NEW ANTIGENS FOR THE SEROLOGY OF LYME BORRELIOSIS

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ACADEMIC DISSERTATION

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To the memory of my sister Taru

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications referred to in the text by their roman numerals:

I Heikkilä T, Seppälä I, Saxen H, Panelius J, Yrjänäinen H, Lahdenne P. Species-specific serodiagnosis of Lyme arthritis and neuroborreliosis due to *Borrelia burgdorferi* sensu stricto, *B. afzelii,* and *B. garinii* by using decorin binding protein A. J Clin Microbiol. 2002;40:453-460.

II Heikkilä T, Seppälä I, Saxen H, Panelius J, Peltomaa M, Julin T, Carlsson S-A, Lahdenne P. Recombinant BBK32 protein in serodiagnosis of early and late Lyme borreliosis. J Clin Microbiol. 2002;40:1174-1180.

III Heikkilä T, Seppälä I, Saxen H, Panelius J, Peltomaa M, Huppertz H-I, Lahdenne P. Cloning of the gene encoding the decorin-binding protein B (DbpB) in *Borrelia burgdorferi* sensu lato and characterization of the antibody responses to DbpB in Lyme borreliosis. J Med Microbiol. 2002;51:641-648.

IV Heikkilä T, Huppertz H-I, Seppälä I, Sillanpää H, Saxen H, Lahdenne P. Recombinant or peptide antigens in the serology of Lyme arthritis in children. J Inf Dis 2003;in press.

In addition, some previously unpublished results are presented.

ABBREVIATIONS

ACA	Acrodermatitis chronica atrophicans
ASO	Antistreptolysin
B. burgdorferi	*Borrelia burgdorferi* sensu lato
BD	Blood donor
BL	Borrelial lymphocytoma
BSA	Bovine serum albumin
BSK	Barbour-Stoenner-Kelly
CDC	Centers for Disease Control and Prevention
CSF	Cerebrospinal fluid
DbpA	Decorin binding protein A
DbpB	Decorin binding protein B
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
EM	Erythema migrans
FP	Facial palsy
GST	Glutathione-S-transferase
HLA	Human lymphocyte antigen
hLFA-1	Human lymphocyte function associated antigen-1
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IL4	Interleukine 4
IR_6	Invariable region 6
LA	Lyme arthritis
LB	Lyme borreliosis
MHC	Major histocompatibility complex
NaCl	Natrium chloride
NB	Neuroborreliosis
OD	Optical density
Osp	Outer surface protein
PAGE	Polyacrylamide gel electrophoresis
PFGE	Pulsed-field gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RF	Rheumatoid factor
RFPL	Restriction fragment length polymorphism
rRNA	Ribosomal ribonucleic acid
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SF	Synovial fluid
SLE	Systemic lupus erythematosus
Th1	Type 1 helper T-cell
VlsE	Variable major protein- like sequence expression site
WB	Western blot
WCL	Whole cell lysate

ABSTRACT

Lyme borreliosis (LB) is an infectious disease caused by the spirochete *Borrelia burgdorferi* sensu lato and characterized by multistage skin, joint, neurologic and cardiac manifestations. In Europe, LB is caused by three different borrelial species, *B. afzelii, B. garinii,* and *B. burgdorferi* sensu stricto. The diagnosis of LB is clinical, but serologic methods are frequently used, especially in disseminated LB, to confirm the diagnosis. The aim of the present study was to evaluate new recombinant proteins DbpA, DbpB, BBK32, and peptide IR_6 as antigens in the serology of LB.

In addition to basic PCR techniques, a novel genome walking method was used to sequence borrelial genes. In comparison of the deduced amino acid sequences, the sequence identity was 43-62% between variant DbpA proteins from Finnish borrelial isolates, representing the three borrelial species. The corresponding sequence identities were 62-68% and 71-95% between variant DbpB and BBK32 proteins. Due to the sequence heterogeneity, the three recombinant variants from each protein were selected for antigens in the enzyme-linked immunosorbent assay (ELISA) or Western blot (WB) of LB.

In patients with erythema migrans, the most sensitive antigen was recombinant BBK32. Of the 23 patients, 17 (75%) had IgG antbodies to BBK32 already at presentation. In the convalescent phase, all 23 patients had antibodies to BBK32, as assessed by ELISA or WB. In IgM serology of acute and convalescent samples, the sensitivity of the BBK32 ELISA was only 4-13%. Of the 23 patients, 6 (26%) had IgG and 4 (17%) had IgM anti-flagella antibodies at presentation and in the convalescent phase, respectively.

In disseminated LB, new recombinant antigens, especially DbpA and BBK32, and to a lesser extent, DbpB and the peptide antigen IR_6 proved to be sensitive and specific antigens. Use of two or three variants of the recombinant proteins increased the number of positive samples. The sequence heterogeneity between immunogenic proteins in borrelial species implies that to cover all relevant species in the serology of LB, several variant proteins in parallel or combined are needed.

Antibodies to recombinant DbpA, DbpB, and BBK32 proteins, peptide IR_6, and flagella were analyzed in detail at diagnosis and during follow-up in Lyme arthritis (LA) patients. In adults, 93% had antibodies to DbpA, and 73% and 100% to DbpB and BBK32, respectively. All

patients also had antibodies to borrelial flagella. In 52 children with LA, the sensitivity of DbpA and IR_6 ELISAs was 98%, of BBK32 ELISA 96%, of DbpB ELISA 77%, and of commercial flagella ELISA 100%. The specificity of the assays varied between 90 and 100%. Of 43 children from whom serum samples at diagnosis were available, the antibody levels were analyzed during the follow-up. The clinical course of these 43 children was defined as acute if there was a single episode of arthritis, as episodic if there were at least two episodes of self-limiting arthritis, or as chronic if the arthritis persisted for three months or longer. Irrespective of the clinical course of the arthritis, the antibody levels to the tested antigens waned slowly, and after 2 years follow-up approximately 80% of patients still had IgG antibodies to the tested antigens. These results imply that, in children with LA, antibodies to IR_6 or to the recombinant proteins DbpA, DbpB, and BBK32 or to flagella do not appear useful as indicators of disease activity or response to therapy.

In conclusion, IR_6 seems to have greatest potential to be used universally in the diagnostic serology of LB. Alternatively, the use of several specific borrelial antigens in parallel might improve the accuracy of serology of LB. In addition, BBK32 seems to be a promising antigen for the serology of early LB.

1. INTRODUCTION

Lyme arthritis (LA) was first described by Steere et al. (1977) after a cluster of cases initially diagnosed as juvenile rheumatoid arthritis was reported in Lyme, Connecticut, USA. The causative agent, a spirochete *Borrelia burgdorferi*, of LB, was not identified until 1982 (Burgdorfer et al. 1982). Later, the multisystem nature of the disease was recognized and the name Lyme disease or Lyme borreliosis (LB) was adopted (Steere 1989).

Currently, LB is the most frequent tick-transmitted infectious disease in North America and Europe. Three species of *B. burgdorferi* sensu lato genogroup, *B. burgdorferi* sensu stricto (North America and Western Europe), *B. afzelii* (Europe), and *B. garinii* (Europe and northern Asia), are known to cause LB.

LB has several clinical manifestations. The hallmark of the disease is an enlarging bluish-red skin rash, erythema migrans (EM). In addition to EM, various skin, neurologic, musculoskeletal, and cardiac manifestations can occur. Distinct clinical manifestations have been associated with different infective borrelial species. *B. burgdorferi* sensu stricto has been associated with LA, *B. garinii* with neuroborreliosis, and *B. afzelii* with acrodermatitis chronica athrophicans (ACA) (Wang et al. 1999).

The diagnosis of LB is clinical but, in disseminated LB, laboratory support of the diagnosis is often needed. The gold standard for microbiological diagnosis would be positive culture, but culturing *B. burgdorferi* from clinical samples other than EM lesions is difficult (Wormser et al. 1998). For routine laboratory testing of LB, PCR-based methods also seem to be too insensitive, probably because of the scarcity of *Borreliae* in clinical samples (Nadelman and Wormser 1998, Sigal 1998). Because these direct assays often fail to demonstrate the presence of the microbe, indirect methods are used. The most frequently used indirect laboratory methods are enzyme-linked immunosorbent assay (ELISA) and Western blotting (WB). These assays measure antibodies to flagella or whole cell lysate (WCL) from various borrelial species. However, the performances of these tests in different laboratories are highly variable (Brown et al. 1999). With flagella, increased sensitivity has been observed as compared with WCL (Hansen and Åsbrink 1989). However, anti-flagella antibodies may not be detected in early LB, and even in disseminated LB, 5-10% of patients may not have anti-flagella antibodies (Oksi et al. 1995). Specificity problems also occur, and viral infections, other spirochetal infections and rheumatoid arthritis may cause false-positive results, especially in immunoglobulin M (IgM) serology. In addition, there is unexplained variation in the performance of antibody tests in different laboratories (Brown et al. 1999). Several recombinant borrelial proteins have been suggested as improved serologic antigens for the serology of LB, but none of them alone has proven superior to the antigens currently used. Some of the recombinant proteins have been applied as antigens in new commercial LB tests.

In this study, genes for the borrelial proteins DbpA, DbpB, and BBK32 were cloned, sequenced, and subsequently expressed as recombinant proteins. These proteins and a peptide antigen IR_6 were evaluated as antigens in ELISA and WB assays for the serology of LB and compared with the current routine flagella ELISA. These assays were performed on human serum samples from patients with various manifestations of LB.

2. REVIEW OF THE LITERATURE

LB is caused by a spirochete *Borrelia burgdorferi*, which is transmitted during the feeding on blood of ticks of the genus *Ixodes*. The disease is a multi-system disorder, which can affect a complex range of tissues, including the skin, nervous system, musculoskeletal organs, heart and rarely other organs, such as the eyes, kidneys and liver.

2.1. HISTORY OF LYME BORRELIOSIS

The first reports on acrodermatitis chronica atrophicans (ACA), a late skin manifestation of LB, were written over 100 years ago (Buchwald 1883, Herxheimer and Hartmann 1902). The association between tick bites and erythema migrans (EM) was described a few years later (Afzelius 1910, Lipschűtz 1913). The connection between tick bites and neurologic symptoms was first reported by Garin and Bujadoux in 1922 (Garin and Bujadoux 1922). Since then, the association of EM with lymphocytic meningitis (Hellerström 1930) and with lymphocytic meningoradiculitis (Bannwarth 1941) were reported. As early as the 1920s, the idea of an infectious origin of these manifestations was suggested by Garin and Bujadoux (Garin and Bujadoux 1922) and Lipschűtz (Lipschűtz 1923). The first medical treatments with antibiotics for the clinical manifestations of LB were done long before the bacterial etiology was discovered (Svartz 1946, Thyresson 1949, Bianchi 1950, Hollström 1951, Hellerström 1951).

In the mid-1970s, the concern of parents in the community of Lyme in Connecticut, USA, promted research, which later led to distinguishing of a new disease named Lyme arthritis. Since the description of LA in 1977 by Steere et al. (Steere et al. 1977), LB has gained increasing interest in medicine. The causative agent of LB, the spirochete *B. burgdorferi*, was first identified in 1982 (Burgdorferi et al. 1982).

2.2. BORRELIA BURGDORFERI

Borrelia is one of the *Spirochaetaceae*, a phylogenetic group of *Spirochaetales* (Paster and Dewhirst 2001). Other members of the *Spirochaeteceae* group are *Brevinema, Cristispira, Spirochaeta,* and *Treponema*. One of the treponema, *T. pallidum*, is a well-known human pathogen causing syphilis. *Borrelia* has been divided into 31 different species (Olsen et al. 2000, Masuzawa et al. 2001). Of these 31 species, 11 belong to the *B. burgdorferi* sensu lato genomic group; *B. afzelii, B. garinii, B. burgdorferi* sensu stricto, *B. valaisiana, B. bissettii, B. lusitaniae, B. japonica, B. andersonii, B. turdi, B. tanukii,* and *B. sinica* (Kurtenbach et al. 2002, Baranton et al. 1998, Postic et al. 1998). *B. burgdorferi* sensu stricto, *B. afzelii,* and *B. garinii* have been shown to be human pathogens (Postic et al. 1998, Baranton et al. 2001).

Figure 1. *Borreliae* spirochaetes in dark-field microscopy.

B. burgdorferi is a mobile spiral-shaped gram-negative bacterium (Figure 1). Borrelial cells are 5-25 µm long and 0.2–0.5 µm wide. An outer membrane surrounds the periplasmic space containing 7-20 flagella. Several borrelial surface molecules have been described, including lipoproteins and lipids (Jones et al. 1995). Living organisms can be seen by dark-field (Figure 1) or phase-contrast microscopy. *B. burgdorferi* can be cultured in special Barbour-Stoenner-Kelly (BSK) medium in microaerophilic conditions (Barbour 1984). Culture is not always successful. This is reflected by the existence of various modifications of the BSK medium and a somewhat different MKP medium (Preac-Mursic et al. 1986, Preac-Mursic et al. 1989). The growth of *Borreliae* is slow in vitro; spirochetes multiply every 8-12 hours.

Members of the genomic group *B. burgdorferi* sensu lato can be typed with phenotypic or genotypic methods. Serotyping represents the most commonly used phenotypic method. Two different serotyping methods are used, based on the heterogeneity of the outer surface proteins A (OspA) and OspC, borrelial membrane lipoproteins. In total, 11 OspA serotypes have been defined with monoclonal antibodies, 8 in Europe and 11 in Japan (Wilske et al. 1993, Wilske et al. 1996, Masuzawa et al. 1996, Yanagihara et al. 1997). OspA serotypes 1, 2 and J10 and J11 correspond to *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. japonica*, respectively, and serotypes 3-8 and J1-J9 correspond to *B. garinii* (Wilske et al.1996, Yanagihara et al. 1997). Sixteen OspC serotypes have been defined (Wilske et al. 1996, Wilske et al. 1995). OspC serotypes are more heterogeneous than OspA serotypes. For *B. burgdorferi* sensu stricto and *B. afzelii* strains, only one OspA serotype, but 6 and 4 different OspC serotypes, respectively, are known.

Genotypic methods provide more precise information on the diversity of *Borreliae*. Several genotypic methods are currently used, including DNA-DNA reassociation analysis, rRNA restriction analysis, pulsed-field gel electrophoresis (PFGE), plasmid fingerprinting, randomly amplified polymorphic DNA, PCR and PCR-based restriction fragment length polymorphism analysis (RFPL), and DNA sequence analysis (Wang et al. 1999).

The DNA of the whole chromosome and of the majority of plasmids of *B. burgdorferi* sensu stricto strain B31 have been sequenced (Fraser et al. 1997, Casjens et al. 2000). The borrelial genome contains a linear chromosome of 911 kb and at least 12 linear and 9 circular plasmids with a combined size of 600 kb (Casjens et al. 2000). The chromosome contains 853 apparent genes and 21 plasmids of different sizes contain an additional 430 genes. Most of the genes do not have known biological functions. In the genome, there are 161 paralogous gene families, each with 2-41 members. Most plasmid genes are members of gene families (Casjens et al. 2000). During long-term culture in vitro, *B. burgdorferi* may lose some plasmids important in virulence (Schwan et al. 1988, Xu et al. 1996, Norris et al. 1995).

2.3. ECOLOGY AND EPIDEMIOLOGY OF LYME BORRELIOSIS

The vector for *B. burgdorferi* sensu lato is a tick of the genus *Ixodes*. In Europe, the main transmitting species is *I. ricinus*. In Northern America, *I. scapularis* and *I. pacificus*, and in Asia, *I. persulcatus* are capable of transmitting *B. burgdorferi* sensu lato to humans (Gray 2002). Occasionally, fleas, mosquitoes, and biting fly may serve as vectors for transmitting *B. burgdorferi* sensu lato (Doby et al. 1991, Magnarelli et al. 1986, Halouzka et al. 1998, Oksi et al. 1994).

The life cycle of the ticks transmitting *B. burgdorferi* sensu lato is 2-6 years, consisting of four stages, egg, larva, nymph, and adult (Gray 1991, Sonenshine 1993) (Figure 2). Of these, nymphs and adult ticks are the principal vectors for humans (Sonenshine 1993, Berger et al. 1995, Gray 2002). After they attach to a host, they crawl to a suitable feeding site. The ticks stay attached at the feeding site for several days. Once fully engorged the tick tumbles to the ground where it begins digesting the blood meal and developing to the next stage (Gray 2002).

Figure 2. Stages of ticks; larva, nymph, adult male, and adult female from left.

B. burgdorferi sensu lato is transmitted to ticks via two specific enzootic cycles (rodent-tick and bird-tick) (Nakao et al. 1994, Humair et al. 1999). Several mammalian hosts and birds have been identified in Eurasia and in the United States (Anderson 1991, Gern and Humair 1998, Nicholls and Callister 1996, Olsen et al. 1995, Gern et al. 1998, Masuzawa et al. 1995). The ticks are infected while engorging a meal of blood from an infected host. In Europe the infestation rates of ticks with *B. burgdorferi* sensu lato have been shown to be 3%, 14%, and 21% for larval, nymphal, and adult ticks, respectively (Hubalek and Halouzka 1998). In the most hyperendemic northeastern part of the USA, infestation rates of 25-50% for nymphal and over 50% for adult ticks have been reported (Bosler et al. 1984, Piesman et al. 1986). In Finland, all the borrelial genospecies pathogenic for humans have been isolated from ticks on sea islands, and only *B. afzelii* and *B. garinii* from the mainland (Junttila et al. 1994, Junttila et al. 1999).

Developing ticks need blood meals for consecutive stages. Occasionally, in the search for a blood meal, ticks bite humans and transmit the spirochete. During feeding, the engorged blood activates *B. burgdorferi* in the tick midgut. *Borreliae* multiply and migrate to the salivary glands, and are expelled further to the human skin. Transmission of *B. burgdorferi* occurs 1-2 days after tick attachment (Piesman 1993, Shih et al. 1995). Transmission may be delayed until 53 hours of attachment (Onishi et al. 2001).

In Europe, all the borrelial species pathogenic for humans, *B. burgdorferi* sensu stricto, *B. afzelii* and *B. garinii*, cause LB. In the United States, the only prevalent pathogenic species is *B. burgdorferi* sensu stricto. LB occurs in Europe, the northeastern, midwestern, and western regions of the United States, and northern Asia (Nadelman and Wormser 1998) (Figure 3). In epidemiological studies, the highest incidencies have been reported in Europe and in the northeastern United States (Table 1). In Europe, highest reported frequencies of LB are found in Scandinavia and in central Europe (Stanek et al. 1996). LB-like illnesses have been reported in Australia, Africa, South America, and the southern part of the United States, but *Borreliae* have not been isolated from the patients. There is no gender preponderance in LB, and people of all ages are affected. In the archipelago of Turku, in Finland, the incidence of EM was reported to be 144 cases per 100 000 inhabitants (Oksi et al. 2001). In southern Sweden, the overall annual incidence of LB was reported to be 69/100 000 (Berglund et al. 1995). In Germany, Huppertz et al. (1999) reported an incidence of 111/100 000 for LB. Strle (1999) reported an incidence of 155/100 000 from Slovenia. Incidences of 20/100 000 and 41/100 000 for LB have been reported in the northeastern United States (Hilton et al. 1999, Lyme disease – United States 1994). The highest reported incidence of LB has been 1198/100 000 in Nantucket, Massachusetts, USA, in 1994 (Lyme disease – United States 1995).

Table 1. Incidence of LB in European countries and in the USA.

Country	Incidence/100000	Reference
Finland	144	Oksi et al. 2001
Sweden	69	Berglund et al. 1995
Germany	111	Huppertz et al. 1999
Slovenia	155	Strle 1999
Bulgaria	4	EpiNorth 2002
Croatia	7	EpiNorth 2002
Czech Republic	36	EpiNorth 2002
Hungary	13	EpiNorth 2002
Poland	6	EpiNorth 2002
Slovakia	12	EpiNorth 2002
Russia	5	Tokarevich et al. 2002
USA	20	Hilton et al. 1999
USA	41	Lyme disease-US 1994

Figure 3. Geographic distribution of LB

2.4. PATHOGENESIS OF LYME BORRELIOSIS

The pathogenetic mechanisms of the various clinical manifestations of LB are not known in detail. To colonize tissues, *Borreliae* have to be able to bind to extracellular matrixes and/or cells. After inoculation into the skin, *B. burgdorferi* migrates through the extracellular matrix and binds to various extracellular matrix components, including heparin, heparan sulfate, and dermatan sulfate (Isaacs 1994), decorin (Guo et al. 1995), glycosaminoglycans (Leong et al. 1995), and fibronectin (Kopp et al. 1995, Guner 1996). *Borreliae* also bind to platelets (Coburn et al. 1994), red blood cells, and dextran (Leong et al. 1995), which may play a role in spirochetemia. When causing neuroborreliosis, *B. burgdorferi* has to be present in the blood and then has to pass through the endothelium and the blood-brain barrier to reach the brain (Garcia-Monco et al. 1990). Penetration to other tissues also occurs. In experimental studies, *B. burgdorferi* has been shown to bind plasminogen (Coleman and Benach 2000), which, after activation to plasmin, may help *Borrelia* to penetrate from the blood to the tissues. In the tissues, *B. burgdorferi* attaches to integrins, matrix glycosaminoglycans, and extracellular matrix proteins (Coburn et al. 1998, Guo et al. 1998, Probert et al. 1998). *Borreliae* may damage tissues both directly and through immunologic mechanisms (Kaiser 1998).

Humoral immunity is considered to be an essential defense mechanism against *B. burgdorferi*. In an experimental mouse model of LB, protective and arthritis- and carditis-resolving immunity is mediated by antibodies (McKisic and Barthold 2000). *B. burgdorferi* elicits specific antibody response that follows the general pattern of an IgM response preceding an IgG response. However, in some patients the detectable antibody response may be delayed (Steere 1989) for unknown reasons. Antibodies may be sequestered in immune complexes (Schutzer et al. 1990). In addition, patients may remain seronegative because of early antibiotic treatment (Dattwyler et al. 1988). In the humoral immune response to *B. burgdorferi*, antibodies function as opsonins (Benach et al. 1984) and activators of complement (Aydintug et al. 1994). However, *B. burgdorferi* is able to circumvent the immune defence for extended periods of time. Most microbes activate the versatile complement system of the host, which usually leads to phagocytosis and/or formation of membrane attack complexes. In recent studies, evidence of the complement resistance of *B. burgdorferi* has been shown (Hellwage et al. 2001, Alitalo et al. 2001). Complement inhibitory factor H binds to the outer surface protein E (OspE) paralogs of *B. burgdorferi* (Hellwage et al. 2001, Alitalo et al. 2002, Stevenson et al. 2002), which may protect *Borreliae* against attack by complement. Antigenic variation by the organism (Zhang et al. 1997) or limited surface exposure of the outer surface proteins (Cox et al. 1996) have also been suggested as possible mechanisms for immune evasion.

In animal models, T-cells have been shown to be involved in the pathogenesis of LB. During early murine LB, type 1 helper T (Th1) cells are predominantly activated (Kang et al. 1997). However, the Th2 cell cytokine response following a Th1 cytokine response has been shown to be important in preventing chronic LB (Kang et al. 1997). In human LB, a predominance of *B. burgdorferi* specific Th1 responses has been shown. However, a Th1 cytokine response alone may not be sufficient to eliminate *B. burgdorferi* in humans (Ekerfelt et al. 1999). Moreover, a Th1 response with a low Th2 response in CSF may cause tissue destruction (Ekerfelt et al. 1997).

The expansion of an EM lesion is thought to be due to centrifugal migration of *B. burgdorferi* in the skin at the inoculation site (Sigal 1997a). In patients with EM and ACA, the expression of MHC markers on Langerhans cells is decreased (Silberer et al. 2000), which may suppress the local immune response and assist in developing the chronic disease.

In experimental models, *B. burgdorferi* can also bind to neural cells, astrocytes, oligodendrocytes (Garcia-Monco et al. 1990), cultured glioma and glial cells (Garcia-Monco et al. 1989). Cytokine production induced by *B. burgdorferi* may cause neural cell damage (Porcella and Schwan 2001). T-cells reactive to the outer surface proteins of *B. burgdorferi*, secreting interferon-γ (INF-γ) have been reported in the CSF of NB patients (Forsberg et al. 1995, Ekerfelt et al. 1998). Local infection causes inflammation of small vessels (Oksi 1996), which may lead to further destruction of the central nervous system (Wokke et al. 1987). In chronic LB cases, with failure to demonstrate bacteria at the site, the tissue destruction has been claimed to be due to immune-mediated or molecular mimicry-based autoimmune mechanisms (Sigal 1997b). Auto-antibodies to axonal proteins, gangliosides, and components of neurons and myelin have also been reported (Garcia-Monco et al. 1988, Fikrig et al. 1993, Kaiser 1995).

In approximately 10% of patients with LA, chronic arthritis develops after apparent eradication of the spirochete (Steere et al. 2001, Gross et al. 1998a). An association of distinct human lymphocyte antigen (HLA) alleles, especially HLA-DR4, with chronic LA has been reported (Steere et al. 1990, Steere and Baxter-Lowe 1998). Conversely, most patients with chronic LA have HLA-DR4 alleles (Steere and Baxter-Lowe 1998). An antibody response to OspA may not develop until prolongation of the arthritis (Kalish et al. 1993). Patients with HLA -DR4 specificity and OspA antibodies seem to be at increased risk for chronic LA (Steere et al. 1994). T-cell reactivity is likely to be important in the synovial inflammation of LA (Steere et al. 2001). In the synovial fluid (SF) of patients with chronic LA, OspA- reactive Th1 cells are detectable years after antibiotic treatment (Gross et al. 1998b). The immunodominant epitope of OspA for T-helper cells has been identified (Gross et al. 1998a). A homology search revealed a peptide from the human lymphocyte function associated antigen-1 (hLFA-1) as a candidate autoantigen. Molecular mimicry between the immunodominant T-cell epitope of OspA and hLFA-1 may be an important factor in the persistence of joint inflammation in chronic LA (Gross et al. 1998a, Trollmo et al. 2001).

2.5. CLINICAL MANIFESTATIONS OF LYME BORRELIOSIS

LB is generally divided in to three stages; early localized, early disseminated and late disseminated disease, with different clinical manifestations at each stage (Table 2) (Sigal 1997a, Steere 1989, Dumler 2001). Early localized disease occurs days to a few weeks after the tick bite, the hallmark being EM. Other manifestations, occurring not only in LB, such as fatigue, malaise, lethargy, headache, fever, myalgia, arthralgia, and lymphadenopathy, have also been described in early LB (Sigal 1997a, Sigal 1998). Early disseminated disease occurs days to several months after the tick bite. At this stage, cutaneous, neurologic, musculoskeletal, cardiac, and ophthalmologic manifestations have been described (Table 2) (Sigal 1997a). If not treated in the early phases, dissemination and late disease may occur months to years after the tick bite, with musculoskeletal, neurologic, and/or cutaneous manifestations (Table 2). The proportions of the various clinical manifestations in published series are shown in Table 3. Manifestations of the clinical stages may overlap. In addition, the infection may not become symptomatic until dissemination has occurred. This phenomenon is analogous to syphilis, another well-known spirochetal disease.

Table 2. Different clinical manifestations of Lyme borreliosis.

Early localized disease:
- Erythema migrans
- Fatigue, malaise, lethargy
- Headache
- Myalgia
- Arthralgia
- Lymphadenopathy

Early disseminated disease:
- Cranial neuropathy
- Meningitis
- Peripheral neuropathy
- Encephalitis
- Myelitis

- Arthritis
- Arthralgia

- Carditis

- Lymphocytoma

Late disseminated disease:
- Arthritis

- Peripheral neuropathy
- Encephalopathy

- Cardiomyopathy

- Acrodermatitis chronica athrophicans

Table 3. Proportions (%) of various manifestations of LB in children and in adults.

Manifestation	Berglund et al. 1995	Huppertz et al. 1999	Cimmino et al. 1998	Williams et al. 1990	Steere 1989	Gerber et al. 1996	Ciesielski et al. 1989
Children							
EM		77%		67%		89%	
NB	28%	7%				5%	
LA		11%				6%	
BL	14%	5%					
Adults							
EM	77%	92%	59%		80%		91%
NB	14%	2%	34%		15%		18%
LA	7%	3%	15%		60%		57%
BL	2%	1%					
ACA	3%	2%					
Carditis	<1%	<1%	2%		5%		10%

EM, erythema migrans; NB, neuroborreliosis; LA, Lyme arthritis; BL, borrelial lymphocytoma; ACA, acrodermatitis chronica atrophicans.

2.5.1. Erythema migrans

Erythema migrans is an expanding bluish-red skin rash. All three species of *B. burgdorferi* sensu lato pathogenic to humans have been cultured from EM skin biopsies (Wang et al. 1999). EM may occur in up to 90% of patients with LB (Nadelman and Wormser 1998), but in many studies of disseminated LB, only approximately one-third of patients have recognized EM. The erythema appears at the site of the tick bite. The EM expands for weeks, as the spirochetes spread through the skin (Nadelman and Wormser 1998). The classical picture of EM is an enlarging, ring-like erythema with a central clearing. However, in recent studies homogeneous redness has also been recognized as a common form of EM (Oksi et al. 2001, Smith et al. 2002). The EM lesions are usually asymptomatic, but pruritus, tenderness, and paresthesias have been described at the site of the primary lesion (Weber and Pfister 1993, Nadelman et al. 1996). Secondary EM lesions may develop after hematogenous spread of spirochetes in untreated patients. The clinical picture of EM in the USA seems to be somewhat different from that in Europe. Patients with multiple EM lesions are more frequent (50%) in the USA (Steere et al. 1983) than in Europe (<10%) (Weber and Pfister 1993). Generalized symptoms, previously listed, in patients with EM have also been reported more often in the USA than in Europe (Nadelman and Wormser 1998).

2.5.2. Other cutaneous manifestations

Borrelial lymphocytoma (BL) is a rare manifestation of early disseminated LB. It is a bluish-red skin infiltrate 1-5 cm in diameter, and usually located on the earlobe, nipple or scrotum. BL seems to be more common in children than in adults (Berglund et al. 1995, Stanek et al. 1996). Even if untreated, it may resolve spontaneously with time (Hovmark et al. 1993).

Acrodermatits chronica atrophicans (ACA) is a late manifestation of disseminated LB. It is a longstanding red or bluish-red atrophic lesion resembling scleroderma. ACA is located most often on the distal extensor surfaces of limbs (Stanek et al.1996). ACA has been associated with *B. afzelii* infection (van Dam et al. 1993, Wienecke et al. 1994), but in a few cases *B. garinii*, or *B. burgdorferi* sensu stricto have also been detected with PCR from ACA lesions (Picken et al. 1998). Thus, *B. afzelii* is the predominant but not the exclusive etiologic agent of ACA.

2.5.3. Musculoskeletal manifestations

Lyme arthritis is the most common musculoskeletal manifestation of LB. In LA, recurrent brief attacks of joint swelling in one or a few of the large joints can occur (Steere 1989, Wang et al. 1999, Stanek et al. 1996, Sigal 1997a). Occasionally, the disease may progress to chronic arthritis. LA has been associated with *B. burgdorferi* sensu stricto infections, which is in line with the predominance of LA in Northern America, where *B. burgdorferi* sensu stricto is the only known infective borrelial species. Other musculoskeletal manifestations, such as arthralgia, myalgia, and tendonitis, do not alone fulfill the diagnostic criteria of LB.

2.5.4. Neurologic manifestations

Neurologic manifestations are common in disseminated LB, especially in Europe (Christen et al. 1993, Garcia-Monco and Benach 1995, Kaiser 1998). Several neurologic manifestations have been described (Table 2). In early neuroborreliosis (NB), cranial and peripheral neuropathies, meningitis, and Bannwarth syndrome are common (Stanek et al. 1996). Bannwarth syndrome is characterized by painful meningoradiculitis with lymphocytic pleocytosis in the CSF with or without peripheral or cranial paresis (Pfister et al. 1993). In children, cranial neuropathy, typically that of the VII cranial nerve, and meningitis are the most common manifestations (Christen et al. 1993). In adults, however, Bannwarth syndrome is the most common neurologic manifestation (Kaiser 1998). Late disseminated NB is a very rare condition and includes long-lasting manifestations such as encephalomyelitis, radiculomyelitis, and chronic meningitis (Stanek et al. 1996, Nadelman and Wormser 1998). NB has predominantly been associated with *B. garinii* infection (Balmelli and Piffaretti 1995, Peter et al. 1997, Ekerfelt et al. 1998), but *B. burgdorferi* sensu stricto and *B. afzelii* may also cause NB (Hubalek and Halouzka 1997, Busch et al. 1996).

2.5.5. Cardiac manifestations

Typical cardiac manifestations of LB are atrioventricular conduction defects, endomyocarditis, and pericarditis (Cimmino 1998, Sigal 1995, Steere et al. 1980, van der Linde et al. 1990), which may develop weeks to months after borrelial infection. A few cases of chronic cardiomyopathy have been reported (Seinost et al. 1998, Sonnesyn et al. 1995, Stanek et al. 1990). However, conflicting evidence regarding the role of *B. burgdorferi* in the development of cardiomyopathy has been reported (Suedkamp et al. 1999). In the USA, carditis was reported in 8% of patients with LB before the use of antibiotics in treatment (Steere et al. 1980). In later studies, the incidence has been <5% both in Europe and in the USA (van der Linde et al. 1990, Gerber et al. 1996, State of Connecticut Department of Public Health 1993).

2.5.6. Ocular manifestations

Ocular findings may include conjunctivitis, keratitis, iritis, uveitis, choroiditis, and vitreitis (Lesser 1995, Karma et al. 1996, Balcer et al. 1997, Mikkilä et al. 1997a, Mikkilä et al. 1997b, Zaidman 1997). The published studies mostly represent single case reports.

2.5.7. Other manifestations

During hematogenous dissemination of *B. burgdorferi*, several organs can be infected. Single cases of hepatitis (Kazakoff et al. 1993), splenomegaly (Nelson and Nemcek 1992), orchitis, microscopic hematuria, and proteinuria (Steere 1989) have been reported in patients with LB.

2.6. LABORATORY DIAGNOSIS OF LYME BORRELIOSIS

The diagnosis of LB should be based on typical clinical features, but laboratory tests, culture, PCR, and serologic assays can be used to support the diagnosis (Sigal et al. 1998, Wang et al. 1999).

2.6.1. Culture

The gold standard for an infectious disease is isolation of the causative agent by culture. Culturing *B. burgdorferi* from clinical samples other than EM lesions is difficult (Wormser et al. 1998), probably because of the scarcity of bacteria in clinical samples and the suboptimal media conditions. Positive culture rates of nearly 90% have been reported for secondary EM lesions, 50% for primary EM lesions, and 48% for large-volume blood or plasma specimens from patients with early LB (Melski et al. 1993, Mitchell et al. 1993, Schwartz et al. 1992, Wormser et al. 2001). Isolation of *B. burgdorferi* has been successful but uncommon from the CSF (Steere et al. 1983, Karlsson et al. 1990), synovial fluid (SF)(Snydman et al. 1986, Schmidli et al. 1988), tendon (Hauptl et al. 1993), myocardium (Stanek et al. 1990), iris (Preac Mursic et al. 1993), and subcutaneous fat (Viljanen et al. 1992).

B. burgdorferi is a fastidious organism. Culture of *B. burgdorferi* requires specific culturing medium, BSK or modifications of it (Barbour 1984, Preac Mursic et al. 1986) for incubation. Detection of *Borreliae* spirochetes from cultures is usually done by dark-field microscopy or by fluorescent microscopy (Reed 2002). Culturing is time-consuming and a negative result does not exclude LB. Therefore, in routine laboratory diagnosis of LB, other laboratory methods are more important than culture.

2.6.2. Polymerase chain reaction (PCR)

PCR-based methods have been used to identify small numbers of *B. burgdorferi* that may be present in various tissues (Dumler 2001). Both single-stage and nested PCR assays have been developed, and detection methods vary from gel electrophoresis and Southern hybridisation to real-time PCR (Morrison et al. 1999, Pahl et al. 1999, Pietilä et al. 2000, Germer et al. 1999). Plasmid and chromosomal targets have been used. Targets from plasmid DNA (*ospA*, *ospC*, and *vlsE*)(Liebling et al. 1993, Priem et al. 1997, Christen et al. 1995, Nocton et al. 1996, Guy and Stanek 1991, Seinost et al. 1999, Iyer et al. 2000) seem to have greater sensitivity, possibly due to multiple copies within each bacterium, than single-copy chromosomal targets (*fla, recA,* and 16S and 23S ribosomal DNA) (Schwarz et al. 1992, Lebech et al. 2000, Oksi et al. 1999, Kruger and Pulz 1991, Lebech and Hansen 1992, Pietilä et al. 2000).

A recent meta-analysis of PCR studies has revealed an overall sensitivity of 68% for skin samples from EM lesions (Dumler 2001). In the SF of patients with LA, a senstivity of 73% and a specificity of 99% was reported. In neuroborreliosis, the sensitivity has remained in the range of 20% from the cerebrospinal fluid samples. In plasma or serum samples, the sensitivity of PCR was 26%. Urine samples have also been analyzed, and the sensitivity of PCR was 68%, with large differences in success rates in different laboratories. Therefore, a negative PCR test does not exclude the diagnosis of LB. Furthermore, borrelial DNA can be detected in skin specimens taken at the site of cured EM lesions 1-6 months after disappearance of the lesion (Kuiper et al. 1994, Strle et al. 1995) and from the CSF and SF specimens of patients with disseminated LB more than 10 years after infection (Bradley et al. 1994, Keller et al. 1992, Lebech and Hansen 1992, Nocton et al. 1994). Thus, a positive PCR test does not always indicate active infection. PCR tests may be most useful in patients with seronegative early LB. However, this would require a skin biopsy from EM lesion, which is not readily feasible in routine clinical practice.

2.6.3. Serologic assays

The mainstay of laboratory diagnosis for LB has been serologic assays measuring antibodies against *B. burgdorferi*, although the performance of these assays in different laboratories is highly variable (Brown et al. 1999). The two most frequently used serologic methods are the enzyme-linked immunosorbent assay (ELISA) and Western blotting (WB). In addition, the indirect immunofluorescent assay has been used. ELISA is widely used as a screening test.

Especially in LB patients with early manifestations, e.g. erythema migrans (EM) and facial palsy, antibody responses to the current antigens may be weak or delayed (Mitchell et al. 1994). However, in disseminated LB, sensitivity is usually not a problem. In Europe, serologic assays using whole-cell lysates (WCL) or flagella as antigens are not standardized, and limitations exist to their sensitivity and specificity (Goossens et al. 1999). False-positive ELISA results are also observed due to cross-reactive antibodies in other illnesses, e.g. other spirochetal infections, Epstein-Barr virus infection, rheumatoid arthritis, and systemic lupus erythematosus (Magnarelli 1995). The Centers for Disease Control and Prevention (CDC) in the USA have suggested a two-test approach, in which the specificity of positive or indeterminate ELISA results are confirmed by WB (Lyme disease – United States 1994). In Europe, the clinical implications of this recommended strategy have remained unclear (Blaauw et al. 1999, Hauser et al. 1999, Robertson et al. 2000b). WB does not seem to discriminate between active and previous *B. burgdorferi* infections (Goossens et al. 1999), although WB is more specific than ELISA (Reed 2002). One factor causing difficulties in serologic tests is the existence of three different pathogenic species of *B. burgdorferi* sensu lato in Europe (Hubalek and Halouzka 1997, Junttila et al. 1999, Baranton et al. 1992). One of the main reasons for these problems is the antigenic diversity due to variations in the sequences and expression of immunogenic proteins in these different borrelial species (Fellinger et al. 1995, Jauris-Heipke et al. 1993, Roberts et al. 1998, Roessler et al. 1997).

In hopes of increasing the specificity of the serodiagnosis, a number of borrelial recombinant proteins have been tested as antigens (a 83/93 kDa protein, flagellins A and B, OspA, OspB, OspC, OspE, OspF, p22, BBK32, BBK50, VlsE, and P39) (Fikrig et al. 1997, Gerber et al. 1995, Hauser and Wilske 1997, Lawrenz et al. 1999, Liang et al. 1999, Magnarelli et al. 1996, Magnarelli et al. 2000, Panelius et al. 2001, Panelius et al. 2002, Rauer et al. 1995, Rauer et al. 1998, Roessler et al. 1997). In addition, recombinant chimeric borrelial proteins, consisting of combinations of partial amino acid sequences from OspA, OspB, OspC, flagella, and P93 proteins, have also been tested in LB serology (Gomes-Solecki et al. 2000, Gomes-Solecki et al. 2002). So far, none of the recombinant or chimeric proteins used as single antigens has proved superior to the current routine serology. However, some of these recombinant protein-based assays seem to be as sensitive and at least as specific as assays based on flagella or whole-cell lysate (Hauser and Wilske 1997, Magnarelli et al. 1996, Magnarelli et al. 2000, Rauer et al. 1998, Gomes-Solecki et al. 2000, Gomes-Solecki et al. 2002). Some ELISA assays using combinations of recombinant antigens are commercially available, but scientific evaluation of their performance is lacking (www.biomedica.co.at/, www.biotest.de).

In addition, peptide-based antigens have been tested in serologic assays. Recently, Liang et al. (2000) have shown that an ELISA based on a peptide antigen corresponding to the invariable region 6 (IR_6) of the borrelial VlsE protein (Liang et al. 1999, Lawrenz et al. 1999) has high sensitivity and specificity. This peptide represents 26 amino acids from the invariable region 6 (Liang and Philipp 2000). In the USA, a sensitivity of 85% or 99% for early or late LB, respectively, and a specificity of 99% of IR_6 (also known as C6) assay have been reported (Liang et al. 1999). A commercial synthetic peptide (C6) ELISA assay (Immunetics, USA), the peptide originating from *B. burgdorferi* sensu stricto, became available in the year 2002. In another peptide-based ELISA assay, a carboxyterminal decapeptide from borrelial OspC was used as an antigen (Mathiesen et al. 1998). The sensitivity of IgM OspC peptide ELISA was 36% or 45% for serum samples from patients with EM or neuroborreliosis, respectively, while IgG antibodies were detected less frequently. Owing to such a low sensitivity, the OspC peptide based method may not be useful in the serology of LB.

In monitoring the treatment response in LB, no generally accepted serologic indicators have been introduced. IgG and IgM antibodies to *B. burgdorferi* seem to persist in the serum for years even after successful treatment of LB (Steere 1993, Craft et al. 1984, Feder et al. 1992). A decline in the anti-flagella antibodies has been observed (Wahlberg et al. 1994), but the antibodies remain positive for several years (Hammers-Berggren et al. 1994a,b). Panelius et al. (1999) showed that a rapid decrease in flagella IgG antibodies could be taken as an indicator for successful treatment of disseminated LB. In a recent study, Philipp et al. (2001) have suggested that IgG antibodies to IR_6 could be used as indicators for the treatment response. They showed a fourfold decline of the antibody titer in successfully treated LB patients.

2.6.4. Other laboratory methods

T-cell proliferative assay (Dattwyler et al. 1986), enzyme-linked immunospot (ELISPOT) T-cell assay (Forsberg et al. 1995), and immune complex detection assay (Schutzer et al. 1999) have been used in laboratory diagnosis of LB. In T-cell proliferative assays, sensitivities of 45% to 77%, and specificities of 78% to 95% have been reported (Dressler et al. 1991, Huppertz et al. 1996). It has been suggested that a T-cell proliferative assay may be useful in patients with seronegative LB (Tugwell et al. 1997, Dressler et al. 1991, Huppertz et al. 1996). However, on account of the laborious assay and variable sensitivity and specificity, this method has not gained widespread interest for clinical use. T-cell immune responses in borrelial infections have been evaluated by detecting secretion of cytokines, INF-γ and interleukine 4 (IL-4), with the ELISPOT method. Assessment of T-cell responses may be important in solving pathogenetic mechanisms, especially in NB (Ekerfelt et al. 1997, Ekerfelt et al. 1999). In addition, asymptomatic borrelial infections may be detected with the ELISPOT assay (Ekerfelt et al. 2001). An assay detecting *B. burgdorferi*-specific antigen–antibody immune complexes has recently been suggested to be sensitive and specific in diagnosing active LB. Moreover, *B. burgdorferi*-specific immune complexes have been suggested to be present before antibodies are detectable in early LB (Schutzer et al. 1999, Brunner et al. 2001).

2.7. TREATMENT OF LYME BORRELIOSIS

In verified LB cases, patients should be treated with antibiotics to prevent disease progression. Most treated LB patients have an excellent prognosis. Treatment can be oral or parenteral, depending on disease severity. Several antibiotics have been used successfully in the treatment of LB. In the USA, practice guidelines for the treatment of LB have been published (Wormser et al. 2000). They recommended various antibiotic treatments for different manifestations of LB. The European Union Concerted Action on Lyme Borreliosis has published similar recommendations for Europe (www.vie.dis.strath.ac.uk/vie/LymeEU). In both of these recommendations EM is treated with oral antibiotics (amoxycillin, doxycycline, penicillin, or cefuroxime axetil) for 14-21 days (Table 4). In Europe, neuroborreliosis is treated with parenteral antibiotics (ceftriaxone, cefotaxime, or penicillin) or with oral doxycycline for 14-28 days. In the USA, the treatment recommendation is otherwise similar, but cranial nerve palsies are treated like EM. In LA, in both recommendations, oral and parenteral antimicrobials are used for 21-28 days. There has been discussion about the use of longer antibiotic treatments. In a recent study, patients with persistent symptoms despite previous antibiotic treatment did not benefit from prolonged antibiotic treatment (Klempner et al. 2001).

Table 4. Recommended antimicrobial therapy for patients with Lyme borreliosis (Wormser et al. 2000).

Erythema migrans	Amoxycillin	2 x 1000mg	p.o.	14-21 days
	Doxycycline	2 x 100mg	p.o.	14-21 days
	Penicillin V	3 x 1 milj IU	p.o.	14-21 days
	Cefuroxime axetil	2 x 500mg	p.o.	14-21 days
Neuroborreliosis	Ceftriaxone	1 x 2000mg	i.v.	14-28 days
	Cefotaxime	3 x 2000mg	i.v.	14-28 days
	Penicillin G	3 x 6 milj IU	i.v.	14-28 days
(in acute NB)	Doxicycline	2 x 1-200mg	p.o.	14-28 days
Arthritis	Amoxycillin	2 x 1000mg	p.o.	21-28 days
	Doxycycline	2 x 100mg	p.o.	21-28 days
	Ceftriaxone	1 x 2000mg	i.v.	14-21 days
	Cefotaxime	3 x 2000mg	i.v.	14-21 days
Carditis	Ceftriaxone	1 x 2000mg	i.v.	14 days
	Cefotaxime	3 x 2000mg	i.v.	14 days
	Penicillin G	3 x 6 milj IU	i.v.	14 days

2.8. PREVENTION OF LYME BORRELIOSIS

The most important way to prevent *B. burgdorferi* infection is to avoid tick-infested areas. When people visit tick-infested areas, they should protect themselves with clothing to avoid tick bites. After a visit to risk areas, the entire body should be inspected for ticks. All ticks should be removed promptly to prevent transmission of *B. burgdorferi* (Piesman et al. 1987, Piesman et al. 1991). Since wearing fully covering clothing is not feasible in many outdoor activities, ticks will continue to have access to human skin. The small nymphs also transmit *B. burgdorferi* (Gray 2002, Robertson et al. 2000a, Maiwald et al. 1998, Berger et al. 1995) impending effectiveness of the skin scrutiny.

There is insufficient evidence that antibiotic prophylaxis after tick bites should be given to asymptomatic individuals (Costello et al. 1989, Shapiro et al. 1992, Agre et al. 1993, Warshafsky et al. 1996). However, in a recent study Nadelman et al. (2001) showed that, in a hyperendemic area of Lyme disease, a single dose of doxycycline (200 mg) given within 72 hours after a tick bite could prevent the development of Lyme disease. Experimental studies have shown that *B. burgdorferi* is rarely transmitted within the first 48 h of attachment (Piesman et al. 1987, Piesman et al. 1991). Thus, individuals who have had attached ticks removed could be observed for signs and symptoms of LB.

Vaccination against LB was possible in the USA with recombinant outer surface proteins A (OspA) vaccine preparation (Steere et al. 1998, Sigal et al. 1998) until February 2002, when it was withdrawn from the market in view of the concern expressed about the low commercial demand (Potera et al. 2002). At present no other vaccines against LB are available. Development of OspC and DbpA vaccines is in progress, but no licensed products are presently available.

3. AIMS OF THE STUDY

The general aim of the study was to improve the serological diagnosis of LB. New borrelial proteins were cloned and sequenced and subsequently produced as recombinant proteins. The new recombinant proteins were then evaluated as antigens in serologic assays, using serum samples from patients with early or late LB.

The specific aims of this study were

1. To clone and sequence borrelial genes for DbpA, DbpB, and BBK32 from local infective borrelial species, *B. afzelii, B. garinii,* and *B. burgdorferi* sensu stricto, and to produce these proteins as recombinant proteins and evaluate them as antigens in the serology of LB.

2. To evaluate new borrelial antigens, recombinant proteins and a synthetic borrelia-specific peptide IR_6, in the serology and follow-up of children with LA.

3. To evaluate new borrelial antigens in the serology of children with neurologic manifestations, possible due to NB.

4. PATIENTS AND METHODS

4.1. PATIENT SAMPLES

Serum samples from altogether 127 patients with LB were used in this study (Table 5). For studies I, II, and III, human serum samples were collected from 23 patients with culture- or PCR-positive EM (Table 5). Sera were collected at the time of diagnosis and 1 to 3 months after antibiotic treatment. All patients were treated with oral antibiotics (amoxycillin or doxycyclin) for 14 days. For studies I to IV, serum samples were collected from 14 adult patients with NB, 15 adult patients with LA, and 52 children with well-characterized LA from Germany (Huppertz et al. 1995). From the adult patients with NB or LA, one serum sample was available. Of the 52 children with LA, in 43 patients the first serum sample had been taken at the time of diagnosis of LA and 2 to 4 convalescent samples were taken during the follow-up. In the remaining 9 patients, the first available sample was drawn 6 to 12 months after the diagnosis of LA. At diagnosis, the clinical course of the 43 patients was defined as acute (n=9) if there was a single episode of arthritis, as episodic (n=17) if there were at least two episodes of self-limiting arthritis, or as chronic (n=17) if the arthritis had persisted for three months or longer (Huppertz et al. 1995). In the patients with disseminated LB, the clinical manifestations agreed with the criteria of the CDC for LB (Wharton et al. 1990). The clinical diagnosis was confirmed by demonstration of antibodies against flagella or WCL in serum. For the children with LA a confirmatory WB analysis was also performed with WCL as antigen. All the patients had been treated with antibiotics.

For studies I, II, and III, serum samples from patients with syphilis (n=10), Epstein-Barr virus infection (n=10), systemic lupus erythematosus (SLE)(n=8), rheumatoid factor positivity (RF+)(n=8), antistreptolysin positivity (ASO+)(n=8), and healthy blood donors (BD)(n=20) were used as controls. For study IV, serum samples from a national study (Huppertz et al. 1995) including 22 children with other arthritides (juvenile rheumatoid arthritis and reactive arthritis) were used as a control group. In study IV, serum samples from 20 healthy blood donors were also used as negative controls.

For studies I and III, plasma samples from mice infected with *B. garinii* strain Å218 were collected. The infection was verified by culturing ear pinnae from each mouse at each time point when groups of five mice were sacrified. Plasma from individual infected mice was pooled

Table 5. Characteristics of patients with Lyme borreliosis and controls.

				Serum samples (n) used in studies				
				I	II	III	IV	Unpublished
Patients	**n**	**age** (years)	**sex** (M/F)					
Adults with EM	23	38-72	11/12	23	23	23		
Adults with NB	14	17-82	5/9	14	14	14		
Children with NB	21	2-13	7/14					21
Adults with LA	15	17-77	8/7	15	15	15		
Children with LA	52	3-17	32/20				52	
Controls								
Syphilis	11	NA	NA	11	10	11		
SLE	8	NA	NA	8	8	8		
RF+	8	NA	NA	8	8	8		
ASO+	8	NA	NA	8	8	8		
EBV	12	NA	NA	10	10	12		
Children with arthritis	22	1-15	8/14				22	
BD	22	NA	NA	20	20	20	22	15

SLE, systemic lupus eythematosus; RF+, rheumatoid factor positivity; ASO+, antistreptolysin positivity; EBV, Epstein-Barr virus infection; BD, healty blood donor; NA, not available.

at time points 2, 4, 8, and 16 weeks post-infection. Plasma samples from an identical set of sham-infected mice were used as control samples.

In addition, serum samples from 21 children with suspected NB were collected. Characteristics of these children are presented in Table 6. All 21 patients had neurologic symptoms with variable laboratory findings, pleocytosis in the CSF in 15, anti-flagella IgG or IgM antibodies in the CSF in 4 or 11, and in the serum in 8 or 15 patients, respectively. All patients were treated with antimicrobials. The patients were retrospectively divided into two groups according to the laboratory findings (Table 7). From each of these 21 children, a sample at diagnosis and 1 to 4 convalescent samples were available.

Table 6. Characteristics of 21 children with suspected neuroborreliosis.

Age	2-13 years (median 7)	
Sex	7 boys/14 girls	
Clinical features	Erythema migrans	5
	Facial palsy	12
	Headache	11
	Ataxia	2
	Back pain	1
	Disturbed vision	1
	Fever	7
	Nausea, vomiting	3
Anamnestic tick bite		5
Time of onset of symptoms	May to December	
Duration of symptoms	1 day to 2 months (median 14 days)	

Table 7. Classification of patients with clinical features indicative of neuroborreliosis.

	Findings
Definite n=7	— elevated anti-flagella IgG antibodies in the CSF (n=4) and/or — elevated anti-flagella IgM antibodies in the CSF (n=7) and seroconversion of serum anti-flagella IgM to IgG antibodies during follow-up and — pleocytosis in the CSF (n=7) and — anti-flagella IgG (n=4) and/or IgM (n=5) antibodies in the serum
Probable n=14	— elevated anti-flagella IgM antibodies in the CSF (n=4) and/or — pleocytosis in the CSF (n=8) and/or — anti-flagella IgG (n=4) and/or IgM (n=9) antibodies in the serum

4.2. BACTERIAL STRAINS

Finnish borrelial strains were received from the National Public Health Institute, Turku, Finland. *B. burgdorferi* sensu stricto strain IA was originally isolated from the CSF of a Finnish patient with neuroborreliosis. Of the *B. afzelii* strains, A91, 1082, and EM9 were isolated from skin biopsy samples of Finnish patients with EM, and 570 and 600 were isolated from ticks. *B. garinii* strains 40, 46, and 50 were isolated from skin biopsy samples of Finnish patients with EM. The genotypes of culture-positive *Borreliae* were confirmed by sequencing a fragment of the flagellin gene (Junttila et al 1999). *Borreliae* were cultivated in BSK-H medium (Sigma, USA) at 33°C in 5% CO_2. The *B. afzelii* strain SK1 was used in an in-house ELISA as a source of borrelial WCL antigen. *Escherichia coli* host cells for cloning and for expression of recombinant proteins were INFαF (Invitrogen, Netherlands) and M15 (Qiagen, Germany), and BL21 (Amersham Pharmacia Biotech, Sweden).

4.3. METHODS OF MOLECULAR MICROBIOLOGY

4.3.1. DNA purification

Borrelial DNA, from five *B. afzelii* strains (A91, 1082, EM9, 570, and 600), three *B. garinii* strains (40, 46, and 50), and one *B. burgdorferi* sensu stricto strain (IA) were purified with a Dneasy Tissue Kit (Qiagen, Germany). Plasmid DNA was purified with a QIAprep-spin plasmid kit (Qiagen, Germany).

4.3.2. PCR amplification

Purified borrelial DNAs were used as templates in cloning experiments. A PCR-based approach was employed to amplify and sequence the *dbpA* and *bbk32* alleles from different isolates of *B. burgdorferi* sensu lato (*B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii*), and *dbpB* from *B. burgdorferi* sensu stricto. Primers for PCR amplification were designed on the basis of published *dbpA*, *bbk32*, and *dbpB* sequences. Several primer pairs were designed and tested to ensure that the entire coding sequences were obtained. To eliminate any errors possibly made by Taq-polymerase, the two strands of each gene were sequenced independently at least twice. Expression primers for each strain encoding the mature portion of the DbpA, DbpB, and BBK32 proteins after cysteine at the site of posttranslational acylation were chosen from the analyzed sequences. Approximately 1 ng of template DNA was used in standard PCR conditions: 30 cycles of 94°C denaturing for 1 min, 50°C annealing for 1 min and 72°C extension for 1 min 30 s with AmpliTaqGold DNA Polymerase (Perkin Elmer, USA).

4.3.3. Genome walking method

The genome walking methodology (Universal GenomWalker Kit, Clontech, USA) was applied to borrelial DNA from *B. afzelii* and *B. garinii* to amplify and sequence the *dbpB* allelles. Firstly, borrelial DNAs from *B. afzelii* strain BaA91 and *B. garinii* strain Bg40 were separately digested with four different restriction enzymes (Dra I, EcoR V, Pvu II, and Stu I). GenomWalker adapters were ligated to both ends of the cut DNA. Then DNA fragments were amplified by PCR, with downstream primers selected from known sequences and the GenomWalker adapter primers as upstream primers. Two PCR amplifications were performed, the second as a nested PCR. To walk the sequence upstream, new primers were designed from the sequences obtained.

4.3.4. DNA sequencing

The full-length or partial genes obtained by PCR were cloned into the pCR 2.1-TOPO vector (Invitrogen, The Netherlands) and sequenced at the Core Facility of the Haartman Institute, University of Helsinki, with a DyePrimer (T7, M13Rev) cycle sequencing kit (Applied Biosystems Inc., USA). Sequencing reactions were run and analyzed with the automated sequencing apparatus model 373A (Applied Biosystems Inc., USA). DNA and protein sequences were analyzed with Lasergene software (DNASTAR, USA).

4.3.5. Cloning and expression of recombinant proteins

For expression of the recombinant DbpA and DbpB, 6 x Histidine-tagged protein constructs were generated. The forward and reverse primers included a BamHI and a KpnI site, respectively. The PCR amplified DNAs encoding the mature portion of DbpA or DbpB were cloned into pCR 2.1-TOPO plasmids (Invitrogen, Netherlands). The recombinant plasmids were purified and digested with BamHI and KpnI restriction enzymes. The cleaved *dbpA* or *dbpB* genes were then ligated to a similarly digested pQE-30 expression plasmid (Qiagen, Germany) and transferred into *E. coli* M15 host cells. The transformation mixture was plated onto Luria-

Bertani plates containing 100 μg ampicillin per ml. Primary cultures for expression of the DbpA or DbpB constructs were started by inoculating a single colony from a fresh transformant plate into 50 ml of Luria-Bertani broth containing 100 μg ampicillin per ml. The culture was incubated at 37°C with shaking overnight. After 1:50 dilution, 1500 ml of Luria-Bertani broth containing 100 μg ampicillin per ml was incubated at 37°C for 3 hours (until growth reached the mid-log phase; the optical density at 600 nm was ca. 0.6). Isopropyl-β-D-thiogalactoside was added to a final concentration of 0.7 mM, and incubated for a further 3 hours. The cells were centrifuged at 8000 rpm in a superspeed centrifuge (Sorvall RC-5B Plus, DuPont Company, USA) for 10 min, washed with phosphate-buffered saline (PBS), sonicated in PBS with a Soniprep 150 sonicator (Sanyo, Japan) for 5 min, and centrifuged at 13000 rpm. The sonicate supernatant containing the recombinant protein was applied to a Chelating Sepharose Fast Flow column (Pharmacia, Sweden) containing Ni^{2+} ions. Recombinant protein was eluted from the column by increasing the amount of imidazole. For expression of the recombinant BBK32, glutathione S-transferase (GST) fusion protein constructs were generated. The PCR-amplified DNA encoding the mature portion of BBK32 was cloned into the pCR 2.1-TOPO plasmid (Invitrogen, Netherlands). The recombinant plasmid was purified and digested with BamHI and XhoI restriction enzymes. The cleaved *bbk32* was then ligated to a similarly digested pGEX-4T-1 expression plasmid (Amersham Pharmacia Biotech, Sweden) and transfered into *E. coli* BL21 host cells. The expression of recombinant GST-BBK32 protein was done according to the manufacturer's instructions (Amersham Pharmacia Biotech, Sweden). The expression and purity of the recombinant proteins were confirmed by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE).

4.4. PEPTIDE IR_6

A 26-amino acid peptide corresponding to the invariable region 6 (IR_6) of the VlsE protein from *B. garinii* strain IP90 (Liang and Philipp 2000) was produced as a synthetic peptide at the Core Facility of the Haartman Institute, University of Helsinki. The aminoterminus of the peptide was biotinylated.

4.5. WESTERN BLOTTING (WB)

Recombinant DbpAs and BBK32s originating from *B.burgdorferi* sensu stricto strain IA, *B. afzelii* strain A91, or *B. garinii* strain 40 were fractionated in 10-12.5% SDS-PAGE and transferred to a nitrocellulose membrane (BioRad, 0.2 µm pore size, USA) by semidry transfer with 40 mM glycine-50 mM Tris (pH 9.0)- 0.375% (w/v) SDS- 20% (v/v) methanol buffer. 12 µg of each recombinant protein was used for one 7-cm-wide nitrocellulose membrane. Two-mm strips of the nitrocellulose membranes were soaked in 0.1% Tween 20, 0.9% NaCl. Serum samples were diluted 1:100 in 0.1% Tween 20, 0.9% NaCl, 0.1 g/l fat-free bovine milk powder (Valio, Finland). Samples were incubated for 2 h. After four buffer rinses for a total of 20 min, the blots were incubated with alkaline phosphatase-conjugated rabbit anti-human IgG (Jackson Immuno Research Laboratories Inc., USA) at 1:5000 for 2 h. The secondary antibody for WB using plasma from mice infected with *B. garinii* was alkaline phosphatase conjugated rabbit anti-mouse IgG (Orion, Finland). After washing, the bands were visualized with 5-bromo-4-chloro-3-indolylphosphate- nitro blue tetrazolium (Sigma Chemical Co., USA). The reaction was terminated 10-15 min later by washing with distilled water. The BBK32 WB strips were scanned with an Agfa Arcus II scanner and Adobe Photoshop software (Adobe Systems Inc., USA) and then analyzed with MacBAS 2.5 (Fuji, Japan) software. The cut-off for a positive IgG WB result was defined as the mean + 3 standard deviations (SDs) of the values of healthy blood donors. For detection of GST, monoclonal anti-GST antibodies (Sigma, USA) were used.

4.6. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

Routine ELISA tests for LB were done as described earlier (Seppälä et al. 1994). Briefly, IgG or IgM antibodies against *B. burgdorferi* were measured with a commercial flagellin-based ELISA kit (Dako, Denmark) modified by titrating the antibodies. Sera were diluted serially in three-fold steps for the test and applied to the plates for overnight incubation. The bound antibodies were detected with biotin-labelled goat anti-human IgG or IgM (Zymed, USA). An end point-titer was obtained at an optical density level determined by a cut-off control provided by the kit. The titer limit for a positive IgG antibody level was 500 and for a positive IgM level 2500. This cut-off control material conformed with the level of the mean + 3 SD of the reference population living in central Finland, an area with low prevalence of LB (Seppälä et al. 1994).

For ELISA assays measuring anti-DbpA, -DbpB, or -BBK32 antibodies, the wells in the microtiter plates were coated with 100 µl (2 µg/ml) of each variant recombinant protein separately overnight. After washing, 100 µl of a diluted serum sample was added to each well, and the wells were incubated overnight. Serum samples were diluted 1:10 (EM) or 1:100 (neuroborreliosis and Lyme arthritis) with 5 mg/ml bovine serum albumin (BSA) in 155 mM NaCl-0.04% Tween 20 buffer (BSA-NaCl-Tween). Mouse plasma samples were diluted 1:100

in 1 M PBS-0.01% Tween 20-0.25% gelatine. For IR_6 ELISA the microtiter plates were first coated with 100 µl of 1 µg/ml recombinant streptavidin (Roche, Germany) over night. The plates were post-coated with 5 mg/ml human serum albumin in 1 M PBS. IR_6 peptide (20 ng/well in 1M PBS- 0.04% Tween 20 buffer) was added to the wells. For serum samples, background wells without IR_6 peptide were also provided. Serum samples were diluted 1:100 in 0.5% human serum albumin-0.04% Tween 20 buffer. After washing, the wells were incubated with the secondary antibody alkaline phosphatase-conjugated rabbit anti-human IgG or IgM (Jackson Immuno Research Laboratories Inc., USA) at 1:5000 or alkaline phosphatase-conjugated rabbit anti-mouse IgG (Orion, Espoo, Finland) at 1:1000 in BSA-NaCl-Tween for 2 h. The reactions were visualised with 1 mg/ml of 4-nitrophenylphosphate (Boehringer Mannheim GmbH, Germany) in diethanolamine buffer pH 10.0. In all ELISAs, the optical density (OD) measurements were made at a wavelength of 405 nm with a Multiscan photometer (Thermo Labsystems, Helsinki, Finland). The cut-off values for human and mouse samples were determined as the means + 3 SD of the respective control samples.

4.7. STATISTICS

Excel 2000 software (Microsoft, USA) was used for calculations of standard statistics in article II. The antibody levels at diagnosis and during the follow-up in study IV were analyzed by ANOVA with GraphPad Prism software (GraphPad Software, Inc., USA). A graphical presentation of the antibodies during the follow-up was created by a locally weighted scatterplot smoother method (Cleveland 1979).

4.8. NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The nucleotide sequences of the *dbpA* genes were submitted to the GenBank under the accession numbers AF441833 for *B. afzelii* A91, AF441832 for *B. garinii* 40, and AF441834 for *B. burgdorferi* sensu stricto IA. Published *dbpA* sequences from *B. afzelii* strains ACA1 (AF069278), BO23 (AF069267), PGau (AF069270), and U01 (AF069284), *B. garinii* strains Ip90 (AF069258), VSBP (AF069272), PBr (AF069281), and JEM4 (AF079362), and *B. burgdorferi* sensu stricto strains 297 (U75866), LP4 (AF069271), MC1 (AF079361), and HB19 (AF069254) were obtained from the GenBank.

The nucleotide sequences of the *dbpB* genes were submitted to GenBank under accession numbers AY083914 for *B. afzelii* A91, AY083915 for *B. afzelii* 1082, AY083916 for *B. afzelii* EM9, AY083917 for *B. garinii* 40, AY083918 for *B. garinii* 46, AY083919 for *B. garinii* 50, and AY083920 for *B. burgdorferi* sensu stricto IA. Published *dbpB* sequences from *B. burgdorferi* sensu stricto strains 297 (U75867), LP4 (AF069264), LP5 (AF069261), LP7 (AF069255), NCH-1 (AF069259), FRED (AF069260), HB19 (AF069254), ZS7 (AF069251), B31 (AF069266) and N40 (AF069252) and *B. garinii* strain 20047 (AF069263), were obtained from the GenBank.

The nucleotide sequences of the *bbk32* genes were submitted to GenBank under accession numbers AF472525 for *B. afzelii* A91, AF472527 for *B. afzelii* 1082, AF472526 for *B. afzelii* 570, AF472528 for *B. afzelii* 600, AF472529 for *B. garinii* 40, AF472530 *B. garinii* 46, AF472531 for *B. garinii* 50, and AF472532 for *B. burgdorferi* sensu stricto IA. Published *bbk32* sequences from *B. afzelii* ACA1 (AF213179), *B. garinii* Ip90 (AF213178), and *B. burgdorferi* sensu stricto strains B31 (AE000788), and N40 (U82107) were obtained from the GenBank.

4.9. ETHICAL CONSIDERATIONS

The Ethics Committee in the Department of Otorhinolaryngology, University of Helsinki, approved the study protocol for the collection of skin biopsies and serum samples from EM patients. Collection of samples in Helsinki University Central Hospital was approved (472/E0/01) by the Ethics Committee of Helsinki University Central Hospital. The study protocol for children with LA (IV)(Huppertz et al. 1995) was approved by the Ethics Committee of the Medical Faculty of the University of Würzburg, Germany.

5. RESULTS

5.1. CLONING OF THE DbpA, DbpB, AND BBK32 GENES

The genes encoding *dbpA* and *bbk32* from *B. afzelii, B. garinii*, and *B. burgdorferi* sensu stricto, and *dbpB* from *B. burgdorferi* sensu stricto were obtained by basic PCR techniques, using several primer pairs. This method was not successful in cloning of the *dbpB* from *B. afzelii,* and *B. garinii*. Instead, a genome walking method was used. The method relies on established sequences in close proximity to unknown DNA. In this case, downstream primers were obtained from *dbpA* sequences published in study I, and genome walker adapter primers were used as upstream primers (Figure 4).

5.2. COMPARISON OF GENE AND PROTEIN SEQUENCES

Genes encoding DbpA, DbpB, and BBK32 were cloned from one *B. burgdorferi* sensu stricto strain (IA), from 1-3 *B. garinii* strains (40, 46, and/or 50), and from 1- 4 *B. afzelii* strains (A91, 1082, 570, 600, and/or EM9). On comparison of the sequences among the three borrelial species, the sequences of the *dbpA, dbpB,* and *bbk32* genes were 32-57%, 78-82%, and 82-100% identical, respectively. Within species, the sequences between different strains were over 95% identical. The deduced amino acid sequences of the mature DbpA, DbpB, and BBK32 proteins were also compared. The sequence identities of DbpA, DbpB, and BBK32 between strains were 43-62%, 62-68%, and 71-95%, respectively (Table 8).

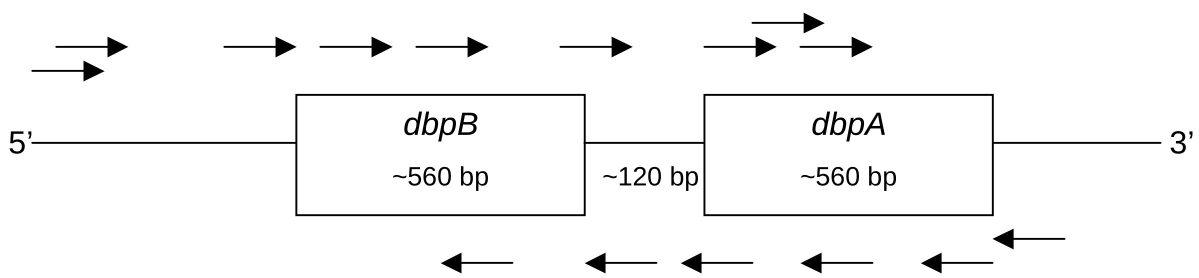
5.3. NEW ANTIGENS IN THE SEROLOGY OF LYME BORRELIOSIS

Eleven different antigens (flagella, IR_6, and three variant recombinant proteins from BBK32, DbpA, and DbpB) were analyzed in ELISA assays, and the recombinant DbpA and BBK32 antigens also in WB assays. Use of variants of the BBK32, DbpA, and DbpB proteins increased the number of positive samples. The positivity for a given antigen in subsequent analyses was based on the highest OD/cut-off value. The sensitivities and specificities of the assays used varied in patients with different clinical manifestations of LB.

5.3.1. Erythema migrans

Serum samples were taken at presentation and 1 to 3 months later from 23 culture- or PCR-positive patients with EM. Of the 23 patients, 6 (26%) had IgG and 4 (17%) had IgM anti-flagella antibodies in ELISA at presentation and in the convalescent samples (Table 9). Of the recombinant proteins, BBK32 was the most sensitive for the serodiagnosis of EM. In 17/23 patients the samples taken at presentation had IgG antibodies to BBK32 in ELISA. In the convalescent phase, all 23 patients had antibodies to BBK32 as assessed by ELISA or WB. The most sensitive antigen was BBK32 from *B. afzelii* strain A91. The specificities for BBK32 ELISA and WB were 89% and 97%, respectively. In IgM BBK32 ELISA, the sensitivity was 4-13% in acute and convalescent samples. With DbpA or DbpB antigens, the sensitivities of IgG ELISAs were 13% or 26% at presentation, and in IgM ELISAs 9% or 4%, respectively.

Figure 4. Schematic figure of *dbpA* and *dbpB* genes and of primers used in cloning and sequencing of these genes.



Species	Primer	Location	Source
	dbpB		
B. burgdorferi	5'-TGC ATA AAA CAA ATT CAC ACT-3'	-208– -187	B31 (AF069269)
sensu stricto	5'-CCG GAT CCA GTA TTG GAT TAG AAA GAA C-3'	64–86	IA (AY083920)
	5'-ATC ATT TTC GTT ATT TGG TTA T-3'	660–639	B31
	5'-CCG GTA CCT TTT TAA TAT TTA TTT CTT TTT TTT GC-3'	628–604	IA
	dbpA		
	5'-ATA TTG AAA ATG GTG GAG AG-3'	-172– -153	B31
	5'-CCG GAT CCG GAC TAA CAG GAG CAA CAA AAA TAA G-3'	76–95	IA (AF441834)
	5'-CAG ATG GAT TTG GTT GGG TAT TGT TTT TA-3'	628–600	B31
	5'-CCG GTA CCC AGA TGG ATT TGG TTG GGT ATT GTT-3'	628–604	IA
	dbpB		
B. garinii	5'-CTT CTC TTT TAT TTT TAA GAC C-3'	-341– -320	40 (AY083917)
	5'-CCG GAT CCA ATT TTG GAT TAA CAG GAG AAG-3'	58–79	40
	5'-GCT TCC TCT GAA ATG GAG CTT TTT ATT C-3'	416–389	40
	5'-CAT TAA ATC AAA CAT AGC CAA GAA TTG AC-3'	348–320	40
	5'-TAT CAG AAG ACG ATT CAA GC-3'	109–90	40
	5'-CCG GTA CCG TTT TTA GCC AAT TCT AAT TAC-3'	594–573	40
	5'-CAT GCT ACT AAC AGG CTA AC-3'	744–725	40 (AF441832)
	dbpA		
	5'-GTC-AAT TCT TGG CTA TGT TTG-3'	-356– -336	IP90 (AF069258)
	5'-TAA ACA CAG CTG AAA GAT TG-3'	-245– -226	IP90
	5'-GTT AGC CTG TTA GTA GCA TG-3'	46–65	40 (AF441832)
	5'-CCG GAT CCG GCT TAA CAG GAG AAA CTA-3'	67–85	40
	5'-CAT GCT ACT AAC AGG CTA AC-3'	65–46	40
	5'-ACT GTT CCT GTC ATT TTT TG-3'	407–388	IP90
	5'-CCG GTA CCT TAT GTA GTA GCA GCA GTG-3'	561–543	40
	5'-ATA AAA ATG TTG TTT ATT ATG TAG-3'	578–554	IP90
	dbpB		
B. afzelii	5'-CCC CTG GCA AAA TAA AAT TC-3'	-458– -439	A91 (AY083914)
	5'-CCG GAT CCA ATT TTG GAT TAA TGG AAG AAA C-3'	58–80	A91
	5'-CTG ATG ATT CCA ATC TAG CTT TTC CTG-3'	765–739	A91 (AF441833)
	5'-TGC TGC TAA CAG GCT AGC AAG TAA AG-3'	728–703	A91
	5'-TTA ACC TCA ATT AAT CTT TCA G-3'	461–440	A91 (AY083914)
	5'-CCG GTA CCT TAT TTT TGA TTT TTA GTT TGT T-3'	513–491	A91
	dbpA		
	5'-ATG ATT AAA TAT AAT AAA ATT ATA C-3'	1–25	BO23 (AF069267)
	5'-CTA GCC TGT TAG CAG CAT GT-3'	44–63	BO23
	5'-TGT AGT TTA ACA GGA AAA GC-3'	61–80	BO23
	5'-CCG GAT CCA GTT TAA CAG GAA AAG CTA G-3'	64–83	A91 (AF441833)
	5'-GCA ACA GAA GAG GAA ACT AT-3'	199–218	A91
	5'-ATA GTT TCC TCT TCT GTT GC-3'	218–199	A91
	5'-TTA TTT TTG ATT TTT AGT TTG TT-3'	513–491	BO23
	5'-CCG GTA CCT TAT TTT TGA TTT TTA GTT TGT T-3'	513–491	A91
	5'-ATA AAA ATG TTG TTT ATT TTT G-3'	529–505	BO23, B31*
GenomWalkerKit	5'-GTA ATA CGA CTC ACT ATA GGG C-3'	Adapter primer 1	
	5'-ACT ATA GGG CAC GCG TGG T-3'	Adapter primer 2	

Restriction enzyme sites of *Bam*HI and *Kpn*I in expression primers are underlined.
* 3' end from BO23

Table 8. Identities (%) of deduced amino acid sequences of BBK32, DbpA, and DbpB among the Finnish isolates of *B. burgdorferi* sensu stricto IA, *B. garinii* 40, 46, and 50, and *B. afzelii* A91, 1082, 570, 600, EM9.

BBK32

Strain	Identity %						
	B. garinii 40	*B. garinii* 46	*B. garinii* 50	*B. afzelii* A91	*B. afzelii* 1082	*B. afzelii* 570	*B. afzelii* 600
B. burgdorferi sensu stricto IA	95.0	92.1	95.0	72.3	72.3	72.0	72.3
B. garinii 40		93.6	100	70.8	70.8	70.5	70.8
B. garinii 46			93.6	71.4	71.4	71.7	72.0
B. garinii 50				70.8	70.8	70.5	70.8
B. afzelii A91					100	99.1	99.4
B. afzelii 1082						99.1	99.4
B. afzelii 570							99.7

DbpA

Strain	Identity %		
	B. garinii 40	*B. garinii* 46	*B. afzelii* A91
B. burgdorferi sensu stricto IA	62.0	62.0	43.0
B. garinii 40		100	45.3
B. garinii 46			45.3

DbpB

Strain	Identity %					
	B. garinii 40	*B. garinii* 46	*B. garinii* 50	*B. afzelii* A91	*B. afzelii* 1082	*B. afzelii* EM9
B. burgdorferi sensu stricto IA	61.7	61.7	61.7	66.9	66.9	66.9
B. garinii 40		100	100	67.5	66.9	67.5
B. garinii 46			100	67.5	66.9	67.5
B. garinii 50				67.5	66.9	67.5
B. afzelii A91					99.4	100
B. afzelii 1082						99.4

5.3.2. Neuroborreliosis

Serum samples from 14 patients with neuroborreliosis were analyzed with recombinant proteins. The diagnoses were based on clinical symptoms and positive IgG anti-flagella antibodies. The sensitivity of DbpA IgG ELISA was dependent on the use of the three variant antigens from *B. afzelii, B. garinii,* and *B. burgdorferi* sensu stricto. The combined sensitivity, with antibodies to at least one variant antigen, was 100% (Table 9), but with variant antigens only 50%, 50%, and 43%, respectively. The specificity was 99%. In DbpB IgG ELISA, the combined sensitivity with three variant antigens was 64%, and the specificity was 93%. In DbpB IgG ELISA the antigen from *B. garinii* was the most sensitive (50%). The same serum samples were also analyzed in BBK32 IgG ELISAs. The sensitivity of ELISAs with variant antigens was between 93% and 100%, and the combined sensitivity was 100%. The specificity of BBK32 IgG ELISA was 93%.

In WB analyses, 9 out of 10 patient samples analyzed were anti-DbpA positive, and 10 out of 10 were anti-BBK32 positive. Specificities of the WB assays were 100% for DbpA, and 91% for BBK32.

Table 9. Number of serum samples, from patients with LB, with antibodies to variant recombinant BBK32, DbpA and DbpB proteins, and flagella in IgG ELISA. Serum samples were from adult patients with erythema migrans (EM), neuroborreliosis (NB), and Lyme arthritis (LA) and children with LA (LA pediatric). Variant recombinant proteins were from *B. afzelii* A91 (*B. afz*), *B. garinii* 40 (*B. gar*), and *B. burgdorferi* sensu stricto IA (*B.b.*ss). **Total** represents the positivity of a given sample for at least one variant recombinant antigen.

Samples	**Antigens**												
	BBK32				DbpA				DbpB				flagella
	B.afz	*B.gar*	*B.b.* ss	**Total**	*B.afz*	*B.gar*	*B.b.* ss	**Total**	*B. afz*	*B. gar*	*B.b.* ss	**Total**	
EM (n=23)	17	6	5	**17**	3	1	ND	**3**	0	1	5	**5**	**6**
NB (n=14)	14	13	14	**14**	7	7	6	**14**	3	7	1	**9**	**14**
LA (n=15)	12	11	14	**15**	12	3	9	**14**	1	9	6	**11**	**15**
LA (n=52) pediatric	50	41	11	**50**	32	38	19	**51**	20	23	30	**40**	**52**

ND, not done

5.3.3. Suspected neuroborreliosis in children

DbpA, BBK32, IR_6, and flagella as antigens were also analyzed in the serology of 21 children with suspected NB (unpublished). In the whole group of 21 patients, antibodies to DbpA, BBK32, and IR_6 at presentation were detected in 48%, 67%, and 67% of patients, respectively. Anti-flagella IgM antibodies were observed in 78%, IgG antibodies in 38%, and IgM or IgG antibodies in 81% of patients. In the first post-treatment sample (at 2-6 weeks), 81%, 75%, 81%, or 69% of patients had IgG antibodies to DbpA, BBK32, IR_6, or flagella, respectively (Table 10). 67% or 62% of 21 patients had antibodies to 2 or 3 new antigens at presentation or during follow-up, respectively. During follow-up for up to 8 months, antibody responses to the studied antigens remained positive (Table 10).

Table 10. Number of patients with IgG antibodies in ELISA at presentation and during follow-up with flagella, IR_6, DbpA, and BBK32 as antigens (positives/ tested samples). Serum samples were from 21 children with neurologic symptoms and signs compatible with neuroborreliosis.

Timepoint	Flagella	IR_6	DbpA	BBK32
At presentation	8/21	14/21	10/21	14/21
2-6 weeks	11/16	13/16	13/16	12/16
2-4 months	10/13	9/13	8/13	7/13
5-8 months	8/9	6/9	3/9	4/9

5.3.4. Lyme arthritis

Serum samples from 15 patients with LA were analyzed in recombinant DbpA, DbpB, and BBK32 IgG ELISAs. The clinical diagnosis of LA was supported by high-titer anti-flagella IgG antibodies. In total, 14 of 15 patients with LA had antibodies to DbpA. In DbpA IgG ELISA the sensitivity was 93% and specificity 99% (Table 9). In variant DbpA ELISAs, with antigens from *B. afzelii, B. garinii,* and *B. burgdorferi* sensu stricto, 12/15, 3/15, and 9/15 patients were positive, respectively. The DbpA originating from *B. afzelii* strain A91 was the most sensitive

(80%) antigen. With DbpB the combined sensitivity was 73% and the specificity 93%. Anti-BBK32 antibodies were detected in 12/15, 11/15, and 14/15 patient samples with the variant antigens from *B. afzelii, B. garinii,* and *B. burgdorferi* sensu stricto, respectively. The combined sensitivity was 100% and the specificity 93%. In WB analyses with DbpA as antigens, the sensitivity was 70% and the specificity 100%, and with BBK32 as antigens the respective figures were 100% and 93%.

In 52 children with LA, IgG ELISAs with recombinant DbpA, DbpB, and BBK32, with peptide IR_6, and with flagella as antigens were performed. The sensitivity with the commercial flagella ELISA was 100%, and the specificity 98% (Table 9). In recombinant protein ELISAs the three variant antigens were tested. The results with the highest OD/cut-off value were used in data analyses. The sensitivity of DbpA ELISA was 98%, and the DbpA from *B. garinii* was the most sensitive (73%) variant antigen. The specificity of the DbpA ELISA was 93%. In the DbpB ELISA the total sensitivity was 77% and the specificity 90%. In the BBK32 ELISA the respective figures were 96% and 90%. In the IR_6 ELISA, the sensitivity was 98% and the specificity 100%.

Of the 43 patients from whom serum samples at diagnosis were available, the antibody levels during the post-treatment follow-up were analyzed against DbpA, DbpB, BBK32, IR_6, and flagella. For the recombinant DbpA, DbpB, and BBK32 ELISAs, the results obtained with the same variant antigen were used throughout the follow-up. The antibody levels in DbpA and BBK32 ELISAs were higher at diagnosis in patients with acute than in those with episodic or chronic LA ($p=0.001$ and $p=0.030$, respectively). In ELISAs with DbpB, IR_6, and flagella, no significant differences between the patient groups were observed. During follow-up in patients with acute, episodic, and chronic LA, all antibody levels waned slowly and the rate of decline did not differ significantly between the patient groups in any of the antibodies analyzed (Figure 2 in study IV). At the 2-year follow-up, 79%, 81%, and 86% of patients had antibodies to IR_6, flagella, and DbpA, respectively.

5.3.5. Species specificity

In WB analyses with mouse plasma, antibody levels to variant DbpAs showed a distinct species-specific pattern. The mice had been infected with *B. garinii* strain Å218. In WB plasma from *B. garinii* infected mice reacted with recombinant DbpA from *B. garinii* but not with that from *B. afzelii* or *B. burgdorferi* sensu stricto.

Of the 79 anti-DbpA positive samples in 81 patients with NB or LA (study I and IV), 39 reacted with one DbpA antigen only. In the remaining 40 sera, positive antibody levels were observed against two or three DbpA variants. In 26 of these 40 sera, the OD/cut-off ratio was twice as high against one antigen as against the other two antigens. In the remaining 14 patients, the antibody levels for the three variant DbpA antigens were indistinguishable. In total, in 65/81 (80%) serum samples, the antibody levels against the DbpA variant antigens suggested that the infective borrelial species was *B. afzelii* in 34, *B. garinii* in 26, and *B. burgdorferi* sensu stricto in 5 patients.

With variant DbpB and BBK32 antigens, species-specific antibody responses in the serum samples were only occasionally detected. With DbpB and BBK32 antigens, antibody responses were of moderate level, and usually antibodies to more than one antigen were positive.

6. DISCUSSION

6.1. IMPLICATIONS OF SEQUENCE HETEROGENEITY TO LYME BORRELIOSIS SEROLOGY

Over 99% of the genome of *B. burgdorferi* sensu stricto strain B31 has been sequenced (Fraser et al. 1997, Casjens et al. 2000). However, it is known that there are marked differences in sequence between borrelial species and strains. Therefore, it is relevant to study borrelial sequences in European conditions, where several borrelial species cause infections (Postic et al. 1998, Baranton et al. 2001). In the present study, a PCR-based approach was used to clone borrelial genes. However, this approach was not always successful. Therefore, a genome walking method, not previously used in borrelial studies, was applied. With this method, the *dbpB* sequences from *B. afzelii* and *B. garinii* were described for the first time.

Previous studies have shown that among individual borrelial proteins in different species, sequence heterogeneity of up to 40% may occur (Fellinger et al. 1995, Jauris-Heipke et al. 1993, Roberts et al. 1998, Roessler et al. 1997). In the present studies, an even higher heterogeneity between sequences of different strains was detected, 57%, 38%, and 27% between DbpA, DbpB, and BBK32 variants, respectively (Table 8). Sequence heterogeneity has major implications for the usefulness of such proteins as antigens in the serology of LB. If the antigenic epitopes in the variant proteins differed, the risk for lower sensitivity would increase in assays where only one variant of a given antigen is used. In the present studies, we demonstrated that, by using variant antigens from all the pathogenic borrelial species, it is possible to maintain adequate sensitivity of WB and ELISA without compromising the specificity.

In this study, with recombinant DbpA variants, species-specific antibodies were observed. The immunoreactivity to DbpA in experimental murine LB in this study also supports the species specificity of DbpA serology. In a previous animal study, some cross-reactivity of antibodies against heterologous DbpA has been observed (Feng et al. 1998). In fact, this is in line with the restriction observed in the immune responses to the DbpA variants in the present study, but also with some cross-reactivity among the variants. The present results also suggest that the recombinant antigens may have some degree of epitope sharing between variants from different borrelial species. This information may be useful at least for epidemiologic purposes. As some borrelial strains or species are associated with more invasive or severe clinical manifestations (Wang et al. 1999), knowledge of the infective subspecies might also have therapeutic implications. Finally, if vaccines against *Borreliae* are to be designed, it is crucial to know which species cause infections. The prevalence of species-specific immune reactions in this series seems to reflect the European epidemiologic situation in general (Hubalek and Halouzka 1997). With variant recombinant DbpB and BBK32 proteins, somewhat similar, but not as species-specific, antibody responses as with DbpA were observed. It is evident that variant recombinant proteins may be needed in parallel or in combination in an immunoassay for LB to cover all the relevant borrelial species. Further studies are needed to evaluate how a combination of variant antigens in the wells of microtiter plates would perform.

6.2. SEROLOGY OF LYME BORRELIOSIS WITH THE NEW ANTIGENS

For reliable serodiagnosis of LB, a confirmatory immunoblotting after the ELISA test has been advocated. However, the use of different species and strains of *B. burgdorferi* sensu lato as sources of antigens, especially in Europe, leads to inconsistent results because of variations in the expression of the immunogenic proteins (Hauser et al. 1998, Robertson et al. 2000b). Furthermore, immunoblotting is not only a tedious procedure in laboratory routine, but is also prone to subjective interpretations of band intensities. A recent European multicenter study formulated a panel of seven test kit-specific immunoblotting rules to be adopted in relation to the characteristics of LB in local areas (Robertson et al. 2000b). In another European study, exclusion of primary EBV and cytomegalovirus infections by appropriate targeted serology gave better predictive power than demanding confirmation with immunoblotting after the positive ELISA test (Goossens et al. 1999). Beyond any doubt, this emphasizes the need for novel methods that should be standardized at least with regard to performance, relevance, techniques, and antigen preparation. Use of new borrelia-specific antigens is an option.

With the current LB serology, based mainly on flagella or WCL as antigens, specificity problems have occurred in disseminated LB (Goossens et al. 1999). In the present studies, novel antigens DbpA, DbpB, BBK32, and IR_6, were analyzed in the serology of LB. In patients with disseminated LB, NB or LA, the sensitivity of ELISA assays using DbpA, BBK32, and IR_6 as antigens was 93-100% and specificity was 90-100%. Furthermore, when using the peptide antigen IR_6, positive antibody levels were proportionally much higher compared with the cut-off level than with other antigens. In previous studies, the sensitivity of BBK32 had been slightly lower (60-92%) (Fikrig et al. 1997, Akin et al. 1999). Only one study has previously evaluated

DbpA in the serology of LB (Cinco et al. 2000). In Italian patients the sensitivity was 35% for disseminated LB. The probable explanation for the low sensitivity is that the DbpA antigen originated from *B. burgdorferi* sensu stricto, whereas *B. afzelii* and *B. garinii* are more prevalent borrelial species in Europe. The sensitivity of DbpA from *B. burgdorferi* sensu stricto in the present study was in accord with the previous results, while DbpAs from *B. afzelii* and *B.garinii* gave frequent positivity. Recently, IR_6 has proved to be a sensitive and specific antigen in LB serology (Liang et al. 1999, Liang et al. 2000, Philipp et al. 2001). In European patients with disseminated LB, the sensitivities reached 70% in Italy and 95% in Austria. All the Austrian patients had ACA, a late manifestation of disseminated LB that is associated with a strong antibody response to the currently used antigens, also. DbpB did not perform as well as the other new antigens. Especially the sensitivity of DbpB ELISA remained at a suboptimal level. In conclusion, the present study shows that DbpA, BBK32, and IR_6 are promising new antigens for the serology of disseminated LB (Table 11). Further studies in unselected patient populations are needed to characterize the details of the antibody responses to these antigens.

Table 11. Performance of the new antigens in IgG ELISA in different clinical manifestations of LB.

	Manifestation		
Antigen	EM	NB	LA
BBK32	**++**	**++**	**+++**
DbpA	**–**	**++**	**+++**
DbpB	**–**	**+**	**+**
IR_6	**NE**	**++**	**+++**

+++ = excellent
++ = good
+ = satisfactory
– = poor
NE = not evaluated

In children, NB may be more frequent than in adults (Berglund et al. 1995). Similar clinical manifestations are seen, but in children the most common findings in NB are facial palsy and aseptic meningitis (Christen et al. 1993). In adults, peripheral nerve disturbances are more common. There are no generally accepted criteria for the laboratory diagnosis of NB in Europe and in the USA. In Europe, demonstration of intrathecal borrelial-specific antibody production and pleocytosis in the CSF are considered essential (Stanek et al. 1996). In the USA, however, less stringent criteria have been used. Seroconversion or a four-fold titer rise of anti-*B. burgdorferi* antibodies in paired sera is accepted as laboratory evidence for NB in the USA (Prasad and Sankar 1999). It is evident that the diagnosis of NB should be based on a combination of clinical features and laboratory parameters. This study shows that assessment of antibodies to several specific borrelial antigens may be useful already at presentation of NB in children. However, in patients with suspected NB the CSF analysis is essential.

The sensitivity of the new antigens was compared to those of the routinely used flagella and WCL antigens. With the recombinant proteins, the sensitivity of ELISAs with single variant antigens was usually moderate to low, but by combining the results with variant antigens higher ELISA sensitivities were reached. However, it is important to keep in mind that, because of the low positivity in culture, there is no “gold standard” concerning the laboratory diagnosis of disseminated LB. The performance of the new antigens was evaluated in patients in whom where the diagnosis of LB was based on clinical features supported by positive serology to flagella or WCL. Therefore the accuracy of original diagnoses may be questionable in some cases, which may cause discrepancies in the serologic evaluations. Given that specificity of serologic testing can be increased by using more than one independent test, it can be speculated that combining several specific assays the diagnostic accuracy could be improved.

6.3. SEROLOGY OF EARLY LYME BORRELIOSIS

In the present study, the performance of the new antigens varied. BBK32 was the only antigen that seemed to function well in early Lyme borreliosis. *bbk32* has been shown to be selectively expressed in vivo like a few other genes (*bbk50, vlsE, ospE* and *ospF* homologs) (Akins et al. 1995, Champion et al. 1994, Das et al. 1997, Fikrig et al. 1997, Fikrig et al. 1999, Probert et al. 1998, Stevenson et al. 1995, Stevenson et al. 1998, Suk et al. 1995, Wallich et al. 1995, Zhang et al. 1997). Moreover, *bbk32* expression is detectable in spirochetes during tick feeding even before transmission to the host, although not in unfed ticks (Fikrig et al. 2000). In addition, in a reverse transcriptase PCR study, expression of *bbk32* in the EM lesion has been demonstrated (Fikrig et al. 1998). Thus, it could be hypothesized that, if BBK32 were immunogenic in humans, it might be useful as an antigen for the serology in early LB. The present study showed that BBK32 has potential as a serodiagnostic antigen for early LB.

In early LB, the ELISA or immunoblot assays using flagella or WCL as antigens do not detect IgM or IgG antibodies until 2 to 4 or 6 to 8 weeks after the onset of the disease, respectively (Craft et al. 1984, Mitchell et al. 1994). During early local LB, the sensitivity of IgM ELISA seldom exceeds 50% (Aguero-Rosenfeld et al. 1993, Engström et al. 1995, Grodzicki et al. 1988, Mitchell et al. 1994). A study on patients with culture-confirmed EM showed that positive serology at presentation and the rate of seroconversion correlated directly with disease duration (Aguero-Rosenfeld et al. 1996). If the EM lesion had emerged less than 7 days prior to sampling, only 10% of the patients showed antibodies in ELISA, whereas, of the patients whose EM had occurred 7 to 14 days earlier, 58% had detectable antibodies. In the present series, the time of occurrence of the EM lesion could not be accurately assessed. However, given the low proportion of anti-flagella antibodies (26%) at presentation of EM and the broad awareness of LB among the general population in regions where LB is endemic in Finland, it can be presumed that, in most cases, the EM lesions represented early disease. In the present study, 74% of patients with acute phase EM had detectable IgG antibodies to BBK32 by ELISA and/or WB. In an early exposure to *Borreliae*, the expected IgM isotype antibodies could be detected only infrequently (4-13%). The reason for this is unknown but low sensitivity of IgM serology has been associated with most of the other new recombinant borrelial antigens as well (Magnarelli et al. 1996, Magnarelli et al. 2000, Panelius et al. 2002). Hence, the present results imply that assessment of IgG, but not of IgM antibodies, to BBK32 proteins may afford a major improvement in the serodiagnosis of early LB (Table 11). In the serology of EM, DbpA and DbpB as antigens in IgM or IgG ELISAs had low sensitivity (26% or less). Therefore, in early LB, assays using DbpA or DbpB as antigens do not seem to be useful (Table 11).

6.4. SEROLOGIC INDICATOR OF DISEASE ACTIVITY

There is a lack of generally accepted serological indicators for monitoring the disease activity or the response to therapy. IgG and, surprisingly, also IgM antibodies to *B. burgdorferi* may persist in the serum even after successful treatment of LB (Kalish et al. 2001, Steere 1993, Craft et al. 1984, Feder et al. 1992). *B. burgdorferi* specific serum immune complexes have been suggested to signify active LB (Brunner et al. 2001, Schutzer et al. 1999), but the results of immune complex assays have not yet been confirmed by others. A rapid decrease of anti-flagella IgG antibodies after treatment of disseminated LB has been suggested to serve as a marker for the cure of the disease (Panelius et al. 1999). In the present study, the majority of patients had anti-flagella antibodies even 2 years after the diagnosis. Philipp et al. (2001) have recently suggested that antibodies to IR_6 could be used as a indicator for treatment response in LB. In the present study, antibody levels to IR_6 failed to predict the clinical outcome of LA. The rate at which antibodies to IR_6 declined failed to differentiate patients with acute LA from those with an episodic or a chronic course of LA. Moreover, the majority of patients were still positive for IR_6 antibodies even 4 years after diagnosis. This indicates that individual elevated antibodies to IR_6 cannot be used as indicators of disease activity. Similar results were recently reported by Peltomaa et al. (2002). The reason for the discrepancy between the present and previous results by Philipp et al. (2001) is not known. Mechanisms for a chronic or treatment-resistant course of LA may, however, be different in European children and American adults. Similarly, in the majority of children with NB, during short-term post-treatment follow-up antibodies to flagella and the new antigens declined but remained positive. Thus, it is unclear, at the moment, whether antibodies to any of the studied antigens could be used to judge the activity of infection in NB.

In conclusion, in the serology of LB, the new antigens DbpA, BBK32 and IR_6, but not DbpB, performed well. Comparison of recombinant DbpA, BBK32, and DbpB proteins, and IR_6 peptide showed that IR_6 may have the best discriminatory power between patient and control samples. Of the recombinant proteins, variant proteins, in parallel or combined, are probably needed to cover all the relevant borrelial species. It is possible that a panel of independent specific antigens may improve the accuracy of serologic tests in LB.

7. CONCLUSIONS

Recombinant BBK32 and DbpA proteins seemed to perform well in IgG serology of patients with disseminated LB, provided that variants from all pathogenic borrelial species were included in the antigen set. With single variant antigen the sensitivity of the serologic assay was considerably lower. This was most probably due to the sequence heterogeneity between variant proteins, which may cause conformationally different antigenic epitopes.

In the serology of EM, the sensitivity of the routinely used flagella ELISA was 26%. With the recombinant BBK32 protein, the sensitivity of IgG serology was 75% already at presentation, and at convalescence all patients had anti-BBK32 antibodies as assessed by ELISA or WB. IgM antibodies could be detected only infrequently (4-13%). The reason for this is unknown but low sensitivity of IgM serology has been associated with most of the other new recombinant borrelial antigens as well. Hence, the present results imply that assessment of IgG, but not of IgM, antibodies to BBK32 proteins may afford a major improvement for the serology of early LB.

The peptide antigen IR_6 seemed to be a sensitive and specific antigen in the serology of children with disseminated LB. In addition, the discriminatory power of the antibody response to IR_6 between LB patient samples and controls was high.

In monitoring the activity of the disease in LB, no generally accepted serologic markers have been introduced. Declines in anti-flagella and anti-IR_6 antibodies have been suggested to be useful indicators for monitoring the treatment response. In children with LA, irrespective of the clinical course of arthritis, antibodies to the recombinant antigens DbpA, DbpB, and BBK32, and to the IR_6 peptide and flagella waned slowly during follow-up. Approximately 80% of the patients had antibodies to the tested antigens 2 years after presentation. In children with suspected NB, the antibody levels declined but remained positive during a short-term follow-up. Thus, it is unclear, at the moment, whether antibodies to any of the studied antigens could be used as indicators for disease activity or treatment response.

In the serology of NB, in preliminary analyses, the new recombinant antigens DbpA and BBK32 and peptide antigen IR_6 seem to be useful. It can be speculated that, supplementary to the CSF findings, assessment of serum antibodies to several specific borrelial antigens may improve the diagnostic accuracy already at presentation of NB.

In the present study, several specific assays for the serology of LB have been introduced. It is possible that a panel of independent specific antigens could improve the diagnostic accuracy and positive predictive value of serologic testing of LB.

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