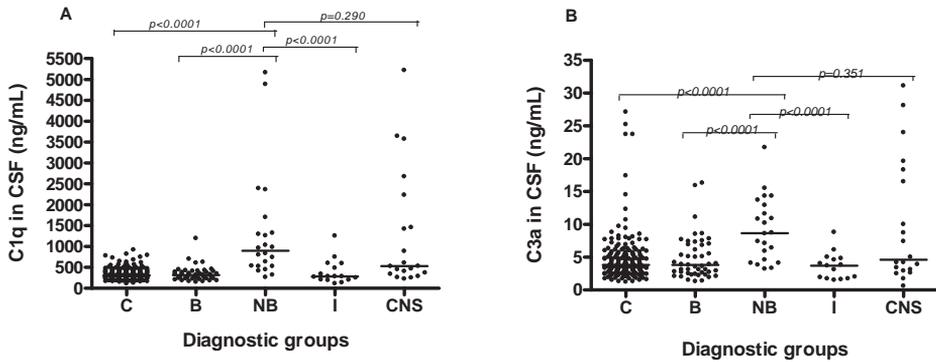


Figure 14. A) C1q and B) C3a in cerebrospinal fluid (CSF) (Paper II).
 Patient groups: C: control patients (n=192). B: other *Borrelia* infection (n=47). NB: neuroborreliosis (n=23). I: other non-CNS infection (n=16). CNS: other CNS, non-*Borrelia* involvement (n=20).

The elevated levels of C1q, C4, C3 and C3a in CSF from NB patients were apparently due to neither systemic synthesis nor systemic activation, since normal levels of all these complement components were found in the plasma, and no systemic complement activation occurred. Of course, leakage of complement from the systemic circulation may have occurred, since raised levels of complement components in the CSF were correlated with damage to the BBB (for C1q in NB: $r=0.685$, $p<0.0001$), as indicated by an elevated CSF-albumin/S-albumin ratio. We also found a correlation between C1q in CSF and CSF pleocytosis (for NB: $r=0.734$, $p<0.0001$), but this correlation could merely be a result of strong inflammation in the CNS. Still, we did not find any significant differences between the study groups in terms of plasma levels of complement components. Furthermore, when we calculated a C1q index in order to compensate for the effect of BBB damage on CSF levels, we still found highly significant differences in the C1q index between the NB and the control groups (Table 15). Also the C3 index was significantly elevated in the NB group as compared to the controls. The fact that we did not see significant differences in the C3a index may be a reflection of the very low C3a levels in the CSF. Surprisingly, the C4 index was increased in those with other *Borrelia* infections not affecting the CNS (group B). However, this increase was probably caused by the low plasma levels of C4 seen in this group, since the C4 level itself was not elevated in the CSF. It is not clear why group B had lower C4 levels in plasma. Since the C4 assay measures total C4 (including activated forms), it is not likely that the low plasma levels are caused by C4 consumption due to complement activation. Thus, the possible impact of complement defects in LB would be an interesting subject for future studies.

Table 15. Complement intrathecal production index (Paper II).

Diagnostic group	C1q index		C3 index		C3a index		C4 index	
	Median, range	p-value* compared to controls						
Neuroborreliosis (NB)	0.10 0.02-0.31	<0.0001	0.06 0.03-0.19	0.032	0.84 0.27-2.07	0.6	0.08 0.05-0.50	0.3
Other borrelia infection (B)	0.06 0.03-0.17	0.6	0.05 0.03-0.14	0.9	0.80 0.07-4.91	0.5	0.11 0.05-0.24	0.015
CNS-involvement, non-borrelia (CNS)	0.15 0.04-0.48	<0.0001	0.05 0.04-0.10	0.2	0.90 0.27-4.08	0.3	0.11 0.07-0.17	0.2
Other non-CNS infection (I)	0.06 0.03-0.14	0.9	0.05 0.03-0.11	0.3	0.65 0.13-1.36	0.2	0.08 0.06-0.18	0.5
Controls (C)	0.06 0.02-0.27	-	0.05 0.02-0.41	-	0.75 0.14-9.30	-	0.09 0.03-0.62	-

* The control group (C) was compared to each of the other four diagnostic groups using the Mann-Whitney test. p-values <0.05 were considered to be significant.

Based on our findings, it is reasonable to believe that complement is synthesized locally within the CNS in NB. This notion is supported by previous studies demonstrating that human glial and neuronal cell lines express and secrete all the components of the CP and the AP, resulting in a functional lytic complement complex (Gasque et al., 1992; Rus et al., 1992; Gasque et al., 1993; Gasque et al., 1995; Francis et al., 2003). Inflammatory cytokines such as IFN- γ , IL-1 β and TNF, have been shown to enhance the synthesis of all complement components, and to induce the synthesis of C1q (Morgan and Gasque, 1996). This finding is particularly interesting because earlier studies have indicated that good prognosis in NB is associated with elevated intrathecal levels of IFN- γ and TNF in the early stage of the disease (Forsberg et al., 1995; Ekerfelt et al., 1998; Widhe et al., 2002; Widhe et al., 2004; Sjowall et al., 2005).

In this study, we were unable to find any convincing evidence that complement levels affect the clinical course of NB. However, the number of NB patients was not very large, and only two patients seemed to have had symptoms for longer than 6 months. In addition, the duration of symptoms after initiation of antibiotic treatment was not systematically documented, since this study was focused on the early phase of the disease. Further studies with long-term follow-up of a larger number of patients are needed to investigate whether the complement activation pattern is associated with active disease and/or clinical outcome. Interestingly, Schutzer et al. have recently published proteome data indicating that patients with neurologic Post-Treatment Lyme Disease display elevated CSF levels of several complement components, such as C1s, C1q and C4b, as compared to patients with chronic fatigue syndrome and healthy

controls (Schutzer et al., 2011), although there may be concerns about the diagnostic criteria used in this study. Aberrant complement activation is thought to play a role in maintaining local inflammation and may contribute to brain tissue damage in neurodegenerative disorders (Gasque et al., 2000; Francis et al., 2003). Moreover, it could also contribute to pathogenesis of long-lasting post-treatment symptoms in NB.

In Paper III, we studied local complement activation in skin biopsies from 24 patients with EM. Both *B. afzelii* and *B. garinii* were visualized in the biopsies taken from the EM (see Appendixes, Paper III, Figure 1). Deposition of C3/C3 fragments was found in connection with the spirochetes, more around *B. garinii* than around *B. afzelii*, illustrating *in vivo* the ability of *B. afzelii* to protect themselves from complement activation. No spirochetes or deposited C3 were detected in the control biopsies taken from healthy skin of the same individuals. IgG was detected in EM from both *B. garinii* and *B. afzelii*, and was found to co-localize with C3, indicating complement activation via the CP. Preliminary, we could not visualize deposition of factor H or leukocyte infiltration in the EM or control biopsies, but this needs to be further investigated after optimization of the method. There were no significant differences regarding age, sex, EM size, EM appearance, reported symptoms or time to recovery related either to the causative genospecies, or to the degree of local complement activation. However, the number of patients was probably too small for reliable statistical analysis.

In general, the diagnostic value of microscope-based assays in the clinical laboratory is limited, as the spirochete density in clinical samples is often very low (Aguero-Rosenfeld et al., 2005; Samuels and Radolf, 2010). Spirochete morphology is also variable, making these microorganisms difficult to distinguish from host tissue structures (Aberer and Duray, 1991). However, visualizing *Borrelia* spirochetes in human EM biopsies has been done by others before (Eisendle et al., 2007). In our study, we selected PCR-positive EM biopsies and an immunological staining method, which improved the sensitivity considerably. In addition, we used control biopsies from healthy skin of the same individuals in order to increase the specificity.

Experimental studies

The different degree of complement activation by *B. afzelii* and *B. garinii* seen in EM biopsies could be confirmed by our *in vitro* experiments (Paper III) with clinical isolates. *B. garinii* LU59 induced higher complement activation than *B. afzelii* K78, measured as the generation of C3a and sC5b-9 in plasma (Figure 15a-b). In contrast, no significant difference was seen between the two isolates in an identical experiment using blood from an individual with anti-*Borrelia* IgG antibodies (Figure 15c-d).

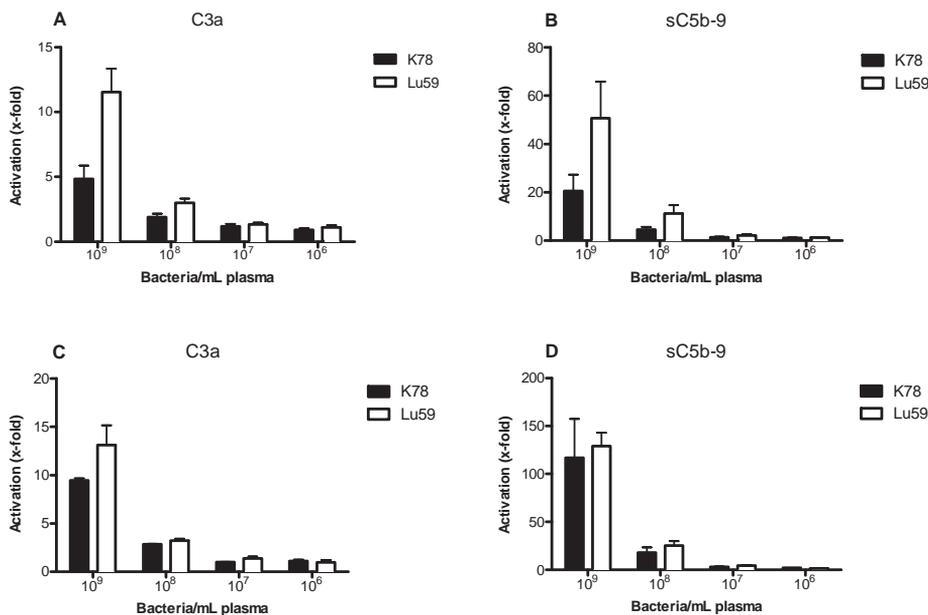


Figure 15. Complement activation in human plasma, measured as generation of C3a (A,C), and sC5b-9 (B, D). Panel A and B: donors without *Borrelia*-specific antibodies ($n=8$). Panels C and D: donor with *Borrelia*-specific IgG ($n=1$), two assays at two different occasions. Data are normalized against plasma incubated without bacteria (Paper III). K78: *B. afzelii* K78. LU59: *B. garinii* LU59.

Binding of factor H and opsonization with C3/C3 fragments on the spirochetal surface were quantified by ELISA, confirming that *B. afzelii* K78 recruited more factor H in order to inhibit complement activation via the AP (Figure 16b). No convincing difference was seen between the two isolates regarding deposition of C3/C3 fragments (Figure 16a). Interestingly, *B. garinii* LU59 was found to bind more C4BP, complement regulator of the CP (Figure 16c), a finding that is congruent with previous data (Pietikainen et al., 2010).

The *Borrelia* spirochetes were phagocytosed both by granulocytes and by monocytes (data only shown for granulocytes), which corroborates previous studies (Benach et al., 1984a; Benach et al., 1984b; Peterson et al., 1984). In addition, we were able to demonstrate that the degree of complement activation plays a crucial role in the process of phagocytosis. Phagocytosis was substantially decreased, but not obliterated, in the presence of compstatin as well as by a C5aRa, while EDTA completely inhibited phagocytosis (Figure 17a). Similar results were obtained regarding phagocytosis by monocytes (data not shown). Compstatin is a synthetic peptide that selectively inhibits proteolytic activation of complement component C3, thereby exerting direct inactivation of the complement system. The C5aRa blocks C5a mediated up-regulation of granulocytes and monocytes, thus suppressing the effects of

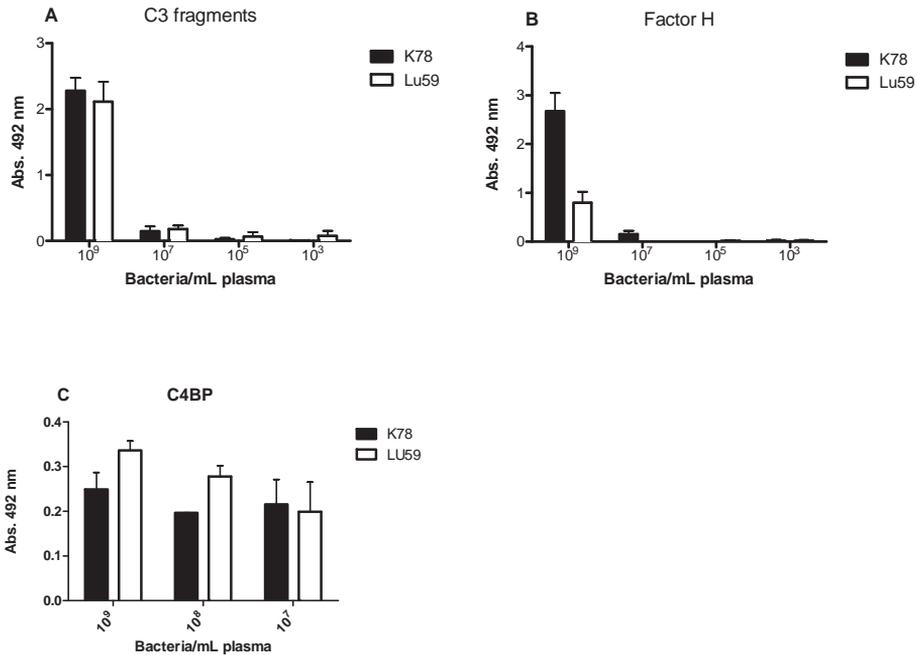


Figure 16. Quantification of C3 fragments (A), factor H (B) and C4BP (C) bound to the surfaces of *Borrelia afzelii* K78 and *Borrelia garinii* LU59. Data are presented as absorbance values of plasma samples incubated with bacteria minus absorbance of plasma incubated without bacteria (Paper III).

complement activation on phagocytic capacity. EDTA acts as a chelator that binds Ca^{2+} and Mg^{2+} , ions that are necessary for a functional complement system, but also for a number of other biochemical reactions. Therefore, the addition of EDTA could possibly affect other mechanisms involved in phagocytosis, and not uniquely the complement system.

Intriguingly, the phagocytosis assay showed that *B. afzelii* K78 was more extensively phagocytosed compared to *B. garinii* LU59. This is contradictory to the results from the complement activation assays where *B. garinii* LU59 was more abundantly opsonized by C3/C3 fragments. One possible explanation could be that *B. garinii* LU59, with an impaired mechanism for efficient factor H recruitment and complement protection, may become lysed and fragmented more rapidly than *B. afzelii* K78. The more intact *B. afzelii* K78 spirochetes would have more C3/C3 fragments on their surface than the *B. garinii* LU59 fragments, and would therefore be more easily phagocytosed.

The presence of specific anti-*Borrelia* antibodies seems to play a crucial role both in complement activation and in the process of phagocytosis. The difference in the degree of complement activation between *B. afzelii* K78 and *B. garinii* LU59 was not

seen when using plasma from a donor with antibodies (Figure 15c-d). Similarly, when blood from a donor with anti-*Borrelia* antibodies was used, there was considerable residual phagocytosis when complement was inhibited (Figure 17b). The difference in the results from the phagocytosis experiments using blood from non-immunized and immunized individuals further emphasizes the central role of complement early in the infection, prior to the generation of specific anti-*Borrelia* antibodies. However, these results need to be confirmed in future experiments.

Based on our findings, it could be speculated that *B. garinii* strains incapable of inactivating the AP of complement by recruitment of factor H, would try to find an immunologically privileged site such as the CNS in order to escape the effects of complement in early infection. This could at least partly explain the neurotropism observed in this genospecies (van Dam et al., 1993). However, this cannot be the full explanation, since *B. garinii* OspA ST4, which has been repeatedly isolated from human CSF (Wilske et al., 1993; Wilske et al., 1996; Marconi et al., 1999), is able to bind FHL-1 quite efficiently (van Burgel et al., 2010). As seen in Paper II, during established CNS borreliosis, there is evidence suggesting complement activation via the classical pathway in CSF. *B. garinii* has previously been shown to be able to bind C4BP (Pietikainen et al., 2010), and this was confirmed by our *in vitro* experiments. Binding of C4BP probably constitutes an important virulence factor for spirochetal survival in the presence of antibodies.

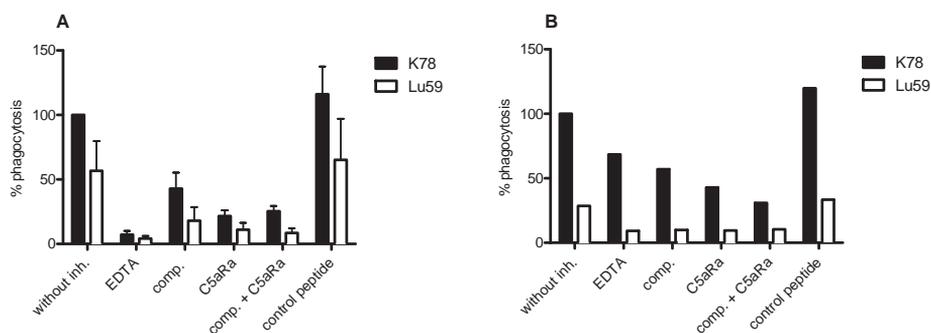


Figure 17. Phagocytosis of *Borrelia afzelii* K78 and *Borrelia garinii* LU59. The mean fluorescence intensity for hirudin blood incubated with *Borrelia afzelii* K78 without complement inhibitors is set to 100%. A: Blood from donors without *Borrelia*-specific antibodies (n=3). B: Blood from donor with *Borrelia*-specific IgG. EDTA: ethylene diaminetetraacetic acid. C5aRa: C5a receptor antagonist. Inh: inhibitors. Comp: compstatin (Paper III).

Relative balance between Th1, Th2 and Th17 responses (Paper III-IV)

Human studies

In Paper IV, we aimed at elucidating the balance between cytokine/chemokines related to different Th cell populations. For this purpose, we used a large number of collected serum and CSF samples from patients investigated for suspected NB. Clinical and laboratory characteristics of the different patient groups are summarized in Table 16. The groups were determined based on CSF findings, and this turned out to be in concordance with the median duration of symptoms before diagnosis. Notably, the patients in group 1 (confirmed NB) displayed marked BBB impairment (CSF-albumin/S-albumin ratio). The age distribution differed significantly among the groups, as described previously in the Materials and Methods section. Radiculitis was most frequently reported in groups 1 (confirmed NB) and 2 (possible late NB), which consisted of more adults, than group 3 (possible early NB), which consisted uniquely of children <15 years of age. Vaguer symptoms, such as vertigo, muscle and joint pain, paresthesias, fatigue, dementia or concentration difficulties, were more common in group 4 (non-NB).

The cytokine and chemokine data from the different study groups are presented in Figure 18a-h. The most prominent differences were found in the CSF. Groups 1 and 3, *i.e.* confirmed NB and possible early NB, showed increased levels of CXCL8 (Figure 18b) and CXCL10 (Figure 18f) as compared to the other groups. CCL22 was particularly elevated in group 1, and moderately elevated in group 3. Here, the time aspect is probably of importance for the chemokine profile. Early in the inflammation process, a Th1-type immune response (CXCL10) dominates (as in group 3, possible early NB), but is then counter-balanced by a Th2 response (CCL22), (as in group 1, confirmed NB). This pattern was also found within group 1, *i.e.* patients with duration of symptoms >2 weeks also tended to have higher CCL22 levels in the CSF than those with shorter symptom duration ($p=0.023$, data not shown in figure). This scenario is in line with previous observations indicating that Th2 controls the primary Th1 response, the latter being crucial in elimination of bacteria but also potentially involved in tissue damage (Oksi et al., 1996; Ekerfelt et al., 1997; Kang et al., 1997; Yin et al., 1997; Widhe et al., 1998; Ekerfelt et al., 2003; Widhe et al., 2004; Jarefors et al., 2007).

Remarkably, half of the patients in group 1, confirmed NB, had elevated levels of IL-17A in the CSF, while it was not detectable in the CSF of the other groups (Figure 18d). The role of the increase in IL-17A is unclear, since there were no significant differences between those with high levels of IL-17A in CSF and those with low levels in terms of sex, age, duration of symptoms prior to diagnosis (see Appendix, Paper IV,

Table 16. Characteristics of the different study groups (Paper IV).

	Group 1 Confirmed NB <i>n</i> =133	Group2 Possible late NB <i>n</i> =19	Group 3 Possible early NB <i>n</i> =15	Group 4 Non-NB <i>n</i> =96	Group 5 Control patients <i>n</i> =17
<i>Borrelia</i> -specific AI or <i>Borrelia</i> -specific antibodies in CSF	+	+	-	-	-
CSF pleocytosis	+	-	+	-	-
CSF-albumin median S-albumin (range)	16*** (3.4-76)	5.5 (2.4-21)	3.5 (0-12)	4.3 (0-22)	n.d.
<i>Borrelia</i> -specific IgG/IgM antibodies detected in serum; <i>n</i> , (%)	115*** (87)	15* (79)	15*** (100)	42 (44)	0*** (0)
Men (%)	80 (60)	14 (74)	9 (60)	52 (54)	9 (53)
Women (%)	53 (40)	5 (26)	6 (40)	44 (46)	8 (47)
Median age; years, (range)	35** (3-87)	52 (18-76)	7*** (2-13)	44 (2-83)	66* (50-72)
Median duration of symptoms before LP; weeks, (range)	2.4 (0-32)	4.0 (0.1-77)	0.7 (0.1-4.0)	3.5 (0-730)	-
Head/neck pain (%)	77 (58)	11 (58)	11 (73)	43 (45)	-
Cranial nerve palsy (%)	70 (53)	4 (21)	11 (73)	13 (14)	-
Radiculitis (%)	53 (40)	7 (37)	0 (0)	3 (3)	-
Other symptom (%)	9 (7)	2 (11)	0 (0)	39 (41)	-

NB: neuroborreliosis.; *n*: number of patients; AI: antibody index; CSF: cerebrospinal fluid; pleocytosis: >5 mononuclear cells/mL CSF; S: serum; LP: lumbar puncture; n.d: not done.

Patients could have one or more of the symptoms head/neck pain, cranial nerve palsy and radiculitis. Patients with none of the above symptoms were classified as "other symptom" (e.g. vertigo, muscle and joint pain, paresthesias, fatigue, dementia or concentration difficulties).

* significant differences compared to the non-NB group (group 4). * $p < 0.01$, ** $p < 0.001$, *** $p < 0.0001$.

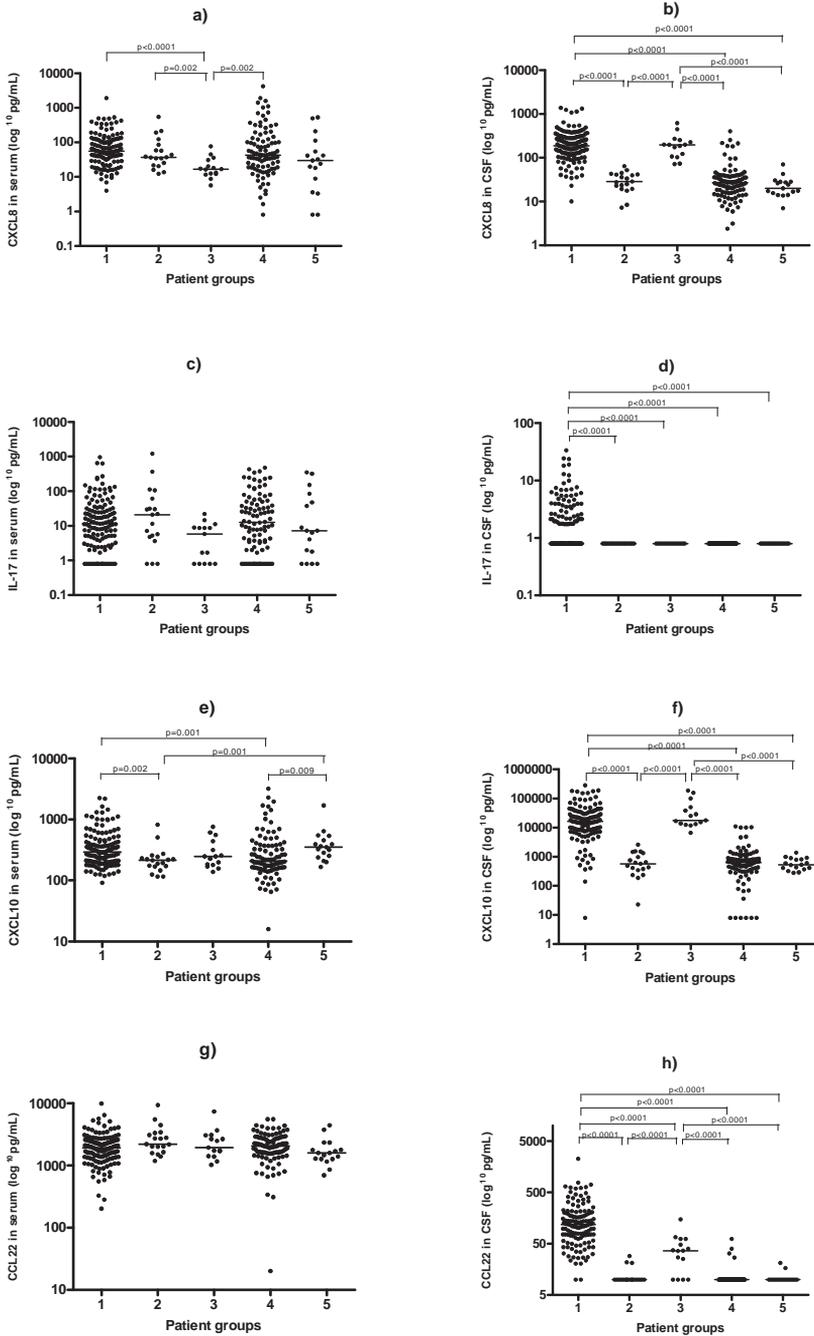


Figure 18. Cytokine/chemokine levels (pg/mL) in serum and in cerebrospinal fluid. Group 1: Confirmed neuroborreliosis (NB); Group 2: Possible late NB; Group 3 Possible early NB; Group 4: Non-NB; Group 5: Control group (Paper IV).

Table 3), or duration of symptoms after treatment (data not shown). There was a slightly more pronounced pleocytosis ($p=0.004$) and a tendency to higher levels of CXCL10 in CSF ($p=0.049$) in patients with elevated IL-17A in CSF compared to those with low levels. The association with pleocytosis suggests that IL-17A is associated with ongoing inflammation, although the absence of IL-17A in group 3 is then puzzling. However, it could be speculated that children may show delayed Th17 responses. The presence of IL-17A in the CSF of NB patients has also been reported from the Islands of Åland (Nordberg et al., 2011). Since IL-17A has proved important in the context of extra-cellular bacteria as well as in the induction of immune-mediated tissue damage (Awasthi and Kuchroo, 2009), further prospective studies on IL-17A and its role in the pathogenesis and clinical outcome of NB are warranted.

We found evidence supporting the intrathecal origin of the induced cytokine/chemokines; (1) no correlations between serum and CSF levels (data not shown), (2) the same magnitude (or higher) of CSF levels compared to serum for CXCL8, CXCL10 and IL-17A (Figure 18), and (3) increased chemokine intrathecal production indices for CXCL8, CXCL10 and CCL22 in groups 1 and 3 (Table 17). The intrathecal production index could not be calculated for IL-17A, due to undetectable levels in CSF in four of the five groups. Yet, it seems that IL-17A in about half of confirmed NB cases is produced intrathecally since neither the serum levels of IL-17A ($\rho=0.09$, $p=0.316$) nor the CSF-albumin/S-albumin ratio ($\rho=0.18$, $p=0.045$) correlated with the CSF levels of IL-17A within group 1.

Table 17. Chemokine intrathecal production indices for the different patient groups[‡] (Paper IV).

		Group 1 Confirmed NB <i>n</i> =133	Group 2 Possible late NB <i>n</i> =19	Group 3 Possible early NB <i>n</i> =15	Group 4 Non-NB <i>n</i> =96
CXCL8 index	median (range)	0.23* (0.01-2.3)	0.11 (0.01-0.5)	4.56*** (0.19-8.63)	0.16 (0-7.40)
CXCL10 index	median (range)	3.13*** (0-76.7)	0.42 (0.02-1.08)	14.4*** (5.21-96.4)	0.59 (0-10.9)
CCL22 index	median (range)	0.30*** (0-7.00)	0.07 (0-0.20)	0.20*** (0-2.00)	0.10 (0-7.00)

Chemokine index: (CSF-chemokine/S-chemokine)/(CSF-albumin/S-albumin); NB: neuroborreliosis; *n*: number of patients; CSF: cerebrospinal fluid; S: serum.

‡: Indices could not be calculated for group 5, since CSF-albumin/S-albumin ratios were not available for all.

The CCL22 index was multiplied by 100 due to much higher concentrations in serum.

The IL-17A index was not calculated since IL-17A was not detectable in CSF in groups 2, 3 and 4.

* significant differences compared to the non-NB group (group 4). * $p<0.01$, ** $p<0.001$, *** $p<0.0001$.

In serum, there were few significant differences across groups; the CXCL8 levels were lower in group 3, children with possible early NB (Figure 18a), whereas the CXCL10 levels were increased in group 1, confirmed NB, possibly reflecting that the

strong Th1 response in the CNS also is associated with a systemic response (Figure 18e). However, CXCL10 was also elevated in serum from group 5, the orthopaedic control group. It could be speculated that this finding might be related to age (Antonelli et al., 2006). Otherwise, no significant differences were found across groups for cytokine/chemokine levels in serum, confirming that the inflammatory process is mainly restricted to the CNS compartment (Ekerfelt et al., 1997).

We also attempted to find correlations between cytokine/chemokine levels and clinical parameters including the course of the disease. No such correlations were found (data not shown). However, we cannot exclude the possibility that such correlations could have been revealed if a standardized follow-up protocol had been applied in a prospective manner. There were no differences in cytokine/chemokine levels in serum or in CSF between men and women.

One limitation to the study (Paper IV) is the difficulty in finding an appropriate control group. The use of completely healthy control patients with no symptoms and no other disorders would of course be desirable, but it is generally very difficult to obtain CSF samples from healthy volunteers. Importantly, we included a control group (group 5) from which CSF was not obtained due to suspicion of NB. We also believe that our non-NB group (group 4) is quite useful, since the patients had neither signs of ongoing *Borrelia* infection, nor any signs of CNS inflammation, and it is our opinion that the inflammation in NB is mainly restricted to the CNS compartment. Furthermore, there were no significant differences between the non-NB group and the control group (group 5), except for higher serum levels of CXCL10 in the control group, as discussed above.

Another possible limitation to the study is the difference in age between patients in group 3 (children with possible early NB) and patients in the other groups. However, when comparing group 3 with the children in groups 1 and 4, we obtained the same results as when including the adults in the analysis (see Appendix, Paper IV, Figure 2).

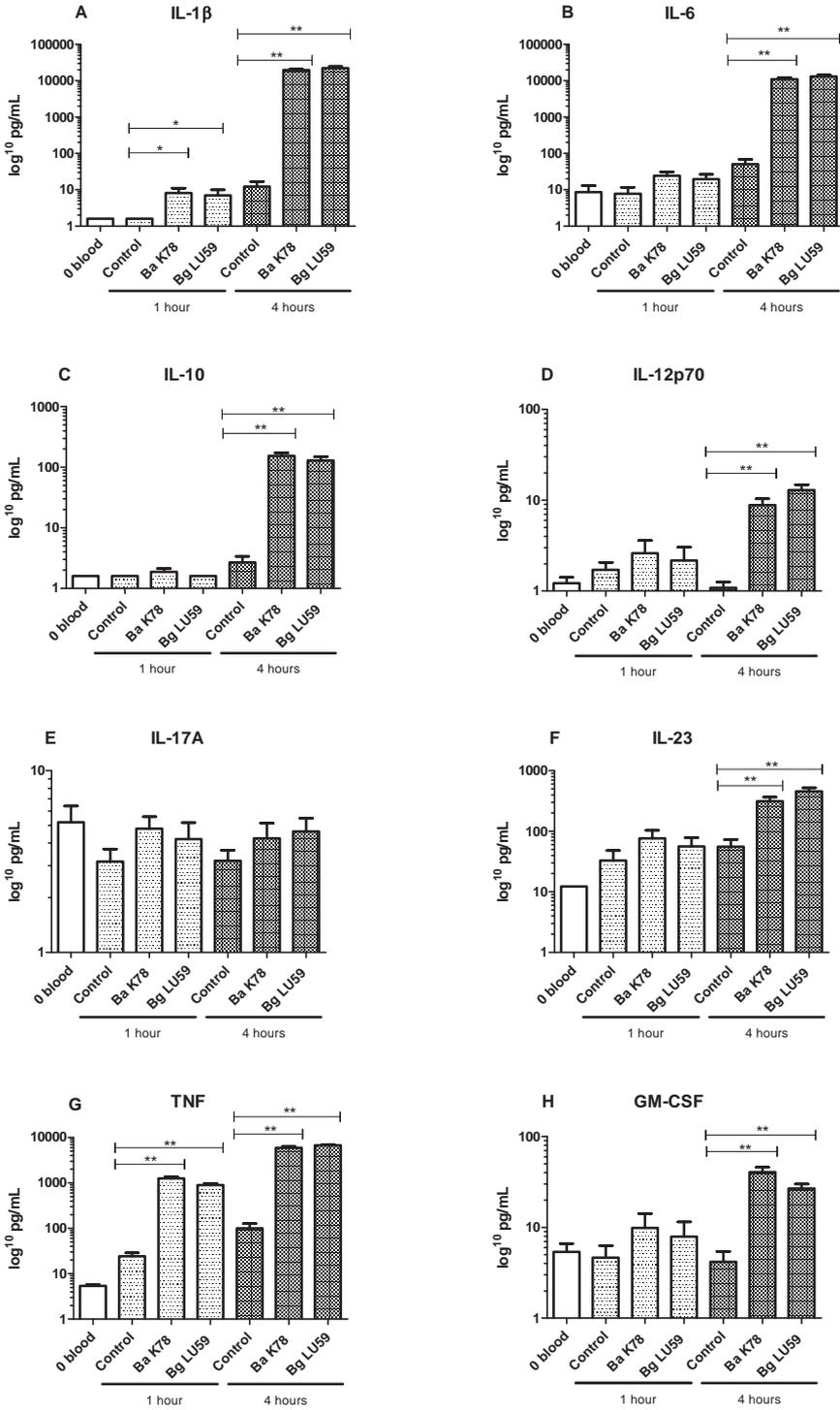
Experimental studies

In Paper III, *B. afzelii* K78 and *B. garinii* LU59 were incubated in human blood from three donors for one and for four hours, respectively. Concentrations of released cytokines and chemokines were then measured, and the results are presented in Figure 19a-n. We found a rapid and pronounced pro-inflammatory response, associated mainly with the Th1- and Th17-types of responses. After just one hour of incubation, significantly elevated levels of IL-1 β , CXCL8, TNF and CXCL1 could be detected in the samples incubated with spirochetes as compared to samples incubated with PBS + Ca²⁺. After four hours of incubation, all analytes were significantly elevated compared to the PBS + Ca²⁺ controls, with the exception of IL-17A and CCL22. The non-elevated levels of IL-17A were expected, since this Th-derived response is likely to occur later than after four hours. However, we did find elevated levels of several other

Th17-associated cytokines and chemokines (IL-6, IL-23, CXCL1, CXCL8, CCL20), suggesting an early Th17 response in LB. The low CCL22 levels indicate a lack of Th2 response in the early phase of infection, a finding that corroborates previous data (Oksi et al., 1996; Ekerfelt et al., 1997; Kang et al., 1997; Yin et al., 1997; Ekerfelt et al., 2003; Widhe et al., 2004) and our studies of NB patients (Paper IV). There were no significant differences between *B. afzelii* K78 and *B. garinii* LU59 in regard to the levels of the released cytokines and chemokines, except for TNF, which was slightly more elevated after one hour of incubation with *B. afzelii* K78 ($p=0.025$), (data not shown).

Interestingly, we found that after four hours of incubation with *Borrelia* spirochetes, there were already significantly elevated levels of IL-10. IL-10 is considered a key negative regulator of pro-inflammatory cytokine release and function. It has been shown that *B. burgdorferi* s.l. induces secretion of IL-10 in human mononuclear cells (Giambartolomei et al., 1998), and that the clearance of *B. burgdorferi* s.l. in IL-10 deficient mice is ten times higher than in wild-type mice (Lazarus et al., 2006). This suggests that *B. burgdorferi* s.l. may induce IL-10 to inhibit the host's immune defence.

Further investigation of the role of complement activation in cytokine and chemokine release in *Borrelia* infection would be an interesting prospect for future studies. Indeed, there are indications that complement activation can initiate Th17 cell differentiation and expansion in certain autoimmune diseases, since C5a has been shown to promote Th17 responses and arthritis in mice, whereas C5a receptor-deficient mice showed inhibited Th17 expansion and no development of arthritis (Hashimoto et al., 2010). Whether complement activation may have a similar role in humans is not known.



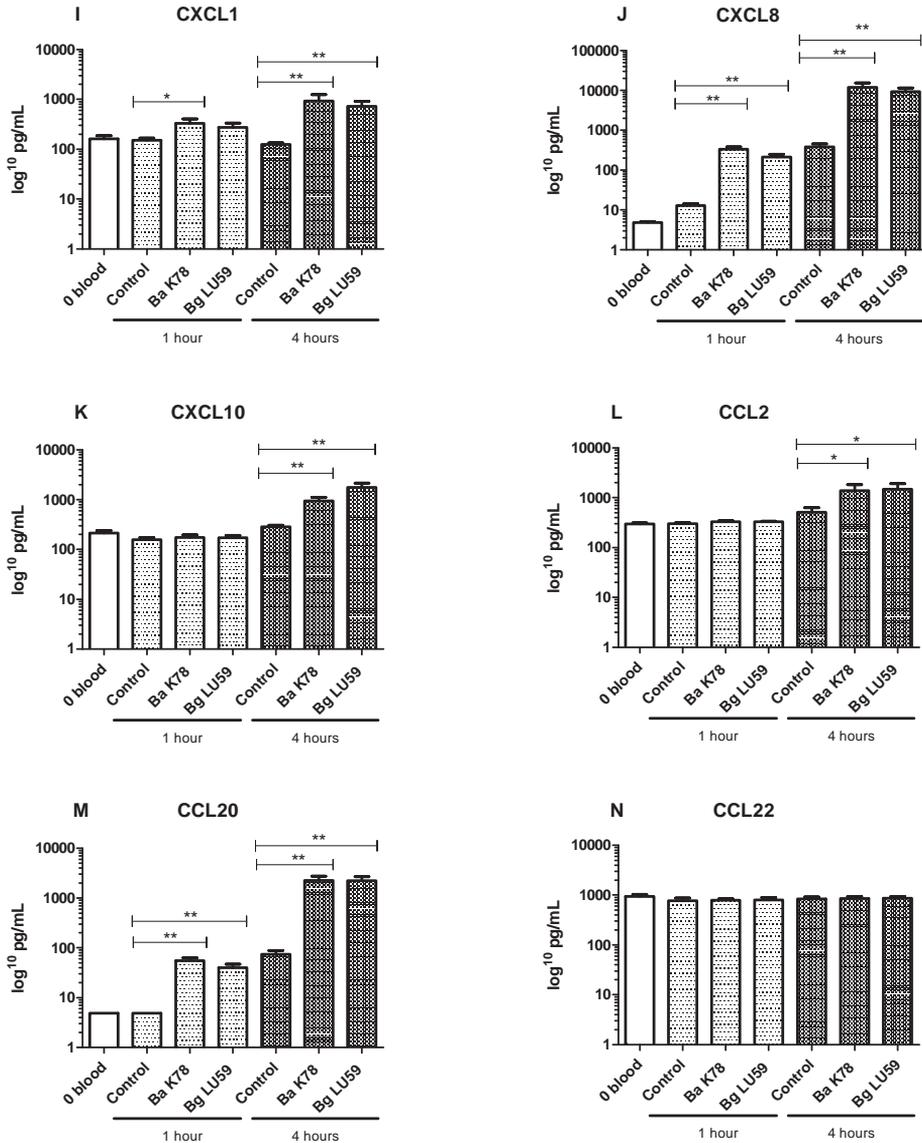


Figure 19. Cytokine and chemokine levels in plasma from three donors after incubation with and without *Borrelia spirochetes*. 0 blood: Plasma collected from hirudin blood, with addition of ethylenediaminetetraacetic acid (EDTA) and frozen immediately after sampling. Control: hirudin blood incubated with phosphate-buffered saline (PBS) + Ca^{2+} . Incubations were performed in hirudin blood in duplicates for 1 and 4 hours (h), respectively, either with PBS + Ca^{2+} , *Borrelia afzelii* (Ba) K78 or *Borrelia garinii* (Bg) LU59. * $p < 0.05$. ** $p < 0.01$ (Paper III).

CONCLUDING REMARKS

We found that NB incidence tended to increase in Jönköping County, Sweden, during 2000-2005. Patients <40 years of age displayed more pronounced CSF pleocytosis and more distinct clinical symptoms than older patients, possibly leading to earlier diagnosis and treatment. In 17 % of the NB patients, anti-*Borrelia* antibodies were detected in CSF but not in serum at the time of diagnosis, emphasizing the importance of CSF analysis for the diagnosis of NB. Post-treatment symptoms persisting for more than six months occurred in 13 % of the NB cases, and the patients concerned were significantly older, had longer-lasting symptoms prior to treatment, had higher levels of anti-*Borrelia* IgG antibodies in CSF, and reported symptoms of radiculitis more frequently. The costs of healthcare and social benefits related to NB are quite important, especially in patients with unspecific and long-lasting symptoms. Early diagnosis of NB would result in reduced human suffering and economic gain.

The complement system is activated locally in the skin in EM, as well as in CSF in NB. No complement activation could be detected in blood in NB patients, indicating a compartmentalization of the immunologic process to the CNS. The NB patients showed elevated levels of C1q, C4, C3 and C3a in CSF, suggesting classical complement activation.

The *in vitro* experiments revealed that *B. garinii* induced higher complement activation compared to *B. afzelii*, which bound more factor H. On the other hand, *B. garinii* bound more C4BP, an ability that is probably important for the spirochetes during later stages of the infection when antibodies are present and complement activation also occurs via the CP. However, the difference in complement activation between *B. afzelii* and *B. garinii* seems to be overruled by the presence of anti-*Borrelia* antibodies, although this finding needs to be confirmed in more studies. The experiments using blood from non-immunized and immunized individuals also illustrate the central role of complement in phagocytosis. It is probably most important early in the infection, prior to the generation of anti-*Borrelia* antibodies.

The results from our studies of NB patients as well as from the *in vitro* experiments, support the notion that early LB is dominated by a Th1-type immune response, eventually accompanied by a Th2 response. In addition, we found indications of a Th17 response *in vitro* and in CSF in half of the NB patients. However, the precise role of Th17 in pathogenesis and clinical outcome in LB remains unclear, and needs to be studied further.

FUTURE PERSPECTIVES

In view of the results of this thesis, it would be most interesting to further evaluate the role of complement activation and Th17 response for the disease course and clinical outcome in NB. A prospective NB study is running, in which blood and CSF samples are collected and the patients followed clinically for one year. In addition to complement and chemokine analyses, it will also be possible to study different immunogenetic polymorphisms in this patient material.

Further *in vitro* experiments comparing more donors with specific anti-*Borrelia* IgG antibodies to donors without such antibodies are needed in order to shed light on the role of specific antibodies for complement activation and phagocytosis. The more precise role of complement activation for cytokine/chemokine release in response to *Borrelia* spirochetes should also be further investigated in experiments using human whole blood and different complement inhibitors.

The possible impact of complement defects should also be addressed in further studies. Interestingly, several of the patients with LB not involving the CNS (Paper II) had low C4 levels in plasma, and it would be interesting to analyze them in more detail. It is also possible to perform *in vitro* experiments with live spirochetes and sera manipulated in order to simulate different complement deficiencies.

Possible coinfection with *Anaplasma phagocytophilum* in more severe LB cases has been little studied so far. The national “STING study”, in which ticks and blood samples are collected from tick-bitten individuals at the time of the tick bite and three months later, makes it possible to study this matter more closely. The study also provides the opportunity to elucidate various epidemiological, microbiological, clinical, diagnostic, immunological and prognostic issues of LB and other tick-borne infections in Sweden and, as the study expands, in Scandinavia.

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