View metadata, citation and similar papers at core.ac.uk

brought to you by CORE

provided by Helsingin yliopiston digitaalinen arkisto

Helsinki Biomedical Dissertations No. 58

# Complement Evasion by *Borrelia burgdorferi* Spirochetes

Antti Alitalo

Department of Bacteriology and Immunology
Haartman Institute
Faculty of Medicine
University of Helsinki
Helsinki
Finland



UNIVERSITY OF HELSINKI

ACADEMIC DISSERTATION

To be publicly discussed, with permission from The Medical Faculty of The University of Helsinki, in Biomedicum Helsinki, Lecture hall 2, Haartmaninkatu 8, 00014 University of Helsinki at 12 o'clock on December 18, 2004.

Supervisor

Professor Seppo Meri, M.D., Ph.D.
Department of Bacteriology and Immunology
Haartman Institute
University of Helsinki
Helsinki
Finland

Reviewers

Professor Helena Käyhty, Ph.D.
Vaccine Development
National Public Health Institute
Helsinki
Finland

Professor Olli Lassila, M.D., Ph.D.
Director, Turku Graduate School of Biomedical Sciences (TuBS)
Department of Medical Microbiology
University of Turku
Turku
Finland

Opponent

Docent Anna Blom, Ph.D.
Department of Clinical Chemistry
Lund University
The Wallenberg Laboratory
University Hospital Malmö
Malmö
Sweden

ISBN 952-10-2240-X (paperback)
ISBN 952-10-2248-5 (pdf)
ISSN 1457-8433

# 1 Table of Contents

# 2 List of original contributions

The thesis is based on the following original publications:

**I** **Alitalo, A.**, T. Meri, L. Rämö, S. T. Jokiranta, T. Heikkilä., I. J. T. Seppälä, M. K. Viljanen, and S. Meri. Complement Evasion by *Borrelia burgdorferi*: Serum Resistant Strains Promote C3b Degradation. *Infection and Immunity* 2001; 69:3685-3691.

**II** Hellwage, J., T. Meri, T. Heikkilä, **A. Alitalo**, J. Panelius, P. Lahdenne, I. J. T. Seppälä, and S. Meri. The Complement Regulator Factor H Binds to the Surface Protein OspE of *Borrelia burgdorferi. Journal of Biological Chemistry* 2001; 276:8427-8435.

**III** **Alitalo, A.**, T. Meri, H. Lankinen, I. Seppälä, P. Lahdenne, P. S. Hefty, D. Akins, and S. Meri. Complement Inhibitor Factor H Binding To Lyme Disease Spirochetes Is Mediated By Inducible Expression of Multiple Plasmid-Encoded OspE Paralogs. *Journal of Immunology* 2002; 169:3847-3853.

**IV** **Alitalo, A.**, T. Meri, T. Chen, H. Lankinen, Z. Cheng, T. S. Jokiranta, I. J. T. Seppälä, P. Lahdenne, P. S. Hefty, D. R. Akins, and S. Meri. Lysine-dependent Multi-point Binding of the *Borrelia burgdorferi* Virulence Factor OspE to the C Terminus of Factor H. *Journal of Immunology* 2004; 172:6195-6201.

**V** **Alitalo, A.**, T. Meri, P. Comstedt, L. Jeffery, J. Tornberg, H. Lankinen, S. Bergström, M. Cinco, D. Akins, and S. Meri. Expression of Complement Factor H Binding Proteins in *Borrelia garinii* Freshly Isolated from Patients with Neuroborreliosis. *Submitted*.

The publications have been reproduced in this thesis with the permission of the copyright holders. The publications are referred to in the text by their Roman numerals.

# 3 List of abbreviations

The following abbreviations are used throughout the text.

| | |
|---|---|
| $^{125}$I | $^{125}$Iodine |
| Ab | Antibody |
| AP | Alternative pathway of complement |
| APC | Antigen presenting cell |
| Ba | Factor B cleavage fragment a |
| Bb | Factor B cleavage fragment b |
| C | Complement |
| C4bp | C4b binding protein |
| CD | Cluster of differentiation |
| CD59 | Protectin |
| CNS | Central nervous system |
| CP | Classical pathway of complement |
| CRX | Complement receptor X, where X=1-3 |
| CSF | Cerebrospinal fluid |
| CX | Complement component X, where X=1-9 |
| CXx | CX cleavage fragment x, where X=2-5 and x=a..g |
| DAF | Decay-accelerating factor (CD55) |
| FB | Factor B |
| FD | Factor D |
| FH | Factor H, beta-1-H globulin |
| FHL-1 | Factor H-like protein 1 |
| FHR | Factor H-related protein |
| FI | Factor I |
| iC3b | Inactivated C3b |
| Ig | Immunoglobulin |
| kDa | Kilodalton |
| LFA | Lymphocyte function antigen |
| LP | Lectin pathway of complement |
| LPS | Lipopolysaccharide |
| mAb | Monoclonal antibody |
| MAC | Membrane attack complex |
| MASP | Mannan binding lectin associated serine protease |
| MBL | Mannan binding lectin |
| DbpA | Decorin binding protein A |
| ErpX | OspEF-related protein X, where X=A..X |

| | |
|---|---|
| GVB | 0.1% gelatin in veronal buffered saline |
| MCP | Membrane cofactor protein (CD46) |
| NHS | Normal human serum |
| OM | Outer membrane |
| ORF | Open Reading Frame |
| OspX | Outer surface protein X of *Borrelia burgdorferi*, where X=A..F |
| P | Properdin |
| PBS | Phosphate buffered saline |
| PC | Protoplasmic cylinders |
| RU | Resonance unit |
| s. l. | sensu lato |
| s. s. | sensu stricto |
| SCR | Short consensus repeat |
| SDS-PAGE | Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis |
| st | Serotype |
| TCC, C5b-8$(C9)_n$ | Terminal complement complex |
| TP | Terminal pathway of complement |
| VBS | Veronal buffered saline |
| VCP | Vaccinia control protein |
| $\Delta$ | Delta, indicates deletion of the consecutive amino acids from the protein |

# 4 Abstract

The human immune system has developed over the course of millennia from the simple innate-type of immune system of simple life forms into a complex system comprising the innate and adaptive arms. The adaptive arm of the immune system is cell-dominated in its actions, while the innate arm consists mostly of phagocytic cells and proteins, including many enzymes. *Borrelia burgdorferi*, the causative agent of Lyme disease (borreliosis), which is a potentially persistent complex multi-system disorder, is an example of a successful human pathogen. It is capable of bypassing the immune defense mechanisms of the human body. The complement system is in the first line of this defense, and thus, for a microbe to be a pathogen and infectious to humans, it must first bypass this system.

Lyme disease is caused by three genospecies that all belong to *Borrelia burgdorferi* sensu lato (s. l.): *B. burgdorferi* sensu stricto (s. s.), *Borrelia afzelii* and *Borrelia garinii*, which share only about 80% of common genetic material. The most prevalent clinical manifestations of borreliosis vary. *B. burgdorferi* s. s. is mostly arthrotropic, *B. afzelii* dermotropic, and *B. garinii* neurotropic. The aim of this study was to characterize the complement evasion features common and unique to each of these strains. More specifically, we wanted to find out, a) by which mechanism *Borrelia burgdorferi* bypasses the complement system, and b) which surface molecules are associated with this mechanism.

It was found that serum resistant *B. burgdorferi* strains bound factor H (FH), an inhibitor of the alternative pathway of complement from their hosts and that this ability correlated with the ability to resist serum-mediated lysis of the bacteria. FH binding promotes C3b inactivation; it prevents C3/C5 activation and membrane attack complex (MAC) formation. A family of surface expressed lipoproteins, the outer surface protein E (OspE)–family was found to be used by *B. burgdorferi* to bind FH. Individual strains expressed multiple OspE-proteins encoded by circular plasmids. The sequences of OspE-proteins from *B. garinii* vs. those of *B. afzelii* and *B. burgdorferi* s. s. were found to vary. These variations may account for the differences in the serum sensitivities of these strains. In addition to OspE, a second FH-binding protein, approximately 35 kDa in molecular weight, was also found.

The OspE-protein family members were found to constitute a repertoire of FH-binding proteins, whose expression depends on signals from the external

environment. The expression of OspE-proteins becomes upregulated at 37°C, which is a key feature making them available when the spirochetes are subjected to the immune attacks of warm-blooded mammals. OspE proteins were thus found to constitute a family of mobile virulence factors, whose expression becomes induced in the host. The binding sites on FH and OspE were found to be located at the C-terminus of FH and at the N- and C-termini of OspE. The essential amino acid residues of OspE in the interaction were found to be lysines.

FH binding apparently has an essential role in the ecology of the enzootic cycle (existence in different hosts) of *B. burgdorferi*. *B. burgdorferi* is able to infect a number of different animal hosts and bind FH from the host environment. There are typically several copies of genes encoding OspE-family proteins in a single bacterial strain, suggesting that their function may be related to different stages of the enzootic cycle. FH binding, therefore, has an essential role in the adaptation of the spirochete to the immune systems of the various hosts.

In conclusion, a new family of *Borrelia burgdorferi* virulence factors, the OspE-protein family, is described in this work. These factors are surface expressed and contain crucial FH-binding determinants. OspE-proteins are tentatively important in the successful persistence of the spirochete in the human body. This new information is of potential use in development of a new multi-strain vaccine effective against all three Lyme disease -causing genospecies.

# 5 Introduction

## 5.1 The human immune system – adaptive and innate arms

The most important first lines of defense of the human body against invading microorganisms are physical barriers, such as skin, and mucous membranes. After pathogenic microbes penetrate these barriers, the immune system steps in. It is customary to divide the immune system into two systems, a nonspecific system, which attacks foreign surfaces and a specific system, which develops diversity, specificity and memory of how to combat the specific microorganism. These are also known as the innate immune system and the adaptive immune system, respectively.

In addition to the aspect of recognition, these terms also encompass the aspect of evolutionary origin. The innate immune system is the more simple one, and more archaic in evolutionary origin (5). The adaptive immune system, which requires the presence of specialized cells and is only present in multicellular organisms, is a later development.

The complement (C) system functions as a cascade of binding interactions and enzymatic events, which help in the processing of foreign material. The lectin (LP) and alternative pathways (AP) of complement (see Fig. 1) are essential components of the innate immune system. The classical pathway (CP) functions as a component of both innate and adaptive parts of the immune system.

## 5.2 Functions of complement as part of the human immune system

In the course of evolution the complement system has developed from a simple protein cascade of single celled organisms into a network of recognition events, interactions and enzymatic reactions. The complement system is one of the most versatile parts of the immune system. Once the mechanical barriers to infection, namely the skin and mucous membranes, are disrupted it is the first line of defense against previously unencountered as well as familiar microbes. The complement system is usually divided into the alternative, classical and lectin pathways (6). The alternative pathway is activated by foreign surfaces and by autoamplification it coats the surfaces of invading bacteria with proteins, which enable their opsonophagocytosis by

neutrophils and other phagocytosing cells. The lectin pathway is also activated by foreign surfaces, but more specifically it recognizes mannose or N-acetylglucosamine-containing carbohydrates, relatively common molecules on bacterial surfaces. The classical pathway is usually seen as part of the adaptive immune system, because it is activated by antibodies recognizing previously encountered microbes, but it may also be activated in the absence of specific antibodies e.g. by bacterial lipopolysaccharides (7). The functions of complement include lysis, opsonization, targeting and enhancement of the immune system, and clearance of foreign material.

### 5.2.1 DIRECT COMPLEMENT-MEDIATED LYSIS

Complement-mediated lysis of target cells is achieved by components of the complement system working in concert. The activation of complement leads to the terminal pathway (TP; Fig. 1), which ends in the terminal complement complex (TCC), or MAC. Formation of MAC results in water permeable hydrophilic pores being inserted into lipid bilayer membranes. The osmotic potential inside the attacked cell thereafter draws fluid inside, and the osmotic pressure eventually results in the rupture of the cell membranes.

### 5.2.2 COMPLEMENT CLEARS DEBRIS AND OPSONIZES BACTERIA

In addition to direct attacks of the complement system against living bacterial cells, it has important functions in clearing debris from the host. The debris may be remnants of lysed bacteria or damaged or dead cells, for example. Complement molecules, such as C1q, C4b, C3b, and iC3b function as ligands for leukocyte receptors, such as complement receptor 1 (CR1) and complement receptor 3 (CR3). The phagocytosis of, for example, leprosy bacilli, is mediated by CR1 and CR3 (8). These receptors are found on antigen presenting cells such as macrophages, and dendritic cells, which phagocytose or pinocytose complement coated (i.e. opsonised) cells or particles.

### 5.2.3 COMPLEMENT ACTIVATION GENERATES INFLAMMATION AND PRODUCES CHEMOATTRACTANTS

Complement-mediated inflammation is produced to a large extent by the release of powerful inflammatory agents C3a and C5a (see Fig. 2). These increase vascular permeability locally at the site of inflammation, resulting in the classical *rubor*, *tumor*, *calor* and *dolor* signs of inflammation. Chemotaxis also results to a large extent from the release of powerful chemoattractants

C3a and C5a, whose concentration gradient serves as a homing signal for leukocytes to sites of inflammation. Histamine is released from mast cells. C5a, and to a lesser extent also C3a, mediates anaphylaxis by binding to specific receptors C5aR, and C3aR. Deposition of MAC also induces inflammatory changes in tissues by activating cells via $Ca^{++}$ influx to release leukotrienes, prostaglandins and other inflammatory mediators.

### 5.2.4 NECROTIC AND APOPTOTIC CELLS ARE CLEARED BY COMPLEMENT

Necrotic or apoptotic cells are found especially in regenerating tissues. This debris is marked by complement for cellular processing. C3b and C4b – molecules, which are recognized by phagocytes, opsonize immune complexes that form when antibodies bind to cellular debris or form other immune complexes. Complement recognizes key structures, such as chromatin, which are normally confined inside of the cells. However, C1q also recognizes apoptotic cells or blebs directly by binding to flip-flopped surface-molecules. This also results in opsonization and phagocytosis. A deficiency or failure in this system results in systemic lupus erythematosus with characteristic symptoms.

### 5.2.5 COMPLEMENT TARGETS AND ENHANCES SPECIFIC IMMUNE RESPONSES

Specific immune system enhancement is achieved when complement components attach to bacteria and other microorganisms to be picked up by cells of the immune system for antigen presentation. Such targeting is achieved primarily by detection of opsonised material using complement receptors (CR1..CR3), which are expressed on specific cells of the immune system. More specifically, CR1 is involved in the transport, CR2 in targeting to B- and dendritic cells, and CR3 in the phagocytosis of antigens. Complement receptor 2 is expressed by B-lymphocytes and dendritic cells and is essential in activating B-cells and reinforcing the immune system, especially by activating and enhancing adaptive immunity (9). This contributes to the specific targeting of the immune system.

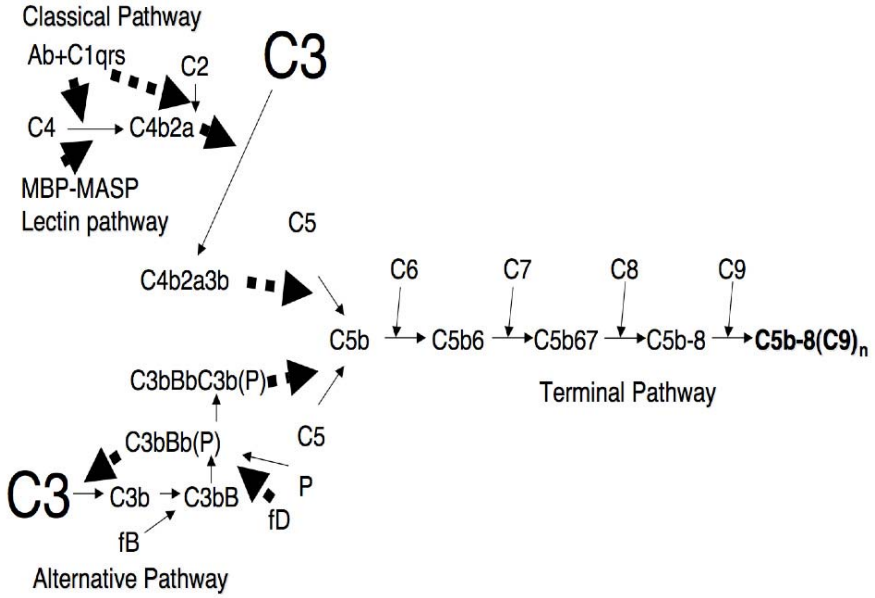


**Figure 1** Activation of the human complement system (1). Thick, discontinuous lines indicate enzymatic activity. The complement system is developmentally an ancient system, with the alternative and lectin pathways, as parts of the innate immune system, being more ancient. Their development dates back to the *Metazoa*, whereas the classical pathway, along with the adaptive immune system, developed in jawed invertebrates (5).

## 5.3 The alternative complement pathway

The AP is a very efficient tool of the immune system in resisting and fighting infection. AP function is not dependent upon previous encounters with the specific pathogen in question, or upon leukocytes, or other cells of the immune system. The liver is the major site of synthesis of all AP components, which are found in serum. The activation of the alternative pathway is triggered by default on a foreign surface and coats it with C3b. This results in efficient opsonization of foreign material. In the presence of phagocytosing cells the material is also engulfed and processed. In addition to opsonization, the activation proceeds to the TCC/MAC and may result in lysis of gram-negative bacteria.

C3 is hydrolyzed in serum. This is known as tick-over activation, which happens at a low rate all the time. Activated C3($H_2O$) binds factor B molecules. After cleavage of B to Bb and Ba by factor D, C3($H_2O$) and Bb together form the initial C3 convertase C3($H_2O$)Bb. If an activating surface is

present nearby, the AP feedback cycle is activated (see Fig. 2). The central reaction in AP activation is the cleavage of C3 to C3b by the AP convertase C3bBb. Because the AP is activated by default and it is efficient, it may become dangerous for cells of the human body if it is not tightly controlled.

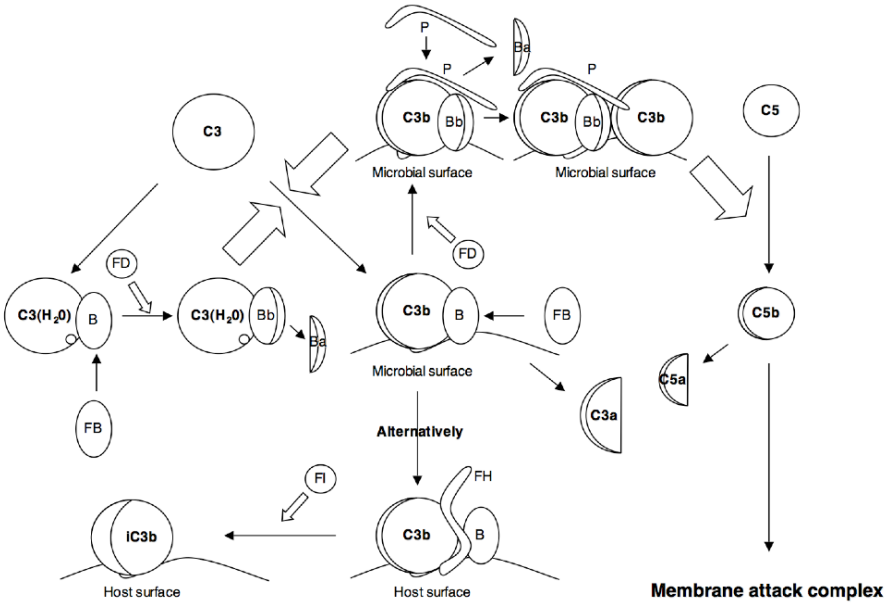


**Figure 2** Activation of the Alternative Pathway of the Human Complement System (6). The pathway starts with tick-over activation of C3 (to the left and down). This refers to the autohydrolysis of an internal thiolester bond of C3 to create reactive groups capable of forming covalent bonds with other molecules.

## 5.4 Complement regulation

Regulation of the complement system is essential, because of the nonspecific nature of AP and LP and to some extent CP activation. In fact, if all complement in the human body were activated on the surfaces of our own cells, it would be able to destroy the entire body in a matter of hours. Fluid-phase and surface regulatory molecules in serum and on cell membranes therefore, regulate complement. They can be divided into AP, CP and terminal pathway (TP) inhibitors and modulators. They all prevent unnecessary consumption of complement and protect our own tissues from complement attack.

### 5.4.1 C1INH

C1-inhibitor is an inhibitor of the CP and lectin pathways inhibiting CP C1r and C1s, and MASP1 and MASP2 of the lectin pathway. Most important in its function is C1s activity regulation, but it also regulates some enzymes of the coagulation cascade and prevents C1r autoactivation. C1INH deficiency results in hereditary angioedema (HAE) (10).

### 5.4.2 C4BP

C4bp is principally a CP regulator and functions as a cofactor in the cleavage of C4b by FI (11). It also inactivates the CP C3-convertase C4b2a by promoting dissociation of C2a. C4bp can act as a cofactor also in the cleavage of C3b.

### 5.4.3 FACTORS H AND I

Factor H (FH) is a 150 kDa AP inhibitor found in serum. It consists of 20

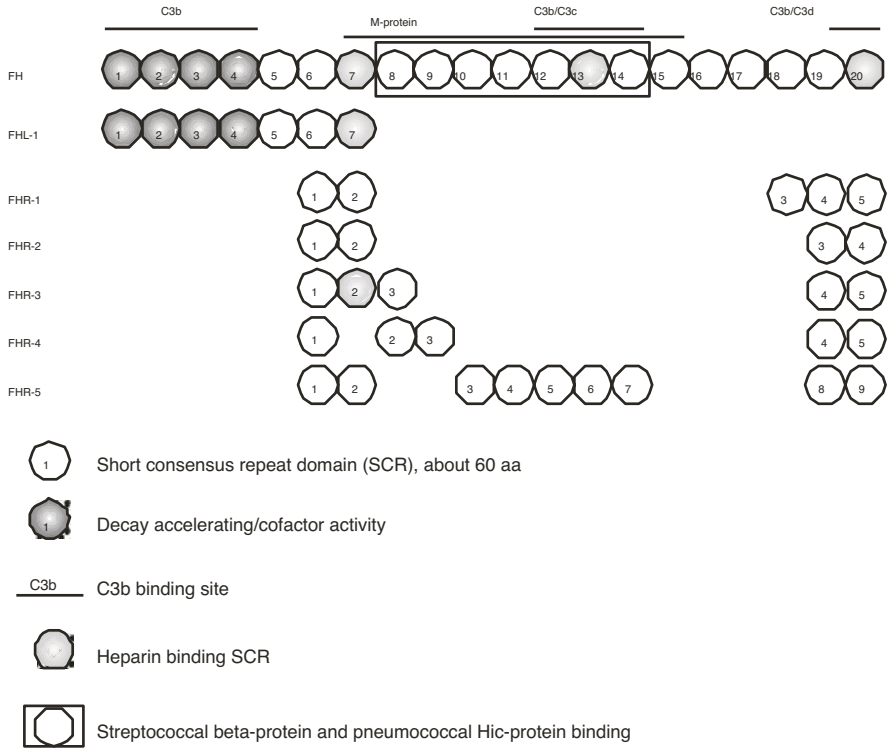


**Figure 3** Schematic diagrams of FH-Protein Family Members FH, FHL-1 and FHR-1. SCR alignment according to regions of homology (1). The figure illustrates binding sites on FH for C3b, C3c, C3d and streptococcal M-protein (2, 3). The group B streptococcal beta-protein and pneumococcal Hic-protein binding SCRs are also indicated (4).

homologous short consensus repeat (SCR) domains containing approximately 60 aa residues each. The structure resembles a string of pearls (see Figs. 2 and 3). FH functions as a cofactor for factor I (FI), which cleaves C3b into iC3b (12). It also prevents the binding of factor B to C3b and accelerates the dissociation of Bb from C3 and C5 convertases. FH-like protein 1 (FHL-1) and FH-related proteins 1-5 (FHR-1..FHR-5) share homologous SCR-domains with FH. FHL-1 shares the first 7 SCR-domains with FH, and thereby lacks the surface-recognition properties, but otherwise has most of the functional activities of FH. In contrast, the FHRs have not been shown to have as efficient C-regulatory functions as FH.

### 5.4.4 MEMBRANE REGULATORS OF COMPLEMENT

The membrane cofactor protein (MCP; CD46) is an AP regulator and functions as a membrane associated cofactor in C3b cleavage. The decay-accelerating factor (DAF; CD55) is an AP and CP regulator and accelerates the decay of both AP and CP C3-convertases. Protectin (CD59) is the only TP–regulator on cell membranes. It prevents formation of the MAC by binding to the C8 and C9 molecules and preventing their insertion into cell membranes (13). Both DAF and protectin are anchored to cell membranes via glycophosphoinositol (GPI) moiety. An acquired deficiency of the GPI-anchor occurs in paroxysmal nocturnal hemoglobinuria (PNH). Complement receptor 1 functions as a receptor for C3b and other complement components, but also functions as a FI-cofactor.

## 5.5 Microbial complement evasion

Infectious microorganisms have evolved many mechanisms of bypassing human immune defenses, especially the complement system. This is an essential feature of pathogenic microorganisms. From an evolutionary aspect the interplay between the immune system and pathogenic bacteria could be described as an interface, where a long-term balance is achieved between fast-evolving bacteria with their ever-developing mechanisms of immune evasion and the ability of the immune system to control them. The occasional imbalance results in the generation of pathogenic microbes, which can cause infectious disease. Well-documented mechanisms of complement evasion include surface structures, like capsules, release of fluid-phase regulators encoded by the bacterial genome, and modulation of bacterial surface protein expression and binding of human complement regulators.

### 5.5.1 LPS AND CAPSULE

Oligosaccharide side-chains of lipopolysaccharides (LPS) and capsules (14) are carbohydrate surface structures on microbial surfaces capable of isolating attacks of the complement system from the lipid bilayer of the bacteria. Especially if these structures contain sialic acid (15), complement attack can be efficiently prevented. *Neisseria meningitidis*, for example, protects itself with a capsule containing sialic acid (16).

### 5.5.2 MODULATION OF SURFACE PROTEIN EXPRESSION

Modulation of surface expressed antigenic determinants is a common mechanism in viruses evolving at a high rate, for example the Influenza-viruses and HIV. Also some bacteria, for example *B. burgdorferi,* have a surface protein repertoire, which is capable of mutating to lead the immune system astray. Changes in expression have especially been found in outer surface protein C (OspC) (17).

### 5.5.3 RELEASE OF FLUID-PHASE REGULATORS

In addition to expressing M-proteins, group A streptococci, more specifically the M1 serotype streptococci, have also been shown to secrete a putative complement control protein, streptococcal inhibitor of complement SIC (18, 19). Some bacteria (like *Pseudomonas* sp. and *Porphyromonas*) have been found to release enzymes that cleave complement components (20, 21).

### 5.5.4 BINDING OF HUMAN COMPLEMENT REGULATORS

Bacteria, as well as viruses and parasites, utilize the binding of human complement regulators for complement evasion. The Human Immunodeficiency virus (HIV), an enveloped retrovirus, captures surface-associated complement control molecules, such as CD59 from the membranes of host cells to prevent complement attack (22-33). Some bacteria (e.g. group A and B streptococci and pneumococci) bind the AP-inhibitor FH using surface proteins, such as, the M-protein (34-40), and the PspC-family protein Hic (41).

## 5.6 Lyme disease - a tick-transmitted zoonosis

Lyme disease (borreliosis) is a spirochetal infection transmitted by ticks of the *Ixodes ricinus*, *Ixodes pacificus*, *Ixodes persculatus,* and *Ixodes scapularis*

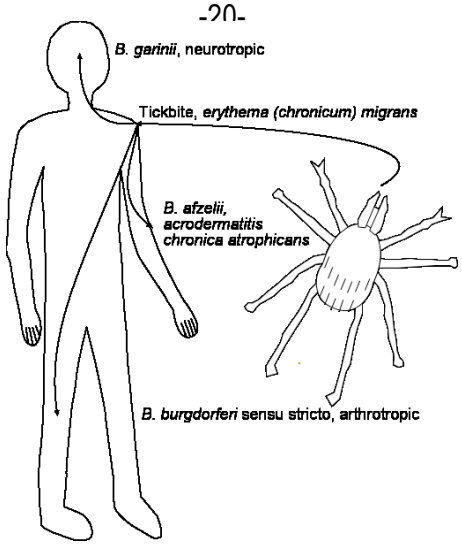


**Figure 4** Lyme disease causing *B. burgdorferi* s. l. spirochetes belong into three distinct genospecies, which tend to cause different clinical manifestations in later stage infection.

species (42-44). The human is essentially a dead end in the enzootic cycle of the causative agents, the *Borrelia burgdorferi* s. l. Lyme disease–causing spirochete bacteria. The natural transmission cycle involves warm-blooded mammals, birds, and rodents as the natural reservoir hosts of the spirochetes. The most important host as well as the most important vector-tick depends on the geographical region. The tick maturation cycle includes the larval stage, nymphal stage, and adult stage of which the nymphal stage is most important in spirochetal transmission. There are three genospecies, which cause Lyme disease: *B. burgdorferi* s. s., *B. afzelii* and *B. garinii*. These genospecies vary somewhat with regard to the most prevalent clinical manifestations (see Fig. 4) they cause.

*B. burgdorferi* is transmitted from the tick mid-gut into the skin of the potential human host when the tick feeds on human blood. Attachment lasting over an average of 53 h (45) increases the likelihood of disease transmission. *Borrelia* bacteria begin to change their surface antigen expression and adapt into the host environment, when key host-associated factors come into contact with the spirochetes (46-50). In the tick midgut, the spirochetes are protected from

host complement by the tick's saliva, which contains complement inhibitory substances (51).

The first symptom of disease and the best hallmark for diagnosis is an enlarging bull's-eye type of annular erythema, *erythema migrans* (EM), which has been reported to occur in 61-100 % of patients confirmed to have the infection (52). However, the prevalence anti-*B. burgdorferi* antibodies in endemic areas as well as selected reports suggest that EM may be more rare or go unnoticed more often than usually thought (53, 54). After 2-3 weeks the disease begins to disseminate into other organs through the bloodstream. Several outer surface proteins induce the formation of antibodies, which are characteristic for each phase of infection (55). A high level of antibodies is typically observed in the infection. However, the level of inflammation in tissues is relatively low. Also, despite the antibodies, the bacteria are often not cleared.

The second stage of infection may involve various tissues and manifests as symptoms from the eye (ophtalmitis), nervous system (Bell's palsy), heart (carditis), joints (arthritis) (56), or skin *erythema*.

In the third stage, the disease seems, at least in most cases, to acquire an immunological nature. The long-lasting high antibody response against spirochetal antigens has been suggested to result in the formation of autoantibodies against cross-reacting human structures, such as LFA-1 (57, 58). This has been suspected to play a role in the formation of long-term Lyme arthritis or damage in nervous system structures, resulting in peripheral neuropathy (59-63). The most likely long-term outcome depends somewhat on the genospecies causing the infection, with *acrodermatitis chronicum atrophicans* being the most prevalent late stage outcome of *B. afzelii* infection. Lyme arthritis and neuroborreliosis are similarly linked to *Borrelia burgdorferi* s. s. and *B. garinii*, respectively.

## 5.7 Special properties of *B. burgdorferi*

*B. burgdorferi* is a 20 µm long cork-screw-like gram-negative spirochete bacterium, which is very motile in phase contrast microscopy. The bacterium has a linear chromosome with 853 open reading frames (ORF) (64). In the *B. burgdorferi* s. s. B31 strain there are also 9 circular and 12 linear plasmids (The Institute for Genomic Research Comprehensive Microbial Resource; (65)). The outer surface of the bacterium is relatively smooth, with 43 proteins being expressed on the surface. Only a subset of these proteins that give the

spirochete a relative advantage in a particular environment, is expressed at any one time at the cost of energy expenditure (66, 67). The membrane consists of two membranes with a periplasmic space in between. Some surface proteins are expressed on both membranes in a dual manner (66).

In Lyme disease pathogenesis the ability of the spirochete to invade tissues is essential. This ability is achieved by utilization of human matrix metalloproteases (MMP's) (68, 69), and plasmin/plasminogen (70-74). By binding these enzymes onto its outer membrane, *B. burgdorferi* can penetrate through connective tissue similarly to leukocytes when they penetrate through blood vasculature. In the pathogenesis of arthritis, the use of MMPs is central to the observed damage in the synovium (75).

## 5.8 Lyme disease prevention by vaccination

The prevention of Lyme disease using vaccination has been the goal of several studies using surface proteins of *B. burgdorferi*. The first widely used vaccine was developed using the OspA surface antigen. However, reports on the cross-reactivity of the antigen with human leukocyte function antigen (LFA-1) have suggested that there may be limitations to its usefulness due to side-effects (58).

## 5.9 Immune evasion and suppression mechanisms of *B. burgdorferi*

*B. burgdorferi* is among the most successful non-commensal pathogens in terms of immune evasion and suppression. The spirochete can persist for years in the human body without a marked inflammatory reaction. The mechanisms of persistence and survival of the spirochete in the human body have sparked research interest since the discovery of the bacterium. Several mechanisms have been reported, including surface antigen modulation, and upregulation of IL-10 expression. These mechanisms may be viewed as active and passive immune suppression/evasion mechanisms (76).

The clinical manifestations and outcome of Lyme disease have long been known to vary widely between different individuals. In most cases the infection is cleared by the immune system with relatively few symptoms and acquisition of immunological memory. Humoral immunity can usually be detected afterwards. In various studies the prevalence of antibodies in the general population in endemic areas has varied from 3.4% (77) to 19.7% (78). On the other hand, the infection may also disseminate and cause symptoms in joints,

the heart, nervous system and skin. Several suggestions have been made as to what determines the clinical outcome. It is generally viewed that humoral (Th2) immunity is more important than cell-mediated immunity (Th1) in clearing the infection. There is evidence to suggest that, of the adaptive immune mechanisms, activation of B-cells is essential for both protective humoral immunity and disease clearance (79), and Th1-responses and activation of CD4+ cells may even exacerbate infection (80). Thus, T-cells are not central in Lyme disease immunity, although they may be important mediators of long-term outcomes. Apparently T-cell independent B-cell activation takes place in Lyme disease (80).

When causing an infection in a previously uninfected individual, the first line of defense, which the bacterium faces, is the alternative pathway and classical pathway of the complement system. The pathogenic genospecies of *B. burgdorferi* (s. s., *afzelii*, *garinii*) have been observed to differ with respect to their sensitivity to nonimmune serum i.e. to the complement system. In this study by Breitner-Ruddock *et al.* it was observed that sensitive strains of *B. burgdorferi* activate primarily the alternative pathway of the complement system, whereas the resistant strains activate the classical pathway in the absence of antibodies (81). Several mechanisms have been described to contribute to the complement evasion property of *B. burgdorferi*.

### 5.9.1 SURFACE ANTIGEN MODULATION

Modulation of surface antigens (e.g. OspC) has been reported by Liang *et al.* (17) as a mechanism for immune evasion by the spirochete. This mechanism reduces antibody binding to the borrelial surface. Also, specific complement acceptor surface proteins have been suggested to play a role in AP evasion (82). Other surface antigens known to undergo genetic variation are OspE, DbpA, EppA, and mlp.

### 5.9.2 PUTATIVE BINDING OF CD59

Pausa *et al.* (83) have reported expression of a CD59-like molecule by serum resistant strains. Because CD59 is a terminal pathway inhibitor, the microbial homolog has been suggested to act by reducing terminal complement complex deposition by complement-activating antibody-molecules. However, no independent confirmation of this observation has yet been made.

### 5.9.3 INDUCTION OF IL-10 SECRETION

Secretion of IL-10, an immunomodulatory cytokine, downregulates the cell-mediated immune response in Lyme disease. The *B. burgdorferi* bacteria are able to induce secretion of IL-10, which results in modulation of the inflammatory response in early infection (76).

### 5.9.4 OTHER MECHANISMS

The above do not offer a full account of the mechanisms *of B. burgdorferi* for AP evasion. Based on the characteristic features of the infection it was hypothesized that additional AP-specific evasion/suppression mechanisms must exist for *B. burgdorferi* to survive in the human host for extended periods of time. Therefore, we presented the necessary questions for further studies.

# 6 Aims

The aims of the present study were to investigate

a) whether the AP inhibitor FH, and/or its homologues is utilized by *B. burgdorferi* for bypassing the complement system,

b) whether there are specific bacterial surface structures associated with this mechanism,

c) what the essential structural features, motifs, or domains of these surface molecules are in the interaction,

d) what homologues of these proteins exist, if any, and

e) why *B. garinii* strains are more sensitive to serum, i.e. complement-mediated killing than *B. afzelii* and *B. burgdorferi* s. s. strains.

# 7 Materials and Methods

## 7.1 The *B. burgdorferi* genome

The *B. burgdorferi* B31 genome has been entirely sequenced (64) and is available online at http://www.ncbi.nih.gov/Entrez/index.html. This information was used frequently for searching candidate proteins, looking for common motifs, and calculating pI-values for proteins to be used in the Biacore analysis. Also, sequence alignments and sequence fragmentations were performed using sequences from NCBI Protein Databases. The plasmid content of the spirochete was analyzed using the information contained in Entrez. *B. burgdorferi* B31 contains 9 circular and 12 linear plasmids. By far, the plasmid-encoded content (600 kb; 670 ORFs) contains more elements (92% vs. 30 %) unique to *B. burgdorferi* than the linear chromosome (1 Mb; 853 ORFs), but has less coding sequences (68% vs. 93%) (84).

## 7.2 Expression and purification of complement components (I-V)

Human complement components were (a) purified from human plasma (85, 86), which was obtained from healthy laboratory workers, or (b) obtained from commercially available sources (Calbiochem, La Jolla, CA, USA). FH and FI were obtained both by purifying (a) and from Calbiochem (b). The latter FH stocks were used for the majority of the experiments, including all Biacore measurements. Human C5 was purchased from Calbiochem (La Jolla, CA, USA). Recombinant proteins FHL-1 (87) (see Fig. 1), SCRs 8-20 and SCRs 15-20 of FH, FH-related proteins FHR-3 and FHR-4 and a construct SCR 1-4 of FHR-3 were expressed with the baculovirus expression system as described earlier (88-91) by Dr. Peter Zipfel's group and Dr. Jens Hellwage and/or Dr. Sakari Jokiranta, and were a generous gift to us. The recombinant proteins had been purified by $Ni^{2+}$-chelate chromatography as described (92). FH and C3 were purified from human plasma (93) and C3b was generated from C3 with factors B and D in the presence of $Ni^{2+}$ ions as described previously (85, 94). C4b was purchased from Quidel Corp. (La Jolla, CA, USA) and Dr. Anna Blom (University of Lund, Sweden) generously donated C4bp. C3b, C4b, FH and FHL-1 were radiolabeled with $^{125}I$ using the Iodogen method (95) (Pierce Chemical Group, Rockford, IL, USA).

The carboxy-terminal part of mouse FH containing SCR domains 18-20 was generated by PCR (see Table 1) from the clone containing full-length cDNA of

mouse FH DNA to amplify the region encoding SCR18-20 of murine FH. Human FH fragments SCR18-20 and SCR19-20 were similarly amplified (Cheng *et al.*, manuscript in preparation) from a human liver cDNA library (Stratagene, La Jolla, CA, USA).

| Target | Primers |
|---|---|
| Human FH SCR18-20 | 5´-GGA TTG GAC CCT GCA GAA GCA AAG ATA A-3´ |
| | 3'-GGT TGA ACA CGT TTT TCT ATC GGC GCC-5' |
| Human FH SCR19-20 | 5´-GGA TTG GAC CCT GCA GAA GCA AAG ATA A-3´ |
| | 3'-GGG TGA ACA CAT GTT TTA GTA CAG CTG-5' |
| Mouse FH SCR18-20 | 5'-GA ATT CAA AAA GAT TCT ACA GGA AAA TGT G-3' |
| | 3'-GGT TGA ACA CGT TTT TCT ATC GGC GCC-5' |

**Table 1** FH primers for generation of SCR18-20 fragments.

The purified PCR products were expressed in *Pichia pastoris* strain X33, which was transformed by electroporation (Bio-Rad Gene Pulser II; BioRad, Hercules, California, USA) using expression vectors pPICZαA and pPICZαB (Invitrogen, Carlsbad, California) (mouse and human FH fragments and a polyhistidine (His-6) tag, respectively). The transformed cells were grown on YPDS (Yeast Extract Peptone Dextrose Medium) plates containing Zeocin (100 µg/ml; Cayla, Toulouse, France) at 30°C for 3 days, and the clones were selected according to the manufacturer's protocol. The recombinant proteins were expressed in BMMY (Buffered Methanol-complex Medium) medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB (Yeast Nitrogen Base), 4 x $10^{-5\%}$ biotin, 1% methanol) with a daily addition of methanol (1% v/v) before harvesting.

The expressed recombinant proteins were purified using $Ni^{2+}$ NTA agarose (Invitrogen, Carlsbad, CA) according to the manufacturer´s protocol. Heparin affinity chromatography (Pharmacia, Uppsala, Sweden) with a Hitrap heparin column and elution with a 50-200 mM salt gradient was used for further purification. The purity of the recombinant proteins was found to be > 95% (moH18-20 and huH18-20 fragments were expressed as glycosylated forms) by sodium dodecyl phosphate polyacrylamide gel electrophoresis (SDS-PAGE). The antigenicity of the fragments was verified by immunoblotting analysis using goat anti-human FH antiserum (Calbiochem, La Jolla, CA). The protein concentrations were determined by BCA Protein assay (Pierce Chemical Corp., Rockford, IL).

## 7.3 Classification and culture of bacterial strains (I, II, V)

The genospecies-classification of the *Borrelia* strains was determined by sequencing PCR-amplified fragments of the flagellin gene of *B. burgdorferi* (92). PCR primers FL7 (biotinylated) and FL59 were used to obtain a 277 bp segment of the flagellin gene (96). The biotinylated PCR products were rendered single stranded using streptavidin-coated Dynabeads according to the instructions of the manufacturer (Dynabeads M-280 streptavidin; Dynal AS, Oslo, Norway). We performed manual sequencing by using Sanger's dideoxynucleotide chain termination method and Sequenase 2.0 (United States Biochemical Corp., Cleveland, Ohio, USA) as described previously (97). The obtained sequences were compared to the flagellin gene sequences

| Strain | Origin | Papers |
|---|---|---|
| *B. burgdorferi* s. s. | | |
| IAI | CSF, LD-patient (98). | II |
| B31 | USA, Patient | III |
| 297 | USA, Patient | III, IV |
| N40 | USA | III, IV |
| *B. afzelii* | | |
| A91 | Skin, LD-patient, 1996 | II |
| 570 | Tick, Helsinki 1996 | II |
| 1082 | LD-patient | II |
| *B. garinii* | | |
| 3/96 | EM-lesion, Turku 1996 | II |
| 5/96 | EM-lesion, Turku 1996 | II |
| 13/96 | EM-lesion, Turku 1996 | II |
| 28/97 | EM-lesion, Turku 1997 | II |
| 40/97 | EM-lesion, Turku 1997 | II |
| 46/97 | EM-lesion, Turku 1997 | II |
| 50/97 | EM-lesion, Turku 1997 | II, IV |
| BITS | University of Trieste, Italy, Gift from Marina Cinco | V |
| PIST | University of Trieste, Italy, Gift from Marina Cinco | V |
| LU59 st 5 | CNS-infection, Umeå University | V |
| LU185 st 6 | CNS-infection, Umeå University | V |
| LU190 st 6 | CNS-infection, Umeå University | V |
| LU222 st 6 | CNS-infection, Umeå University | V |
| FAR03 | Puffin blood, reisolated from C3H/HeN mice, Umeå University | V |
| FAR04 | Puffin blood, reisolated from C3H/HeN mice, Umeå University | V |

**Table 2** *B. burgdorferi* strains used. Most strains were obtained from The National Public Health Institute (NPHI), Department in Turku.

of the type strains of *B. afzelii* Bo23 and *B. garinii* 387. *B. afzelii* A91/96 and *B. garinii* 40/97 are low-passage (< 5 *in vitro* passages) strains and *B. burgdorferi* s. s. IA is a high-passage strain.

The strains used and their sources are shown in Table 2. Four *B. garinii* strains (LU59 serotype (st) 5, LU185 st 6, LU190 st 6, LU222 st 6) isolated from patients with CNS-infection and two additional strains originally isolated from puffin blood (FAR03, FAR04) and reisolated from C3H/HeN mice were obtained from Pär Comstedt and Sven Bergström, Umeå University, Sweden. All isolates were grown in BSK-H medium containing 6% heat-inactivated rabbit serum (Sigma Chemicals, St. Louis, MO, USA) at 33°C in 1-5% $CO_2$ atmosphere for two weeks or until the cultures reached late exponential phase. The concentration of bacteria was estimated by counting at least three microscope fields at 40-fold magnification. Cultures were harvested by centrifugation, washed and diluted to a final concentration of 2 x $10^7$ - 1 x $10^9$ ml in veronal buffered saline (VBS), pH 7.4 or 1/3 GVB (1/3 x VBS with 0.1% gelatin), and subsequently used in experiments.

*B. burgdorferi* strain 297 and *B. garinii* strain 50 were cultivated in BSK II medium supplemented with 6% rabbit serum as previously described (114). Cloning and protein purification experiments were performed using *E. coli* DH5$\alpha$. Plasmid pGEX-4T-3 (Pharmacia Biotech Inc, Piscataway, N.J.) was used for the generation of glutathione *S*-transferase (GST)-fusion proteins.

## 7.4 Serum sensitivity test (I, II, V)

*Borrelia* bacteria were cultured until a density of at least $10^7$/ml was reached. We extracted serum from a healthy laboratory worker without *B. burgdorferi* antibodies. A vial of blood covered with Parafilm was let to stand still for 30 min and centrifuged. The separated serum was then aliquoted, and frozen (-70°C) in plastic tubes. Bacteria were centrifuged (6000 *g*) and washed three times with BSK-II buffer. For the serum sensitivity test, the reaction mixtures were set up to a volume of 100 $\mu$l consisting of bacteria (50 $\mu$l/reaction) and diluted serum. Serum was diluted with the BSK-II medium to set up five different reaction mixtures for each strain: 10% normal human serum (NHS), 20% NHS, 40% NHS, and heat inactivated serum. The mixtures were incubated at 37°C with shaking for a total of 16 hours and 10 $\mu$l aliquots were pipetted onto microscope slides for analysis at 2, 5 and 16 hours using an Olympus microscope with dark field. The number of bacteria and the percentages of live (mobile) bacteria were estimated by counting several fields. Survival was categorized into one of the following groups: 100%, 75%,

25%, 5% or 0% live bacteria at each time point. The strains were classified as either sensitive or resistant based on the endpoint category at 16 hours. The serum sensitivities of a *B. garinii* 50 and a *B. garinii* 50 strain (T50) transfected with a plasmid containing the *ospE*-gene from strain 297 and confirmed to be expressing OspE-297 were compared as previously described (I, V). The *ospE*-297-containing plasmid was maintained using kanamycin-selection in culture until commencement of the experiment (V).

## 7.5 FI cofactor assay (I)

*Borrelia* bacteria (approximately 4 x $10^7$) were incubated in the presence of FI (500 ng) and radiolabeled C3b or C4b (50,000 cpm) at 37°C for 1.5 h in 40 µl of VBS containing 0.1% gelatin (GVB) to prevent adhesion of the reactants to surfaces of the reaction tube. After incubation, the samples were electrophoresed in 8% SDS-PAGE gels under reducing conditions. The gel was fixed in 5% acetic acid for 30 min, dried and autoradiographed using the Fujifilm BAS 2500 instrument (Fuji Photo Film, Tokyo, Japan).

The assay included positive and negative controls. As negative controls the C3b and C4b degradation assays were carried out in the absence of *Borrelia* bacteria, or in the presence of selected strains of *Escherichia coli*, *Staphylococcus aureus* and *Enterococcus faecalis* instead of *B. burgdorferi*. The control bacteria were isolated at the Helsinki University Central Hospital diagnostics laboratory and selected randomly for the assay. The positive control mixture consisted of FH (5 µg/ml or 0.5 µg/ml), FI and $^{125}$I-C3b or C4bp (10 µg/ml), FI and $^{125}$I-C4b. In addition, a sample of $^{125}$I-C3b or $^{125}$I-C4b without FI and FH/C4bp were used to monitor for any spontaneous degradation under the experimental conditions.

## 7.6 Binding of FH and FHL-1 to whole bacteria

The *Borrelia* spirochetes were cultured as described above. *B. burgdorferi* s. s., *B. afzelii*, *B. garinii* or *S. aureus* (used as a negative control) were used at a concentration of 2 x $10^7$ cells /ml in 1/3 x VBS, pH 7.4 containing 0.1% gelatin (GVB). In the binding assay, the indicated amounts of bacteria were incubated for 20 min at 37°C with the $^{125}$I-labeled FH or FHL-1 (60,000 cpm) in a total volume of 100 µl of 1/3 x GVB (90). Particle-associated FH/FHL-1 was separated from unbound ligand by centrifuging the mixtures through 20% sucrose in 1/3 x GVB. The tube was then frozen at –80°C to avoid mixing the supernatant with the pellet. After cutting off the pellets, the binding of the FH/FHL-1 proteins was calculated as a percentage of the total radioactivity

input. All experiments were performed a least in duplicate. For the heparin inhibition assay, varying amounts of heparin (Sigma Chemicals, St. Louis, MO, USA) were added to the reaction mixture simultaneously with FH/FHL-1.

## 7.7 SDS-PAGE and Western blot analysis (I, V)

In SDS-PAGE analysis proteins were separated through 4% stacking and 10% separating gels. The proteins were transferred to nitrocellulose membranes for Western blot analysis. The membranes were blocked with 5% (w/v) dried milk in phosphate buffered saline (PBS) for 30 min and incubated at room temperature for 1 h with polyclonal goat anti-human FH antibody (Calbiochem, La Jolla, CA; diluted 1:5000). This antibody also recognizes human FH-like protein 1 (FHL-1) and the family of FH-related proteins (FHR-1 to FHR-5). A donkey anti-goat IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania; diluted 1:5000) was used as the secondary antibody.

$5 \times 10^7$ organisms of *B. burgdorferi* strain 297, *B. garinii* strain 50 and 200 ng of recombinant OspE protein were boiled for 10 min in final sample buffer (62.5 μM Tris-HCl [pH 6.8], 10% [vol/vol] glycerol, 5% [vol/vol] -mercaptoethanol, 5% SDS, 0.001% bromophenol blue) and subjected to SDS-PAGE through a 12.5% separating gel before being electroblotted onto nitrocellulose membrane (Schleicher and Schuell, Keene, N.H.). To determine the reactivity of *B. burgdorferi* strain 297 and *B. garinii* with OspE antibody, the membrane was first blocked in blocking buffer (5% non fat dry milk in PBS, 0.2% Tween-20) overnight at 4°C before being washed and incubated with a 1:1000 dilution of rat anti-OspE serum for 1 hr. This was followed by three washes with blocking buffer before a 1:5000 dilution of HRP-conjugated goat anti-rat antibody (Zymed, San Francisco, CA) was added for 45 min. The nitrocellulose was then washed three times prior to detection by enhanced chemiluminescence according to the manufacturer's recommendations (Amersham Pharmacia). To ensure equivalent loading of whole cell lysates, the same membrane was subsequently blotted with rabbit anti-FlaB antibodies as described above using goat anti-rabbit HRP as the conjugate.

## 7.8 Immunoblotting and ligand blotting analysis of FH binding (I, V)

FH binding by *Borrelia* strains directly from the growth medium or from human plasma was also analyzed by an immunoblotting assay. *B. burgdorferi* strains

that had been grown in the BSK-H medium or incubated in human EDTA plasma and washed for 3 times with VBS were mixed with a reducing SDS-PAGE sample buffer. The samples containing $10^7$/ml bacteria were incubated at 37°C for 20 min on a shaker, and aliquots were run in an 8% SDS-PAGE gel. The samples were electrotransferred onto a nitrocellulose membrane and the membrane was blocked with 5% fat free milk in PBS. Polyclonal goat anti-FH antibody (1:5000 dilution) was added and the membrane incubated for 12 hours at +4°C. The membrane was washed 5 times, a secondary peroxidase-conjugated donkey-anti-goat antibody (1:5000 in 1% BSA/PBS) was added and incubated with the membrane for 60 min at RT on a shaker. The membrane was washed with PBS and positive reactions were visualized by the electrochemiluminescence (ECL) method (Amersham, Amersham, UK). To analyze binding of radiolabeled FH or FHL-1 to borrelial proteins separated in an SDS-PAGE gel a radioligand blotting analysis was employed. For the radioligand blotting analysis of FH and FHL-1 binding, outer membranes (OM) and protoplasmic cylinders (PC) of one strain of each genospecies of *Borrelia* bacteria were isolated using a method described previously (I). The OM and PC specimens were run in an 8% SDS-PAGE gel under non-reducing conditions and transferred onto a nitrocellulose membrane. The membranes were blocked with 5% fat free milk in PBS for 60 min at RT. $^{125}$I-labeled FH and FHL-1 ($1x10^6$ cpm/membrane) were incubated on the membranes for 24 hours at +4°C on a shaker. The membranes were washed five times with PBS and analyzed by autoradiography.

## 7.9 Immunofluorescence microscopy analysis of complement deposition (II, III)

*B. burgdorferi* s. s. (strain IA), *B. afzelii* (strain 1082) and *B. garinii* (strain 50) were cultured as described above. Freshly harvested (s. s. IA, $6.4 \times 10^7$) or frozen bacteria (*garinii* $5.4 \times 10^9$ and *afzelii* $6.4 \times 10^9$) were washed with GVB and incubated 1:2 with non-immune NHS (no IgG or IgM -antibodies against *B. burgdorferi* were detected by ELISA (99) or EDTA-plasma from the same donor for 60 min at +37°C. Bacteria were washed three times with GVB and incubated with a monoclonal antibody against iC3b (Quidel Corp., La Jolla, CA, USA). For detection of FH and FHL-1, we used the 196x mAb recognizing the N-terminus (SCR 1) of FH and mAb VIG8 (kindly provided by Dr. Wofgang Prodinger, University of Innsbruck) (89) for specific detection of the C-terminus (SCRs 19-20) of FH. An unrelated B-cell lymphoma idiotype-specific AF1-antibody served as a control. Samples were incubated for 30 min at +37°C with primary antibodies (10 µg/ml) and washed three times with GVB before adding a polyclonal FITC-conjugated goat anti-mouse IgG

(Alexa 488, Molecular Probes, Oregon, USA) used in 1:200 dilution. The stained samples were mounted with Mowiol (100) and examined on an Olympus BX50 standard microscope equipped with a filter specific for FITC fluorescence. The samples were photographed with a Spot RT Slider digital camera and processed with Spot RT Software 3.0 (Diagnostic Instruments, Sterling Heights, MI, USA).

## 7.10 Analysis of borrelial surface protein expression by immunofluorescence microscopy

To analyze expression of surface proteins of *B. burgdorferi* within ticks, mid-guts were dissected out of *B. burgdorferi* strain 297 infected ticks (nymphal stage) prior to feeding (*n* = 12) or after feeding to repletion (*n* = 10). Extracted mid-guts were diluted in 20 μl PBS (pH 7.4) before being disrupted by repeated pipetting. Ten μl of each sample (mid-guts from unfed or fed ticks) were spotted onto fluoro-slides (Erie Scientific) and indirect immunofluorescence assays were performed as described previously (101). For these experiments, rat polyclonal antiserum directed against OspA, OspC, OspE, ElpB1, or OspF was used as the primary antibody at 1:25 dilutions. Alexa 488 labeled goat anti-rat IgG (heavy and light chain specific) was diluted in PBS-0.2% BSA at 1:1000 and used as the secondary antibodies. Slides were washed and fixed in acetone before fluorescent imaging was performed. For each protein analyzed, multiple fields were viewed before images of representative fields were captured using a Spot digital camera and associated software (Diagnostic Instruments, Sterling Heights, Michigan).

## 7.11 Expression and purification of recombinant borrelial surface proteins

*B. burgdorferi* s. s. strain N40 OspE protein (Table 3; (102)) DNA corresponding to the mature portion of the OspE gene was cloned into a pGEX-2T vector (Pharmacia, New Jersey, USA) in Michael Norgard's lab, Dallas, USA. We expressed the GST-OspE fusion protein in *E. coli* DH5-alpha host cells. Fresh transformants selected from a single colony were cultured in 50 ml of Luria-Bertani (LB) broth containing 100 mg/ml ampicillin at 37°C with shaking overnight. The culture was diluted 1:100 to 1500 ml of LB broth containing 100 mg/ml ampicillin, incubated at 37°C for 3 hours to reach the mid-log phase with an optical density of approximately 0.6 at 600 nm. The culture was incubated for an additional three hours in the presence of Isopropyl-β-D-thiogalactoside (IPTG) 0.6 mM. The GST-OspE fusion protein

was then purified from harvested, washed and sonicated cells by affinity chromatography on an agarose-glutathione matrix according to the instructions of the manufacturer (Amersham Pharmacia Biotech, Sweden). Bovine thrombin was used to cleave OspE from GST (Sigma, USA) in thrombin cleavage buffer (150 mM NaCl, 100 mM KCl, 2.5 mM $CaCl_2$, 1 mM DTT, 20 mM Hepes pH 7.6) for 3 h. and. The cleaved OspE was then eluted with TCB and dialyzed in an 11 mm Spectrapore membrane tube (Spectrum Medical Industries, Rancho Dominguez, CA, USA) against 10 mM diaminopropane pH 8.6 for 20 h. The dialysed OspE was applied on a MonoQ (Pharmacia, Uppsala, Sweden) column and eluted using NaCl. The purity of the OspE protein was thereafter confirmed by SDS-PAGE.

*B. burgdorferi* s. s. 297 OspA, and *B. burgdorferi* s. s. N40 OspD (Table 3) were cloned originally in Michael Norgard's lab, Dallas Texas. Outer surface lipoproteins (Table 3) from *Borrelia burgdorferi* s. s. 297 and B31 strains were expressed in *E. coli* as GST or polyhistidine-tagged fusion proteins and purified as previously described (101, 103, 104). Polyhistidine-tagged ErpA, ErpP, ErpL and ErpX proteins (Table 3) were generated by cloning PCR-amplified products (Table 4) representing the mature portion of each protein using the respective primers. The GST moiety was removed from all fusion proteins with thrombin prior to use in the binding assays. All resulting constructs were sequenced to confirm that the inserted DNA was in frame and that no errors were incorporated during PCR amplification. Fusion constructs were grown in tryptone-yeast broth supplemented with 100 µg of ampicillin to an $OD_{600}$ of 0.7 before arabinose was added to a final concentration of 0.2% to induce expression of the fusion proteins. Remaining proteins were hyperexpressed like OspE. Polyhistidine-tagged proteins were affinity-purified using nickel chromatography as instructed by the manufacturers (Amersham Pharmacia, Piscataway, NJ, USA and Invitrogen, Carlsbad, CA, USA). The hexa-His fusion proteins were purified by affinity chromatography using a chelating Sepharose Fast Flow matrix containing $Ni^{2+}$-ions and eluted from the column by increasing the amount of imidazole. SDS-PAGE was used to confirm the purity of the proteins. Other proteins were purified similarly to OspE.

## 7.12 Generation and purification of P21 deletion mutants and synthesis of C-terminal peptides (IV, V)

P21 is an OspE-paralog from the *B. burgdorferi* s. s. 297 strain. The truncated versions of P21 lacking the N-terminus (P21Δ1-35) or C-terminal regions of varying lengths (P21Δ105-166, P21Δ143-166 and P21Δ152-166) were made

| Strain | Protein | Vector (Manufacturer) | Construct | Host cell |
|---|---|---|---|---|
| *B. burgdorferi* s. s. 297 | OspA | | GST | *E. coli* XL1Blue |
| | OspE | | | |
| | P21 | pGEX-4T-2 (1) | | |
| | P21-mutants | | | |
| | OspF | | | |
| | Bbk2.10 | | | |
| | Bbk2.11 | | | |
| *B. burgdorferi* s. s. N40 | OspE | pGEX-2T (2) | | *E. coli* DH5-alpha |
| | OspD | | | |
| *B. burgdorferi* s. s. B31 | ElpA1 | | | |
| | ElpA2 | | | |
| | ElpB1 | | | |
| | ElpB2 | | | |
| | ErpA | pBAD-TOPO (3) | Poly-Histidine | |
| | ErpP | | | |
| | ErpL | | | |
| | ErpX | | | |
| *B. burgdorferi* s. s. IA | DbpA-1 | pQE-30 (4) | Hexa-His | *E. coli* M15 host cells |
| | OspC | | | |
| | P35/BBK32 | | | |
| *B. garinii* BITS | OspE | pCR-TOPO (5) | | |
| *B. garinii* 40/97 | OspE | | | |

**Table 3** *B. burgdorferi* surface proteins expressed in recombinant form. Vector sources: (1), (2) Pharmacia, NJ, (3), (5) Invitrogen Life Technologies, Carlsbad, CA, (4) Qiagen, Valencia, CA.

| Target | Primers |
|---|---|
| erpA | 5'-TGCAAAGCAATGGAGAGGTAAAGGTC-3' |
| | 3'-CTTCTCGAATTTTCTTTAAATTTT-5' |
| erpP | 5' TGCAAAATTCATACTTCATATGATGAG-3' |
| | 3'-GGTGATCTTCACGAATTTTTTTTAAATTTT-5' |
| erpL | 5'-TGCAAGAATTATGCAAGTGGTG-3' |
| | 3'-CCCTTATCTTCTTCTATTTTTTCTT-5' |
| erpX | 5' TGCAAGATTGATGCAACTGGTAAAGATGC-3' |
| | 3'-CTTTAATTTCCTATGTAGTCAATGTCAGTC-5' |

**Table 4** Primers used in expression of Erp-proteins in recombinant form.

| Target | Primers |
|---|---|
| P21Δ1-35 | 5'-GCG GAT CCA AAT TTA CTG TAA AAA TTA AAA-3' |
| | 3'-CTA GAA TTT TTT TTA AAT TTT CAG CTG CCG-5' |
| P21Δ1-45 | 5'-GCGGGATCCGAGTTAAAACTTAAAAAAATAGAATT-3' |
| | 3'-TTCTAGAATTTTTTTTAAATTTTATCATCGAGCTCGCG-5' |
| P21Δ105-166 | 5'-GCG GAT CCT GCA AAA TTC ATA CTT CAT ATG-3' |
| | 3'-CCA AGC AAA TTT TGA CTA CTC CAG CTG CCG-5' |
| P21Δ143-166 | 5'-GCG GAT CCT GCA AAA TTC ATA CTT CAT ATG-3' |
| | 3'-ATA TAA TGT AAA AGT CCT TTA CAG CTG CCG-5' |
| P21Δ152-166 | 5'-GCG GAT CCT GCA AAA TTC ATA CTT CAT ATG-3' |
| | 3'-C CTA AGG CCC CTA TTT CAA CAG CTG CCG-5' |

**Table 5** Primers used for construction of P21-mutants. Sites added for cloning are underlined.

## 7.13 Surface plasmon resonance assays (III, IV, V)

Protein-protein interactions were analyzed in real time using surface plasmon resonance (SPR) with a Biacore 2000 instrument as previously described (90, 91). FH or recombinant surface proteins (P35/BBK32, OspC, OspE) were coupled via a standard amine-coupling procedure to flowcells of a sensor chip (carboxylated dextran chip CM5; Biacore AB, Uppsala, Sweden). Flowcells were activated with 35 µl of a mixture of 0.2 M N-ethyl-N'-(dimethyl-aminopropyl)-carbodiimide and 0.05 M N-hydroxysuccinimide (NHS-EDC; Biacore AB). The protein to be immobilized was dialyzed against 10 mM acetate buffer (pH 4.8 – 5.5) and 20 µg (> 150 µg/ml) were injected into one of the flowcells until an appropriate level of coupling for the binding experiments (> 4.000 resonance units (RU)) was reached. A control flowcell with no protein was similarly activated. Unreacted groups in both flowcells were inactivated by a standard ethanolamine-HCl (Biacore AB) injection (35 µl). After the coupling procedure, the flowcells were washed thoroughly with a sequential injection of 1/3 x VBS (50 mM VBS pH 7.4) and 3 M NaCl in 10 mM acetate buffer, pH 4.6.

First, the binding of a panel of *Borrelia* surface proteins (OspA, OspC, OspD, OspE, P35/BBK32, DbpA) to FH was tested. The proteins were dialyzed against 1/3 VBS and protein concentrations were measured using the BCA Protein Assay (Pierce Chemical Company, Rockford, IL). Each ligand was injected separately to a blank control flowcell and the flowcell with the analyte using a flow rate of 5 µl/min at +22°C. The final concentrations of the fluid-phase ligands in the binding assay ranged from 125 to 200 µg/ml. In a second set of experiments, a reverse setting was used. The *Borrelia* surface proteins were immobilized to the sensor chip surface as described above. The binding of FH, recombinant deletion constructs of FH or FH-related proteins was tested using by injection of the proteins to a flowcell with *Borrelia* surface protein and to a blank channel. Binding was assayed at least in duplicate using independently prepared sensor chips.

| Strain | Protein | Sequence |
|---|---|---|
| *B. burgdorferi* s. s. 297 | OspE | Biotin-GABA-KVAEYAISLEELKKNLK |
| *B. burgdorferi* s. s. 297 | P21 | Biotin-GABA-KIKNSKNKVAEYAILLENLKKNLK |
| *B. burgdorferi* s. s. B31 | ErpP | Biotin-GABA-KINNGVGGNKTAEYAIPLEVLKKNLK |
| N/A | S-ErpP | Biotin-GABA-KINNGVGGNKTAEYAIPLEVLKKKFK |
| *B. garinii* BITS & *B. garinii* 40/97 | OspE | 5'-TGTGGAAATTTTAGAAGTAGTTTAAGTG-3' |
| | OspE | 3'-CTTGTATAATGTAAAAGTCCTCTATTTAA-5' |

**Table 6** *B. burgdorferi* C-terminal peptides from OspE-related proteins and primers used for amplification of *B. garinii* OspE for sequencing. N/A, not applicable, S-ErpP, scrambled ErpP sequence.

For coupling of OspA, OspE, P21, OspF, Bbk2.10, Bbk2.11, OspE, OspD, ElpA1, ElpA2, ElpB1, ElpB2, ErpA, ErpP, ErpL, ErpX, OspE-40/97, and OspE-BITS the buffer was chosen according to the pI of the analyte: maleate and sodium acetate buffers were used for proteins whose calculated pI values were over or below 6, respectively. If poor results were obtained, the buffer pH was lowered. The coupling levels of proteins varied between 500-3000 resonance units (RU). The binding of complement FH (Calbiochem, San Diego, California; purity > 95% by SDS-PAGE) to immobilized proteins was assayed at 22°C and 37°C or only at 25°C. Recombinant OspE- OspF- and Elp-family proteins were coupled to a CM5-chip and the binding of fluid phase FH was analyzed. FH at different concentrations in 50 mM VBS was used as the ligand. A set of adjusted pH-values and a series of different FH concentrations in 150 mM PBS were used to examine the pH-dependence of binding. The binding kinetics was fitted to the simultaneous ka/kd determination feature of the BiaEvaluation 3.1 software package and a natural logarithmic Langmuir binding (1:1) and mass transfer model. For OspE-40/97 and P21-297 comparison strain the calculations were made using the SigmaPlot 8.0 software package. The sequences of the proteins used in the

Biacore assays and peptide mapping were retrieved from the NCBI sequence database and aligned using the ClustalX 1.8 software package (106) (http://www-igmbc.u-strasbrg.fr/BioInfo/clustalx, ftp://ftp-igmbc.u-strasbrg.fr). A phylogenetic analysis was performed based on sequence comparisons of the proteins; ClustalX was used to generate an N-J-tree, which was plotted using the Phylip software package (107).

For P21-mutants, the following method was used. Purified mutant P21-297 proteins (5.6 µM) were amine-coupled to carboxyl groups of CM5 sensor chips according to the manufacturer's instructions. The buffer was chosen according to the pI of the analyte: maleate and sodium acetate buffers were used as above. The binding of complement FH (Calbiochem, San Diego, California; purity > 95% by SDS-PAGE) to immobilized proteins was assayed at 25°C in 50 mM VBS. Similar binding tests were performed with the biotin-GABA-linked C-terminal peptides of OspE proteins. The peptides were coupled to an SA-chip and tested for FH binding ability (see Table 7). The binding of recombinant human FH fragments huH18-20 (0.044 mM) and huH19-20 (0.10 mM) and the recombinant mouse FH fragment moH18-20 (0.061 mM) to P21-297 was analyzed similarly using VBS as a buffer. The bound proteins were removed with a regeneration buffer containing 3.0 M NaCl (pH 4.7). To see if binding of FH could be inhibited by heparin, FH (300 nM) binding to P21 was assayed in the presence of 0.3 or 3.0 µM heparin 17/19 kDa (Sigma Chemicals, St. Louis, MO). Similar inhibition analyses were performed with the deletion mutants of P21 and soluble C-terminal P21 peptides (see below). P21 1-45 and Bba68 were coupled in sodium acetate, pH 4.0-5.0 to a CM5-chip to achieve coupling levels of several thousand RU.

The proteins were amine-coupled and the binding of complement FH was assayed as above. *B. garinii* OspE proteins from the BITS and 40/97 strains were tested for FH binding activity and compared to P21-297. Also, FH subunits containing only SCRs 18-20 or 19-20 were used as ligands in the Biacore assays. Biacore testing was done similarly to OspE-proteins (see above). FH was applied to the chip in PBS, pH 7.48.

| Protein | P21 amino acids | CM5 coupling level (RU) | Paper |
|---|---|---|---|
| P21 | 1-166 | 2468; (Figs. 2, 4A) | IV |
| P21 | | 3403; (Figs. 4B, 5) | IV |
| P21Δ1-35 | 36-166 | 1990 | IV |
| P21Δ1-45 | 46-166 | 970 | V |
| P21Δ105-166 | 1-104 | 7439 | IV |
| P21Δ143-166 | 1-142 | 2293 | IV |
| P21Δ152-166 | 1-151 | 9284 | IV |

**Table 7** P21-mutants and their coupling levels in Biacore .

## 7.14 Peptide mapping and alanine scanning (III, IV)

The candidate protein sequences chosen for peptide mapping analysis from the NCBI protein sequence databank (http://www.ncbi.nlm.nih.gov) (108) are shown in Table 8. P21-DK1 is an OspF family member from *B. afzelii* DK1. OspE-IP90 is a C-terminally truncated sequence from the *B. garinii* IP90 strain that does not bind FH. All sequences were fragmented into 15 amino acid (aa) peptides with a 3 aa transition and a 12 aa overlap. The peptides were synthesized as spots onto polyethylene glycol -derivatized cellulose membranes (AIMS Scientific Products, Braunschweig, Germany; http://www.aims-scientific-products.de) using the peptide scanning instrument Auto-Spot Robot ASP222 (Abimed Analysen-Technik Gmbh, Langenfeld, Germany). Subsequently, a protein overlay assay with radiolabeled FH (II, (91)), I) was carried out. The membranes were washed and exposed on a phosphoimager plate. Amino acid sequences of the tested proteins were aligned using the Megalign and ClustalX programs.

| Strain | Protein | NCBI Reference |
|---|---|---|
| *B. burgdorferi* s. s. 297 | OspE (cp32-2) | AAC34953 (103) |
| | P21 (cp18-2) | AAC34957 (103) |
| *B. burgdorferi* s. s. B31 | ErpA (cp32-1) | AAF07400 (109) |
| | ErpP (cp32-9) | AAF07678 (109) |
| *B. burgdorferi* s. s. N40 | OspE | AAA22959 (110) |
| *B. afzelii* DK1 | P21 | CAA69689 |
| *B. garinii* IP90 | OspE | AAC62927 |

**Table 8** OspE-related and control proteins screened for FH binding using Biacore and peptide scanning (III).

The Megalign and ClustalX-programs were used to align the amino acid sequences of the tested proteins (Table 8). Sequences from five putative FH-binding regions (III) were chosen from ErpA and from the proteins giving the strongest FH-binding signal strength for each region. We incorporated single alanine mutations into all aa-positions in the sequences in separate peptides. The membranes were washed and exposed on an X-ray film (Fuji Photo Film Co Ltd, Tokyo, Japan), which was thereafter scanned and processed using Adobe Photoshop 6.0.

## 7.15 The GST-fusion protein pull-down technique (IV)

To identify potential *Borrelia* OspE -interacting proteins in human serum, a GST-fusion construct of *B. burgdorferi* s. s. strain N40 OspE (Table 3) was generated as previously described (I) and used as an analyte in a GST-OspE

pulldown technique (111). Both NHS and serum from a volunteer deficient in the FH-related protein 1 (FHR-1DS) were used. Serum samples were incubated with glutathione-sepharose 4B (Amersham Biosciences, Freiburg, Germany) beads (1 ml serum + 50 µl of 50% slurry) for 2 h at 4 ºC with gentle mixing and then centrifuged (20,800 *g*, 2 min) to collect the supernatants. Pre-cleared sera were used at dilutions 1:2 and 1:10. GST (10 µg) was added to the control tube of and GST-OspE (~16 µg) was added to the other tubes, followed by gentle mixing for 2 h at 4 ºC. The beads were washed three times with PBS and bound proteins were eluted by adding 50 µl of 20 mM reduced glutathione. After centrifugation, the supernatants containing the eluted proteins were collected for further analysis.

## 7.16 Sequencing and sequence comparisons (V)

Amplicons from *B. garinii* BITS and *B. afzelii* 40/97 were generated using PCR-primers (Table 6). The resulting amplicons were cloned into the pCR-TOPO vector (Table 3) (Invitrogen Life Technologies, Carlsbad, CA) and individual clones for each strain were subjected to nucleotide sequence analysis. Sequencing was performed with the universal T7 and M13 reverse sequencing primers at the OUHSC, core DNA sequencing facility using an Applied Biosystems Inc. model 373A automated DNA sequencer and PRISM ready reaction DyeDeoxy terminator cycle sequencing kit according to the manufacturer's instructions (Applied Biosystems Inc., Foster City, CA). All nucleotide and deduced amino acid sequence analyses were performed using the MacVector version 6.5.3 software package (Oxford Molecular Group, Campbell, CA). Multiple sequence alignments and phylogram analyses were performed using the ClustalW multiple sequence alignment program of the MacVector version 6.5.3 software package.

Sequence comparisons were carried out between OspE-related proteins from serum sensitive *B. garinii* IP90 and serum resistant *B. burgdorferi* s. s. 297 and *B. afzelii* strains using the GoCore sequence analysis tool (112), which highlighted sequence differences most likely associated with observed differences in serum resistance. Also, most likely antibody binding sites were screened using GoCore.

# 8 Results

## 8.1 Serum sensitivities of *Borrelia* species (I, V)

The serum sensitivities of various strains of *B. burgdorferi* were tested by incubating the bacteria in 10%, 20%, and 40% NHS for 16 hours at +37°C. Samples from the reaction mixtures were taken at 2, 5 and 16 hours for microscopy, and the survival percentages of bacteria were estimated. *B. burgdorferi* s. s. IA, *B. afzelii* 1082 and *B. afzelii* A91 were classified as resistant and *B. garinii* 3/96, 5/96, and 46/97 as sensitive at the 16 hour timepoint. We also analyzed a tick isolate of *B. afzelii* 570, but we included only the patient isolates in the final analysis. The tick strain was resistant at the first two timepoints, but its survival declined at the 16-hour timepoint, possibly due to a lower adaptation to survival in serum.

Because of the observed differences in serum sensitivity, we next compared the ability of the various *Borrelia* genospecies to promote C3b cleavage. When $^{125}$I-C3b was incubated with *B. afzelii* (A91) and FI for 1.5 h at 37°C, a distinct C3b degradation pattern was observed. The $\alpha$'–chain of C3b became cleaved into 68 kDa, 46 kDa and 43 kDa fragments. The amount of cleavage products showed a dose response with an increasing concentration of bacteria and the pattern of cleavage resembled that obtained with FH and FI. In the absence of FI or bacteria, no cleavage of C3b took place. As controls, we tested other bacteria. *Enterococcus faecalis*, *S. aureus* or *E. coli* did not exhibit C3b degrading activity in the presence or absence of FI.

When a larger panel of *Borrelia* strains was tested, it was observed that *B. burgdorferi* s. s. (IA) and *B. afzelii* strains (A91, 570, 1082) promoted cleavage of C3b in the cofactor assay, whereas the seven strains of *B. garinii* (3, 5, 13, 28, 40, 46, 50) did not. A similar cleavage pattern was observed with all *B. afzelii* strains. The cleavage pattern obtained with *B. burgdorferi* s. s. was somewhat different from that obtained with the *B. afzelii* strains. Incubation of the *B. burgdorferi* s. s. IA strain with $^{125}$I-C3b and FI produced a fourth $\alpha$-chain 41 kDa band in addition to the 68, 46 and 43 kDa bands. This cleavage pattern possibly indicated a more efficient C3b inactivation by *B. burgdorferi* s. s. than by *B. afzelii*. No cleavage occurred with either *B. burgdorferi* s. s. or *B. afzelii* in the absence of FI, indicating that the cleavage was not due to a protease on the borrelial surface.

## 8.2 Complement deposition on serum-incubated *Borreliae* analyzed by microscopy (I)

In order to examine complement deposition and regulation on the borrelial surface, we stained spirochetes of the three subspecies with specific antibodies after incubation in normal human non-immune serum. Deposition of iC3b was detected on all three strains. An antibody specific for the C-terminal domains SCR 19-20 of FH showed strong signals for the B. burgdorferi s. s. and even stronger for the *B. afzelii* strain, but weaker staining for the *B. garinii* strain. However, incubation of the *Borrelia* bacteria in NHS may lead to complement activation on the borrelial surface. The FH from the serum may then bind rather to C3b deposited on the *Borrelia* surface than to a specific ligand on the spirochete. Thus when NHS-EDTA was added, in which the alternative pathway activation is inhibited, signals were only obtained for the serum resistant s. s. and *afzelii* strain. The deposition on *B. garinii* was much weaker. The staining was repeated with an antibody specific for the N-terminus of FH. In contrast to the other antibody, this antibody detects FH and the smaller FHL-1 protein, which represents SCR 1-7 of FH. The N-terminal antibody showed a weaker staining, although the signals for the *B. garinii* strain were stronger than with the other antibody. A non-complement antibody, serving as a negative control, showed no signals at all.

## 8.3 Acquisition of FH and FHL-1 from growth medium by *Borreliae* (I)

In order to determine the mechanism responsible for the observed cofactor activity, we tested the possibility that the bacteria had acquired FH from their growth medium. The presence of FH was analyzed by immunoblotting using a goat antibody that recognizes both human and rabbit FH. Two different preparations of *B. afzelii* A91, two *B. garinii* strains (3/96, 46/97) and *B. burgdorferi* s. s. IA that had been grown in the BSK-H medium were washed and incubated in SDS-PAGE sample buffer and run on an SDS-PAGE gel. Immunoblotting with the polyclonal anti-FH antibody revealed FH on the surfaces of *B. afzelii* A91 and *B. burgdorferi s. s.* IA strains. In addition, *B. burgdorferi* s. s. IA appeared to have bound a lower molecular weight protein that corresponded to FHL-1, an alternatively spliced 43-kDa product of the FH gene. No bound FH or the putative FHL-1 on either of the *B. garinii* strains was observed.

## 8.4 Binding of FH and FHL-1 to different strains of *B. burgdorferi* (I)

To directly analyze FH binding to *B. burgdorferi*, radiolabeled proteins FH and FHL-1 were incubated with six different strains of the three pathogenetically relevant *B. garinii, B. afzelii,* and *B. burgdorferi* s. s. strains. The bound proteins were separated by centrifugation through a 20 % sucrose solution and the binding quantified with a gamma counter. Sedimentation of radioactivity in the absence of bacteria was taken as background (always below 0.3 % of total offered radioactivity). Both FH and FHL-1 bound to the *B. burgdorferi* s. s. IA strain (I). The values were ten times higher for FH and five times higher for FHL-1 when compared to the *B. garinii* and *B. afzelii* strains. *S. aureus* and *S. epidermidis* were used as controls. Neither FH nor FHL-1 bound to these bacteria.

## 8.5 Binding of human FH and FHL-1 to borrelial surface proteins (II, III)

*B. burgdorferi* s. l. has a two-layer outer membrane with very few proteins on the outermost membrane. In order to localize the FH binding molecule(s), outer membranes of strains of all three genospecies were isolated by ultracentrifugation through sucrose density gradients. OMs and protoplasmic cylinders (PC) were collected, run in SDS-PAGE and transferred onto nitrocellulose membranes. FH and FHL-1 binding was analyzed by incubating the membranes with radiolabeled FH and FHL-1. FH binding was observed to *B. burgdorferi* s. s. IA OM and PC fractions suggesting that a FH binding molecule is present on the outer membrane. The FH-binding proteins had approximate molecular weights of 19 and 35 kDa (I). FHL-1 binding was observed to the 35 kDa protein on *B. afzelii* A91 OM and PC fractions as well as on the *B. burgdorferi* s. s PC fraction. This suggests that a molecule binding FHL-1 can be found on the outer membrane of at least *B. afzelii* strains. No binding of either FH or FHL-1 to *B. garinii* proteins was observed. The results suggested that FH and FHL-1 selectively bound to the outer membranes of both *B. burgdorferi* s. s. and *B. afzelii* strains. This accounted for the observed differences in serum sensitivity and C3b degradation promoting activity.

Given the interaction between FH and the whole *Borrelia* bacteria, we wanted to identify potential ligands on the *Borrelia* surface. To this end we screened a set of six recombinantly expressed surface proteins of *Borrelia*: the outer surface proteins OspA, OspC, OspD and OspE, the Decorin-binding protein

DbpA and the protein P35 (also known as BBK32). This set represents a number of prominent proteins on the borrelial surface. For most of these proteins immune responses in the human host have been reported.

As a screening method for the putative protein-protein interaction we used the Biacore biosensor method. FH was immobilized to the surface of the sensor chip by standard amine coupling. The various *Borrelia* surface proteins were injected to the flow cell containing FH and to a blank flow cell used as an internal control. One of the outer surface proteins, OspE, bound to FH while OspA, OspC and OspD did not bind. The P35/BBK32 protein showed some binding to FH but interacted also with the dextran-matrix of the control blank channel.

To confirm the result and to simulate a more physiologic situation (borrelial protein on the surface and FH in the fluid phase), the analysis was repeated in a reverse setting. The *Borrelia* surface proteins OspC, OspE and P35 were immobilized onto the chip surface and FH was used as an analyte in the fluid phase. In this approach the binding of OspE to FH was confirmed, while no binding could be observed between FH and OspC or the P35 protein. Thus, FH interacts with OspE regardless of which of the binding partners is immobilized. OspE was hereby identified as the first specific receptor in the interaction with the complement regulator FH (II).

A similar analysis was performed with complement components, C3b and C5. The P35 protein bound to both C3b and C5, when the latter proteins were coupled to the chip (data not shown). Thus, the interaction between P35/BBK32 and FH/C3b/C5 seems to be more due to a general 'stickiness' of the protein rather than to a specific interaction. Next we analyzed a set of OspE-related borrelial surface proteins for FH binding using SPR. We found OspE-related proteins to constitute a family of FH-binding proteins (III). The observed binding affinities are shown in Table 9.

| *B. burgdorferi* OspE-protein | Coupling level/RU | Buffer | t/°C | Affinity |
|---|---|---|---|---|
| OspE | 140 | 1/3 VBS | 25 | 8.23 ± 0.27 nM |
| P21 | 359 | 1/3 VBS | 25 | 8.36 ± 0.25 nM |
| ErpA | 1930 | PBS | 22 | 102 ± 7 nM |
| ErpP | 2843 | PBS | 22 | 129 ± 13 nM |
| OspE 40/97 | 1538 | PBS | 25 | 268 nM $r^2$0,922 |
| P21 | 1522 | PBS | 25 | 20 nM $r^2$0,990 |

**Table 9** Measured affinities of *B. burgdorferi* FH-binding proteins for FH. The calculations have been made with either BiaEvaluation 3.0 of SigmaPlot 8.0. $R^2$-values are a measure of the accuracy of the result when using SigmaPlot.

## 8.6 Mapping of the OspE binding site on FH (IV)

To localize the binding site on FH responsible for the interaction with OspE, we tested a set of recombinant constructs of FH and FH-related proteins. OspE was immobilized on the Biacore chip surface and six different recombinant constructs were tested. The recombinant proteins were expressed in the baculovirus system and their functional activity had been shown earlier (e.g. binding to the complement component C3b). We tested the following constructs of FH: SCR 1-7 (FHL-1), SCR 8-20 and SCR 15-20. Surprisingly, the construct SCR 1-7 did not bind to OspE. This construct contains the domain responsible for the basic complement regulatory functions of FH in SCRs 1-4 and a domain, which has been shown to interact with heparin, streptococcal M protein and the C-reactive protein. A construct consisting of the SCRs 8-20 clearly bound to OspE. A construct SCR 15-20 of FH also bound to OspE, suggesting that the interacting site is located in the C-terminal region of the protein.

## 8.7 Binding of the most C-terminal SCR-domains of human and mouse FH to OspE (IV)

FH from multiple animal species binds to the borrelial outer surface protein OspE (II, IV, 115, 116). Thus, we wanted to localize the borrelial OspE binding sites on both human and mouse FH. As a representative of the OspE-family of proteins we used the OspE-paralog P21 that is encoded by the *B. burgdorferi* s. s. strain 297. Since our earlier studies suggested a binding site in the SCR15-20 region (II), we first tested binding of human FH constructs containing SCRs 18-20 or 19-20 to surface-coupled P21 using the surface plasmon resonance technique. A human FH construct containing SCRs 18-20 bound to the P21-protein with a high affinity. For the shorter construct huH19-20, both P21 association and dissociation occurred more slowly, indicating that the FH19-20 fragment remained strongly bound to P21. Further experiments showed that the mouse C-terminal FH construct moH18-20 also binds to P21-297.

## 8.8 Binding of FHR-1 to OspE (IV)

Since human blood plasma contains several FH-related proteins (FHR-1, -2, -3, -4 and -5) whose C-terminal SCRs resemble those of FH, we analyzed whether OspE could bind to serum proteins other than FH. To identify potential OspE interacting proteins, NHS (1:2 or 1:10 dilution) and serum from an individual deficient in FHR-1 (FHR-1DS) was first precleared from any

GST-binding proteins and thereafter incubated with the GST-OspE fusion protein and glutathione-sepharose beads. The beads were separated by SDS-PAGE and analyzed by silver staining and Western blotting. FHR-1 was found to bind to OspE in a specific manner. The binding of FHR-1 to OspE is probably due to the three most C-terminal domains (SCRs 3, 4, and 5) of FHR-1, which are very homologous (100%, 100%, and 97% respectively) to FH SCR domains 18, 19 and 20.

## 8.9 FH binding by truncated P21-297 proteins (IV, V)

The FH sites responsible for OspE binding were analyzed among the five putative sites previously characterized by peptide mapping (III). We therefore constructed N- or C-terminal deletions of varying lengths of P21 and tested FH binding of the mutant-proteins in Biacore analysis. The P21-297 mutants were coupled to the sensor chip and human FH was used as the fluid phase ligand. Of the mutants, P21Δ1-35 showed a virtually identical FH binding to the full-length P21 ($K_d$ ~8.4 nM), indicating that the N-terminal amino acid residues 1-35 of P21 are not required for FH binding (IV). Removing aa's 1-45 from P21-297, however, abolished binding activity present in P21Δ1-35, which confirmed our earlier hypothesis (V). This suggests that P21 aa:s 35-45 contains crucial binding determinants (4 K residues in P21, 2 K residues in OspE-BITS). The mutant P21Δ105-166 showed no binding of FH, while the mutant P21Δ143-166 as well as the deletion mutant P21Δ152-166 that lacked only 15 C-terminal residues had only a minimal ability to bind FH.

Next we tested, whether the shortest FH constructs with OspE binding activity would bind to the C-terminally truncated P21-proteins. Some binding of the FH constructs SCR18-20 and SCR19-20 occurred to the C-terminally truncated mutant P21-proteins P21Δ143-166 and P21Δ152-166, although the binding was significantly reduced when compared to full-length P21. This indicated that region V consisting of the C-terminal 15 amino acid residues is critical for FH binding. Importantly, however, this does not exclude the possibility that additional sites contribute to the binding. This is because weak residual binding of FH and of the huH18-20 and huH19-20 fragments was observed to the C-terminally truncated mutants of P21.

## 8.10 FH binding by C-terminal peptides and peptide mapping analysis (III, IV)

Since analysis of the deletion mutants suggested that the C-termini of OspE proteins are important for FH binding, we analyzed whether surface-

associated peptides representing the C-terminus of OspE are sufficient for the binding. However, biotin-GABA-coupled peptides synthesized from the C-termini of OspE paralogs OspE-297, P21-297 and ErpP-B31 did not bind FH in Biacore analysis. Also, soluble forms of the individual peptides did not inhibit the binding of FH to OspE (data not shown). This suggested that either the peptides did not adopt the correct conformation in solution or that the binding requires multiple binding sites on P21 for FH. The latter is compatible with the presence of a binding site in the 36-45 aa region at the N terminus of P21.

Since the C-terminal OspE peptides immobilized on cellulose membranes bound FH, we analyzed the characteristics of FH binding to OspE by performing an alanine scanning analysis on peptides from all the five putative binding domains of OspE. To scan for potentially important amino acids of OspE in its interaction with FH, Ala was substituted for each amino acid of each of the regions I-V separately in peptides synthesized on a cellulose membrane. Radiolabeled FH was applied on the membrane and binding was detected using autoradiography. In this assay, FH bound to peptides I, III, IV and V. The replacement of any of the three lysines (Lys-162, -163 and -166) in peptide V completely abolished FH binding, indicating that these residues were crucial for FH binding. Similarly, the replacement of two (out of three) lysine residues in peptide IV (Lys-143 and -145) inhibited FH binding. Lysines appeared to contribute to binding also in peptide III and weakly in peptide I, while other amino acid replacements had no appreciable effects on FH binding. In conclusion, in this assay, lysine residues, particularly those close to the C-terminus of OspE, constituted the FH binding determinants.

## 8.11 Inhibition of FH binding by heparin and by truncated mutants of P21 (II, IV)

To test whether binding of FH to the *Borrelia* surface is dependent on charge, the effect of heparin on the interaction between FH/FHL-1 and *Borrelia* was investigated. Radiolabeled FH and FHL-1 were incubated with whole *Borrelia burgdorferi* s. s. bacteria and the influence of heparin on the binding was analyzed. At lower concentrations of below 30 µg/ml, a small enhancing effect on FH binding to the spirochetes was observed. At higher heparin concentrations the binding of FH to *Borrelia* decreased. In contrast, the binding of FHL-1 showed only a small reduction when the heparin concentration increased. These results were in accordance with the fact that the C-terminal heparin/sialic acid -binding site of FH is involved in the interaction with *Borrelia burgdorferi*.

Fluid-phase P21 and mutants thereof were tested for their ability to inhibit FH binding to surface coupled P21. For comparison, heparin was tested as a control. Inhibition of FH binding was seen with P21 and its N-terminal mutant but not with the mutants with deletions in the C-terminus. This further confirmed that the C-terminal binding site in P21 was the most essential one for FH binding. Heparin inhibited binding to FH already at an equimolar concentration (300 nM), which is in agreement with the ionic nature of the interaction between P21 and FH. On FH a heparin-binding site is located in SCR 20 (36). Thus, binding of heparin to SCR20 could hinder the interaction between SCR18-20 and FH. On the other hand, heparin could also bind to positively charged residues in P21.

## 8.12 FH binding by *B. garinii* OspE-BITS and OspE-40/97 (V)

When we compared different *Borrelia* strains for their resistance to serum, consistently with previous results, we found that *B. garinii* strain 50 was more sensitive than the other strains to complement-mediated lysis as judged from the final experimental time-point (17 h). However, transfecting a plasmid from *B. burgdorferi* 297 containing *ospE*-297 to *B. garinii* strain 50 (a transfected *B. garinii* T50 strain) made the strain more resistant at the first two time-points of the experiment.

Because of the lack of OspE-sequences from serum sensitive strains (only OspE-IP90 from *B. garinii* available from NCBI), two new sequences were obtained from serum sensitive *B. garinii* BITS and *B. garinii* 40/97 by sequencing products obtained by PCR. The sequences were compared to OspE-297 and P21-297. The sequence was hypothesized to contain the crucial N-terminal binding determinant (…SEFTVNIKNKK…).

Sequence comparisons were carried out using the GoCore structural alignment tool and by comparing binding determinant -regions. The program predicted differences, which might cause the observed differences in serum resistance. We found crucial differences in the sequences of OspE-proteins from serum resistant and sensitive *Borrelia*-strains, especially near the N-terminus. The 15 amino acids in the C-termini, which contain sequences crucial for FH binding (III), were found to be nearly identical in the full-length *B. garinii* 40 and *B. burgdorferi* s. s. N40 strains. Thus, in addition to regions predicted by GoCore, there are also other potentially important differences between *B. garinii* OspE-proteins and OspE-297/P21-297 near the N-terminus.

Since *B. garinii* strains grown *in vitro* were sensitive to C attack, we wanted to analyze reasons for this, which possibly included lack of OspE genes or expression thereof. Alternatively the OspE proteins lacked FH-binding activity. To examine this possibility FH binding by OspE BITS and OspE 40/97 was analyzed by surface plasmon resonance. The OspE proteins were coupled to the chip and assayed with fluid phase FH. Interestingly, the *B. garinii* OspE proteins showed clearly less FH binding activity than P21-297.

## 8.13 FH binding by neuroborreliosis-causing *B. garinii* strains

Unlike *B. garinii* strains grown for prolonged periods *in vitro,* we found by ligand blotting that three out of four strains freshly isolated from CSF of patients with neuroborreliosis (LU59, LU165 and LU190) bound FH. The main FH-binding protein was most likely OspE. We also saw binding to an approximately 35-kDa protein similarly to that in study II. The control strains IAI and B31 showed clearly stronger binding of OspE. We further explored candidate proteins to find new FH–binding proteins. Based on preliminary data, we selected Bba68 from the *B. burgdorferi* s. s. 297 strain as a candidate protein. However, when the neuroborreliosis-causing strains were analyzed with a polyclonal antibody against Bba68, they were not found to express this protein. In summary, *Borreliae* bind FH with at least two families of FH-binding proteins (OspE- and Bba68-proteins). OspE-proteins seem to contribute also to the pathogenesis of *B. garinii* strains.

# 9 Discussion

Lyme borreliosis occurrence has been increasing in Finland over the last decade in part due to increased awareness of health-care workers on the diagnosis. Lyme disease is the most common vector-borne infection in Finland and is spread by *Ixodes ricinus* ticks. In other parts of the world other ticks are important vectors. The occurrence of Lyme disease is mostly concentrated in coastal areas and archipelagos, but is found throughout the country. Early treatment with antibiotics is usually effective, whereas several vaccine development programs have ended in failures. Therefore, a clear need for more accurate knowledge on the pathogenesis of the disease existed, which motivated this work. Lyme disease may become a chronic condition with long-term effects on the life of the individual, which has raised also public concern about ways of prevention and treatment.

## 9.1 Need for complement evasion by the Lyme disease spirochetes

Lyme disease was first described in the 1970's, when a novel juvenile rheumatoid arthritis-like condition was observed to occur in Lyme, Connecticut, USA (117). The causative microbe was soon described (1982) (118) and documented. The Lyme disease -causing spirochetes have since been found to include three genospecies, *Borrelia burgdorferi* s. s., *Borrelia afzelii*, and *Borrelia garinii* strains. These subspecies are very diverse in their genetic content and manifest differently during infection (119). Since all strains are invasive, they must be able to evade the immune system, including complement attack. The complement system is active in serum, in which cell-mediated actions of the immune system are absent and the serum sensitivity is variable between different *B. burgdorferi* subspecies (81). *Borrelia burgdorferi* s. l. complex Lyme disease spirochetes are microbes that successfully suppress and evade the human immune system. Therefore, they cause persistent infections with immune-related long-term sequelae. Several microbes utilize FH binding in complement suppression. The aims of this study were to find out the whether binding of FH and its homologues is utilized also by *B. burgdorferi* strains for complement evasion, which molecules are associated with this mechanism, which primary structural determinants are essential in the tentative interaction, what homologues, if

any, of these proteins exist and whether these proteins are potential vaccine targets.

## 9.2 Cofactor activity for C3b cleavage in *B. burgdorferi* s. s. strains

It was first observed that *B. burgdorferi* s. s. strain cleave C3b into inactive iC3b fragments (I). The activity required the presence of FI, but no added FH was required. However, it turned out that the cofactor activity was dependent upon growth medium for the spirochetes. Rabbit factor H was indeed present in the culture medium BSK-H. Thus, the most likely explanation for the observation was utilization of this FH by the spirochetes.

## 9.3 Serum resistant strains of *B. burgdorferi* bind FH

The observation of factor H binding by *Borrelia* spirochetes resulted in this work, which comprises a description of a novel complement suppression mechanism. Factor H binding to *B. burgdorferi* was found to be due to OspE-proteins (I, II, III) on the surfaces of *B. burgdorferi* spirochetes (I) and contribute to their virulence (IV). In addition, a 35 kDa protein was found to bind FHL-1 and FH. OspE proteins with comparatively low FH-binding affinity were later found to tentatively contribute to the virulence of *B. garinii* strains (V).

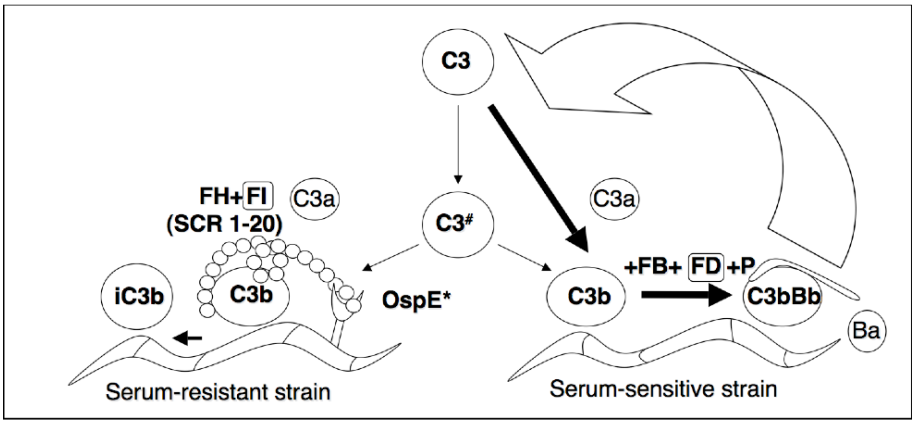


**Figure 5.** The current concept of *B. burgdorferi* complement evasion. FH is bound by OspE and/or by other FH-binding proteins (II, III). Complement becomes inactivated on the surface of serum resistant *Borrelia* strains, but is activated normally on serum sensitive strains. By binding FH *B. burgdorferi* can avoid opsonophagocytosis and complement killing.

## 9.4 FH binding is mediated by OspE-proteins

A family of OspE-related plasmid-encoded proteins was found to bind FH with high affinity in the serum resistant strains of *B. burgdorferi* (III). The FH-binding capacity of serum sensitive *B. garinii* strains was found to be limited compared to the serum resistant strains (V). This can be tentatively accounted for by the following: a) a lower affinity of OspE proteins in *B. garinii* -strains for FH (V), b) lower quantity of OspE-expression on *B. garinii* spirochetes, or possibly no surface expression (IP90, (120)) (V), c) better ability of OspE-specific antibodies to block FH binding to *B. garinii* OspE-proteins in competition for binding sites. The affinities of the OspE-297- and P21-297-proteins for FH were approximately 10 nM, ErpA-B31- and ErpP-B31-proteins for FH approximately 120 nM (IV). This suggests a relatively high affinity interaction. For *B. garinii* OspE an affinity of 268 nM for FH was recorder (V).

The affinity of antibodies for antigens generally ranges from $10^{-4}$ M to $10^{-11}$ M (121) and varies with antigen and phase of infection. Thus, the affinity of antibodies covers the range of observed OspE-FH affinities, suggesting that high affinity antibodies may, at least partially, block the interaction between OspE-proteins and FH. This is true especially regarding the OspE-proteins of *B. garinii* strains, which have a lower affinity for FH (268 nM for OspE-40/97). During *B. burgdorferi* infection in humans, a marked antibody response is formed against OspA, -B, and -C (55, 122). In related studies our research group has observed the presence of an OspE-response in a proportion of human patients with borreliosis (Panelius *et al.*, unpublished). The immunogenicity of OspE in mice and rats has also been observed (unpublished). Whether these antibody responses can neutralize the virulence effect of OspE remains to be studied.

## 9.5 OspE-proteins are encoded by plasmids

OspE-related proteins are encoded by genes in circular plasmids (104), which contain more genetic material unique to *Borrelia burgdorferi* than the linear chromosome (84). This is consistent with the adaptive nature of the complement evasion mechanism. Stevenson *et al.* found Erp-proteins (OspE-family proteins) to bind FH in various animal species that belong to the enzootic cycle of the *B. burgdorferi* spirochete (115). Recently it was reported that bacteriophages could transfer these mobile genetic elements between bacterial cells (123). Thus, the adaptive mechanism has an extra tool for creating diversity achieved via a form of symbiosis.

The genes encoding the OspE-protein family, or OspE paralogs are a subset of the upstream homology box (UHB) gene family, which also includes genes encoding OspF paralogs (120).

## 9.6 Five putative FH binding regions are found in OspE-proteins

The putative FH-binding regions in the OspE-proteins were located by peptide scanning and using deletion mutants lacking binding regions in various combinations. P21, an OspE-homologue from the *B. burgdorferi* s. s. 297 strain, was modified by constructing deletion mutants using PCR-cloning. Altogether, the P21-protein contained five putative FH-binding regions. One essential FH-binding region located within the amino acids 36-45 was in the N-terminus (V) and the remaining sites were closer to the C terminus of the primary structure of P21.

These regions were found to have corresponding regions in OspE-proteins from *B. garinii* strains in a sequence alignment (V). When the five regions were reanalyzed, region II was not found to interact with FH demonstrating one of the limitations of the peptide scanning method. In addition the folding structure of the full-length protein is not taken into account when short peptides are analyzed. In the three-dimensional structure of the full-length protein the different binding regions may orient differently and/or may be sterically blocked from the interacting protein.

Peptides synthesized from these five putative binding regions did not inhibit the interaction between FH and P21 in the fluid phase when examined in the Biacore. This suggested the relevance of analyzing the whole three-dimensional structure of P21 as the individual peptides did not have sufficient electrostatic attraction for FH in the fluid phase. The individual binding regions are, therefore essential, but not sufficient for binding FH in the whole protein.

## 9.7 The primary structure FH-binding regions contain crucial lysine-residues

Lysine-residues in the OspE-related proteins were found to be essential for the FH-interaction, which involved the C-terminus of FH (SCR18-20) and the N- and C-termini of OspE-proteins (III, V). Positively charged lysine-residues form sites of electrostatic attraction between negatively charged regions in FH and zwitterionic OspE-proteins. The observed ability of heparin to inhibit this

interaction is consistent with the observed ionic nature of the interaction (III) and tentative dependence of the affinity of OspE for FH on the ionic strength of the environment (V). FH interactions with short peptides are generally dependent upon lysines (Jokiranta *et al*., unpublished). The C-termini of OspE-proteins are relatively conserved across strains.

The *B. garinii* OspE-proteins contain only two lysines in the N-terminal 36-45 region, where the P21-297 protein has altogether four lysine-residues. Lysines 38 and 42 were found to be replaced by glutamic acid and asparagine in the three studied *B. garinii* OspE-proteins. This, in conjunction with the crucial role of lysine-residues, is consistent with the lower FH-binding affinity observed for the *B. garinii* OspE-proteins. The ability of *B. garinii* strains to cause infection despite their relative serum sensitivity may be related to their transient expression of FH-binding proteins and other virulence factors. Another contributing factor could be their ability to invade the CNS, where cytotoxic complement is more scarce than elsewhere in the body (124).

## 9.8 OspE binding regions in FH

Using FH-deletion constructs we mapped the OspE binding site on FH to the C-terminal SCR18-20 region. FH constructs containing SCR18-20 and SCR19-20 only were amplified from a human liver cDNA-library and PCR-cloned into plasmid vectors. Using the expressed form of the protein it was found in Biacore that the binding site is contained within SCR18-20 with the removal of SCR18 reducing the observed $R_{max}$. In comparison the binding $R_{max}$ of SCR18-20 was comparable to that of the interaction of the full length P21 protein with full-length FH (IV). This suggests that the binding site may be the only relevant region in the interaction with OspE-proteins making the interaction distinct from FH interactions with streptococcal M-proteins and Bba68. The latter proteins have been reported to bind to the SCR7 domain of FH.

## 9.9 FHR-1 binding to OspE

Several FH-related proteins that are found in human plasma were also tested to find out whether they bind to OspE. The representative FHR-protein, FHR-1 was later observed to also bind to OspE. FHR-1 contains three domains (3-5), which have a high degree of similarity with the FH SCR18-20 (Fig. 3). Therefore this observation is consistent with those made with FH-constructs. The physiological functions of FHR-proteins are under ongoing research and

as of yet unknown. Therefore, the relevance of FHR-1 binding to OspE is not yet known.

## 9.10 Binding of mouse FH construct by OspE-related proteins

An SCR18-20 construct from mouse FH was made similarly to the human FH SCR18-20 construct. We observed binding of the SCR18-20 fragment to P21 (IV), implying that the full-length mouse FH-protein would also interact with P21. Therefore, the *B. burgdorferi* spirochetes most likely utilize this mechanism also in mouse and the binding sites in human and mouse FH are analogous. This information is important e.g. in considering murine animal models of *Borrelia* infections.

## 9.11 Transfer of complement resistance to *B. garinii* 50 by transfection with *ospE*

To analyze whether complement resistance can be transferred with OspE and the complement-sensitive phenotype of *B. garinii* 50 changed to a complement-resistant one, transfection studies were carried out. A *ospE*-containing plasmid with a kanamycin cassette was used to transfect a complement sensitive *B. garinii* 50 strain and the plasmid was maintained in the strain with kanamycin-selection until the serum sensitivity was tested and compared to *B. garinii* 50 and another control strain. The transfected strain (T50) expressed OspE on its surface. The functional test showed an increased resistance of the T50 strain to complement-mediated killing at 2 hours (V). This suggests that the transfected plasmid did not decrease the viability of the T50 strain compared to the 50 strain, but increased its complement resistance. This is consistent with other observations and is the first evidence to directly show the effect of OspE on complement resistance. This also suggests the wider importance of this mechanism.

Neither strain died in the experiment. We observed a form of biofilm-formation of large *Borrelia burgdorferi* bundles (see cover; unpublished), which likely served to protect *Borrelia* bacteria from complement. This may be analogous to attack of the complement system on tumors, where the outer cell-layers are attacked, but the inner layers protected (128). This mechanism has also been documented elsewhere (76). MMPs (68, 75, 129) enable the spirochetes to invade tissues and when they are able to grow into a larger population, they may be able to shield each other by forming a form of biofilm. Because there was a very large amount of bacteria, the majority of the cells were not attacked. In a physiological sense this may partially explain the difficulty in

treating persistent long-term infections. Transfecting a serum sensitive *B. garinii* strain with a plasmid containing OspE from a serum resistant 297 strain, increased the serum resistance of the *B. garinii* strain (V). This suggests that in *in vitro* cultured *B. garinii* spirochetes, the expression of OspE-proteins is suppressed.

## 9.12 The relative roles of various FH-binding proteins

Other authors recently presented an argument for a key role of CRASP-1, a 25.9 kDa *B. burgdorferi* outer surface protein (125) in FH binding to Lyme disease spirochetes (126). CRASP-1 corresponds o the Bba68-family of proteins. The binding of the CRASP-1-protein is mediated by FH SCR7, which is distinct from the SCR18-20 region mediating OspE binding. Also, CRASP-1 is encoded by lp54, a linear plasmid (126). The CRASP-1 protein probably corresponds to the 35 kDa FH-binding proteins found in study I. In addition, FH binding was seen to a factor H-binding protein, which most likely is OspE (V) in the CNS-infective *B. garinii* strains. The relative roles of these and possible other FH-binding proteins (125) remain to be elucidated. However, the temporal division of expression is likely the key to their central functions, with OspE-family proteins being expressed in a wide variety of hosts. One of the key differences between the Bba68- and OspE-proteins may be related to the relative mobility of the latter (123).

## 9.13 The expression of FH-binding proteins is regulated by external factors

This work has pinpointed crucial virulence proteins and primary structures, whose expression is regulated by external factors. It was observed that OspE-expression is upregulated upon temperature change from 22°C to 37°C (III). Mammalian-specific expression of OspE-proteins has been reported also elsewhere (101, 103, 127). Future studies on the relative roles and ubiquity of OspE-proteins and other FH-binding proteins in *B. burgdorferi* and other pathogenic microbes will shed more light into the importance of this mechanism at the interface between the human immune system and pathogens.

The temporally controlled expression of virulence factors may also explain the virulence of *B. garinii* strains in infecting the CNS. It was observed that *B. garinii* strains have a higher sensitivity to complement *in vitro* compared to *B. burgdorferi* s. s. and *B. afzelii* strains. Nevertheless, they seem to enter the CNS where they can escape complement, which is relatively scarce inside the

CNS. This may require the temporally regulated expression of virulence factors, such as OspE or Bba68-proteins or other FH-binding proteins preceding entry. As observed in study V, the OspE proteins are expressed in *B. garinii* strains freshly isolated from the CNS.

## 9.14 Key mechanisms of pathogenesis and future studies

This work has answered some of the most central questions in Lyme disease pathogenesis. Apparently, the utilization of the FH-binding proteins, which include the OspE, and Bba68 families of proteins are important to the virulence of *Borrelia burgdorferi* spirochetes. A rigorous proof for this necessitates animal model studies. The generation of e.g. OspE-transfected strains of *B. garinii* and other analogous gene-manipulated *Borreliae* will be instrumental for studies of this kind.

At least two proteins (130, 131) have been studied as vaccine candidates against Lyme disease. A vaccine using OspA as antigen was sold for some time in North America, but it was withdrawn because of arthritogenic side-effects (132). Subsequently, the vaccine was redesigned to contain a recombinant antigen lacking a potentially cross-reactive site (58) and currently exists as a second-generation rOspA vaccine (133). However, similarly to the first-generation OspA vaccine, the rOspA vaccine is only effective against North-American strains. In the Åland islands, Finland, a polyvalent vaccine (134), utilizing the OspC antigen (135) was undergoing clinical testing but this testing has been discontinued. Therefore, no currently available vaccine exists for the European market.

Computer prediction studies indicate that OspE has an overall coiled-coil-type structure. In this regard, it resembles other FH-binding proteins, e.g. M-protein of group A streptococci (136) and PspC-family proteins of pneumococci (41). Future studies regarding the three-dimensional structure of OspE and Bba68-family proteins would be of potential interest for docking analysis of the FH-OspE interaction, which would enable the design of targeted antigen design for vaccines and/or pharmacotherapy.

# 10 Conclusions

This work has demonstrated that Lyme disease spirochetes utilize FH binding as a complement evasion mechanism. The complement inhibitor factor H binds with its C terminus to plasmid-encoded OspE surface proteins of *B. burgdorferi*. The OspE-family of FH-binding proteins, therefore, constitutes a family of virulence factors, which affect the complement sensitivity of *B. burgdorferi* s. l. spirochetes. In addition, another FH-binding protein family was observed and identified by others as the Bba68-family of proteins. This information may enable the development of new, effective vaccines against a wide range of strains

# 11 Acknowledgments

*Coworkers.* The Department of Bacteriology and Immunology at The Haartman Institute provided the environment for this work. I would like to kindly thank Prof. Seppo Meri, M.D. Ph. D. for wonderful supervision of my work and Dr. Sakari Jokiranta, M.D. Ph.D. for teaching, advice and coauthorship of several papers. I also thank Ms. Taru Meri, M.Sc. for collaboration, coauthorship, good company during the long hours it took to complete the serum sensitivity analyses, listening, all good care and for her comments and ideas in designing the cover of this book. Dr. Ilkka Seppälä, M.D. Ph.D., Dr. Pekka Lahdenne, M.D. Ph.D., Dr. Tero Heikkilä, M.D. Ph.D. and Dr. Jaana Panelius, M.D. Ph.D. provided their expert assistance, coauthorship and comments on my papers, which I am very grateful for. Dr. Zhu-Zhu Cheng, M.D. made the mouse and human factor H constructs containing only SCRs 18-20 and 19-20, which added an interesting aspect to the study. Dr. Tong Chen, M.D. made the GST-OspE-pulldown technique come to life in paper IV. I would also like to thank the various people I have had the opportunity to share my working environment with, including special thanks to Dr. Riina Rautemaa, DDS for listening to all of my babbling, Mr. Jorma Tissari, M.Sc. and Dr. Juha Hakulinen, Ph.D. for giving advice when it was most needed, Dr. Hanna Jarva, M.D. Ph.D. for teaching, advice and nice chats, Dr. Antti Harjunpää, M.D. for giving the lab a joyous presence and laughter every now and then. Johanna Tornberg, a medical student, came into this project during its last year; she was a great help in expanding the implications of the findings in this thesis. Finally, I thank the secretaries Kirsi Udueze and Carina Wasström for their help with the red tape of the academic environment and for their hospitality during my visits to the department office.

*Facilities.* The Haartman Institute and University of Helsinki with the expertise and volume of a world-class academic institution have provided the necessary general connections, funding and facilities for this type of project, which could not have been completed without the aforementioned tangible as well as intangible capital. I want to extend special thanks to Dr. Hilkka Lankinen, Ph.D., the laboratory head of the Peptide and Protein laboratory and core services at the Haartman Institute, for her contributions, and many conversations, which functioned as sieves for all the ideas that surfaced during this project.

*Collaborators and colleagues.* Firstly, I would like to thank Dr. Darrin Akins, Ph.D. for his contribution and help with this project. When Prof. Meri was

away Darrin could usually be reached by e-mail, even in the evenings. This also added several potential hours of time to my working days. I also thank all other coauthors for their comments and contributions. Special thanks go to Dr. Jens Hellwage, Ph. D., who, during his postdoctoral visit at our laboratory, was mainly responsible for studies in paper II. I would like to thank Dr. Jarmo Oksi, M.D. Ph.D. and Prof. Matti Viljanen, M.D. Ph.D. (University of Turku) for borrelial strains, and Sven Bergström, Pär Comstedt Umeå University for neuroborreliosis strains. The work of Dr. Miikka Peltomaa, M.D. Ph.D. in coordinating the collection of ticks in the Helsinki area added a local flavor to this study. The possibility of using locally occurring Finnish strains made the work more motivating. Dr. Peter Zipfel, Ph.D. kindly gave the FHL-1 protein and Mr. Jussi Hepojoki synthesized the membrane bound peptides.

*General thanks.* General thanks go to my parents, sister and other relatives as well as all of the fellow students who contributed to my research either directly or indirectly with their stimulating presence.

*Funding support.* I thank all institutions and foundations, which have provided financial support for this project: The Helsinki Biomedical Graduate School, The Biomedicum Helsinki Foundation, The Finnish Medical Society Duodecim, The Emil Aaltonen Foundation, The Research and Science Foundation of Farmos, The Scandinavian Society for Immunology and the European Union (for traveling support). The Sigrid Jusélius foundation, The Helsinki University Central Hospital (EVO-Funds) and The Academy of Finland have provided general support to our laboratory.

# 12 List of references


1. Zipfel, P. F., T. S. Jokiranta, J. Hellwage, V. Koistinen, and S. Meri. 1999. The factor H protein family. *Immunopharmacology 42:53*.
2. Junnikkala, S. 2002. Complement resistance of human tumor cells. In *Department of Bacteriology and Immunology*. University of Helsinki, Helsinki, p. 106.
3. Giannakis, E., T. S. Jokiranta, D. A. Male, S. Ranganathan, R. J. Ormsby, V. A. Fischetti, C. Mold, and D. L. Gordon. 2003. A common site within factor H SCR 7 responsible for binding heparin, C-reactive protein and streptococcal M protein. *Eur. J. Immunol. 33:962*.
4. Jarva, H., J. Hellwage, T. S. Jokiranta, M. J. Lehtinen, P. F. Zipfel, and S. Meri. 2004. The group B streptococcal beta and pneumococcal Hic proteins are structurally related immune evasion molecules that bind the complement inhibitor factor H in an analogous fashion. *J. Immunol. 172:3111*.
5. Warr, G. 2002. A Survey of the Immune system. A. Alitalo, ed. Scandinavian Society for Immunology (SSI), Bergen, Norway, p. Oral communication at the annual Scandinavian Society for Immunology 33rd Annual Meeting and 18th Summer school 2002.
6. Morgan, B. P., and C. L. Harris. 1999. *Complement Regulatory Proteins*. Academic Press.
7. Loos, M., D. Bitter-Suermann, and M. Dierich. 1974. Interaction of the first (C1), the second (C2) and the fourth (C4) component of complement with different preparations of bacterial lipopolysaccharides and with lipid A. *J. Immunol. 112:935*.
8. Schlesinger, L. S., and M. A. Horwitz. 1990. Phagocytosis of leprosy bacilli is mediated by complement receptors CR1 and CR3 on human monocytes and complement component C3 in serum. *J. Clin. Invest. 85:1304*.
9. Bohnsack, J. F., and N. R. Cooper. 1988. CR2 ligands modulate human B cell activation. *J. Immunol. 141:2569*.
10. Davis, A. E., 3rd. 2003. The pathogenesis of hereditary angioedema. *Transfus. Apheresis Sci. 29:195*.
11. Dahlback, B., and B. Hildebrand. 1983. Degradation of human complement component C4b in the presence of the C4b-binding protein-protein S complex. *Biochem. J. 209:857*.
12. Fontaine, M., M. Daveau, and D. Gilbert. 1983. Regulation of the binding of C3-coated particles to human lymphocytes by human complement component H. *Scand. J. Immunol. 17:463*.

13. Meri, S., B. P. Morgan, A. Davies, R. H. Daniels, M. G. Olavesen, H. Waldmann, and P. J. Lachmann. 1990. Human protectin (CD59), an 18,000-20,000 MW complement lysis restricting factor, inhibits C5b-8 catalysed insertion of C9 into lipid bilayers. *Immunology 71:1*.
14. Schiller, N. L. 1988. Characterization of the susceptibility of Pseudomonas aeruginosa to complement-mediated killing: role of antibodies to the rough lipopolysaccharide on serum-sensitive strains. *Infect. Immun. 56:632*.
15. Wetzler, L. M., K. Barry, M. S. Blake, and E. C. Gotschlich. 1992. Gonococcal lipooligosaccharide sialylation prevents complement-dependent killing by immune sera. *Infect. Immun. 60:39*.
16. Jarvis, G. A., and N. A. Vedros. 1987. Sialic acid of group B *Neisseria meningitidis* regulates alternative complement pathway activation. *Infect. Immun. 55:174*.
17. Liang, F. T., M. B. Jacobs, L. C. Bowers, and M. T. Philipp. 2002. An immune evasion mechanism for spirochetal persistence in Lyme borreliosis. *J. Exp. Med. 195:415*.
18. Akesson, P., A. G. Sjoholm, and L. Bjorck. 1996. Protein SIC, a novel extracellular protein of *Streptococcus pyogenes* interfering with complement function. *J. Biol. Chem. 271:1081*.
19. Frick, I. M., P. Akesson, M. Rasmussen, A. Schmidtchen, and L. Bjorck. 2003. SIC, a secreted protein of *Streptococcus pyogenes* that inactivates antibacterial peptides. *J. Biol. Chem. 278:16561*.
20. Kharazmi, A. 1991. Mechanisms involved in the evasion of the host defence by *Pseudomonas aeruginosa*. *Immunol. Lett. 30:201*.
21. Schenkein, H. A., H. M. Fletcher, M. Bodnar, and F. L. Macrina. 1995. Increased opsonization of a prtH-defective mutant of *Porphyromonas gingivalis* W83 is caused by reduced degradation of complement-derived opsonins. *J. Immunol. 154:5331*.
22. Chong, Y. H., and M. J. Lee. 2000. Expression of complement inhibitor protein CD59 in human neuronal and glial cell lines treated with HIV-1 gp41 peptides. *J. Neurovirol. 6:51*.
23. Aries, S. P., B. Schaaf, F. Hansen, K. Weyrich, V. Kurowski, R. Dennin, and K. Dalhoff. 1997. Expression of complement receptors and regulatory proteins on alveolar CD4+ lymphocytes from human immunodeficiency virus-1 infected individuals. *Eur. Respir. J. 10:1736*.
24. Saifuddin, M., T. Hedayati, J. P. Atkinson, M. H. Holguin, C. J. Parker, and G. T. Spear. 1997. Human immunodeficiency virus type 1 incorporates both glycosyl phosphatidylinositol-anchored CD55 and CD59 and integral membrane CD46 at levels that protect from complement-mediated destruction. *J. Gen. Virol. 78 ( Pt 8):1907*.

25. Sullivan, B. L., E. J. Knopoff, M. Saifuddin, D. M. Takefman, M. N. Saarloos, B. E. Sha, and G. T. Spear. 1996. Susceptibility of HIV-1 plasma virus to complement-mediated lysis. Evidence for a role in clearance of virus *in vivo*. *J. Immunol. 157:1791*.

26. Nakamura, M., H. Okada, H. Sasaki, K. Yoshida, M. Kamada, N. Okada, M. Terada, and T. Ohno. 1996. Quantification of the CD55 and CD59, membrane inhibitors of complement on HIV-1 particles as a function of complement-mediated virolysis. *Microbiol. Immunol. 40:561*.

27. Spear, G. T., N. S. Lurain, C. J. Parker, M. Ghassemi, G. H. Payne, and M. Saifuddin. 1995. Host cell-derived complement control proteins CD55 and CD59 are incorporated into the virions of two unrelated enveloped viruses. Human T cell leukemia/lymphoma virus type I (HTLV-I) and human cytomegalovirus (HCMV). *J. Immunol. 155:4376*.

28. Schmitz, J., J. P. Zimmer, B. Kluxen, S. Aries, M. Bogel, I. Gigli, and H. Schmitz. 1995. Antibody-dependent complement-mediated cytotoxicity in sera from patients with HIV-1 infection is controlled by CD55 and CD59. *J. Clin. Invest. 96:1520*.

29. Saifuddin, M., C. J. Parker, M. E. Peeples, M. K. Gorny, S. Zolla-Pazner, M. Ghassemi, I. A. Rooney, J. P. Atkinson, and G. T. Spear. 1995. Role of virion-associated glycosylphosphatidylinositol-linked proteins CD55 and CD59 in complement resistance of cell line-derived and primary isolates of HIV-1. *J. Exp. Med. 182:501*.

30. Marschang, P., J. Sodroski, R. Wurzner, and M. P. Dierich. 1995. Decay-accelerating factor (CD55) protects human immunodeficiency virus type 1 from inactivation by human complement. *Eur. J. Immunol. 25:285*.

31. Montefiori, D. C., R. J. Cornell, J. Y. Zhou, J. T. Zhou, V. M. Hirsch, and P. R. Johnson. 1994. Complement control proteins, CD46, CD55, and CD59, as common surface constituents of human and simian immunodeficiency viruses and possible targets for vaccine protection. *Virology 205:82*.

32. Saifuddin, M., M. Ghassemi, C. Patki, C. J. Parker, and G. T. Spear. 1994. Host cell components affect the sensitivity of HIV type 1 to complement-mediated virolysis. *AIDS Res. Hum. Retroviruses 10:829*.

33. Weiss, L., N. Okada, N. Haeffner-Cavaillon, T. Hattori, C. Faucher, M. D. Kazatchkine, and H. Okada. 1992. Decreased expression of the membrane inhibitor of complement-mediated cytolysis CD59 on T-lymphocytes of HIV-infected patients. *AIDS 6:379*.

34. Bisno, A. L. 1979. Alternate complement pathway activation by group A streptococci: role of M-protein. *Infect. Immun. 26:1172*.
35. Carlsson, F., K. Berggard, M. Stalhammar-Carlemalm, and G. Lindahl. 2003. Evasion of phagocytosis through cooperation between two ligand-binding regions in *Streptococcus pyogenes* M protein. *J. Exp. Med. 198:1057*.
36. Kotarsky, H., J. Hellwage, E. Johnsson, C. Skerka, H. G. Svensson, G. Lindahl, U. Sjobring, and P. F. Zipfel. 1998. Identification of a domain in human factor H and factor H-like protein-1 required for the interaction with streptococcal M proteins. *J. Immunol. 160:3349*.
37. Johnsson, E., K. Berggard, H. Kotarsky, J. Hellwage, P. F. Zipfel, U. Sjobring, and G. Lindahl. 2000. Role of the hypervariable region in streptococcal M proteins: binding of a human complement inhibitor. *Eur. J. Immunol. 30:1243*.
38. Peterson, P. K., D. Schmeling, P. P. Cleary, B. J. Wilkinson, Y. Kim, and P. G. Quie. 1979. Inhibition of alternative complement pathway opsonization by group A streptococcal M protein. *J. Infect. Dis. 139:575*.
39. Horstmann, R. D., H. J. Sievertsen, J. Knobloch, and V. A. Fischetti. 1990. Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H. *Infect. Immun. 58:2535*.
40. Hong, K., T. Kinoshita, J. Takeda, H. Kozono, P. Pramoonjago, Y. U. Kim, and K. Inoue. 1990. Inhibition of the alternative C3 convertase and classical C5 convertase of complement by group A streptococcal M protein. *Infect. Immun. 58:2535*.
41. Jarva, H., R. Janulczyk, J. Hellwage, P. F. Zipfel, L. Bjorck, and S. Meri. 2002. *Streptococcus pneumoniae* evades complement attack and opsonophagocytosis by expressing the pspC locus-encoded Hic protein that binds to short consensus repeats 8-11 of factor H. *J. Immunol. 168:1886*.
42. Anderson, J. F. 1989. Epizootiology of *Borrelia* in *Ixodes* tick vectors and reservoir hosts. *Rev. Inf. Dis. 11:S1451*.
43. Eisen, L., R. J. Eisen, and R. S. Lane. 2002. Seasonal activity patterns of *Ixodes pacificus* nymphs in relation to climatic conditions. *Med. Vet. Entomol. 16:235*.
44. Steere, A. C., J. Coburn, and L. Glickstein. 2004. The emergence of Lyme disease. *J. Clin. Invest. 113:1093*.
45. Ohnishi, J., J. Piesman, and A. M. de Silva. 2001. Antigenic and genetic heterogeneity of *Borrelia burgdorferi* populations transmitted by ticks. *Proc. Natl. Acad. Sci. U. S. A. 98:670*.

46. Anguita, J., M. N. Hedrick, and E. Fikrig. 2003. Adaptation of *Borrelia burgdorferi* in the tick and the mammalian host. *FEMS Microbiol. Rev. 27:493*.
47. Yang, X., M. S. Goldberg, T. G. Popova, G. B. Schoeler, S. K. Wikel, K. E. Hagman, and M. V. Norgard. 2000. Interdependence of environmental factors influencing reciprocal patterns of gene expression in virulent *Borrelia burgdorferi*. *Mol. Microbiol. 37:1470*.
48. Das, S., S. W. Barthold, S. S. Giles, R. R. Montgomery, S. R. Telford, 3rd, and E. Fikrig. 1997. Temporal pattern of *Borrelia burgdorferi* p21 expression in ticks and the mammalian host. *J. Clin. Inves. 99:987*.
49. Carroll, J. A., N. El-Hage, J. C. Miller, K. Babb, and B. Stevenson. 2001. *Borrelia burgdorferi* RevA antigen is a surface-exposed outer membrane protein whose expression is regulated in response to environmental temperature and pH. *Infect. Immun. 69:5286*.
50. Revel, A. T., A. M. Talaat, and M. V. Norgard. 2002. DNA microarray analysis of differential gene expression in *Borrelia burgdorferi*, the Lyme disease spirochete. *Proc. Natl. Acad. Sci. U. S. A. 99:1562*.
51. Ribeiro, J. M. 1987. *Ixodes dammini*: salivary anti-complement activity. *Exp. Parasitol. 64:347*.
52. Berglund, J., R. Eitrem, K. Ornstein, A. Lindberg, A. Ringer, H. Elmrud, M. Carlsson, A. Runehagen, C. Svanborg, and R. Norrby. 1995. An epidemiologic study of Lyme disease in southern Sweden. *N. Engl. J. Med. 333:1319*.
53. Reik, L., Jr., W. Burgdorfer, and J. O. Donaldson. 1986. Neurologic abnormalities in Lyme disease without *erythema chronicum migrans*. *Am. J. Med. 81:73*.
54. Oksi, J., and M. K. Viljanen. 1995. Tick bites, clinical symptoms of Lyme borreliosis, and *Borrelia* antibody responses in Finnish army recruits training in an endemic region during summer. *Mil. Med. 160:453*.
55. Batsford, S., C. Rust, and U. Neubert. 1998. Analysis of antibody response to the outer surface protein family in lyme borreliosis patients. *J. Infect. Dis. 178:1676*.
56. Steere, A. C., and L. Glickstein. 2004. Elucidation of Lyme arthritis. *Nat. Rev. Immunol. 4:143*.
57. Trollmo, C., A. L. Meyer, A. C. Steere, D. A. Hafler, and B. T. Huber. 2001. Molecular mimicry in Lyme arthritis demonstrated at the single cell level: LFA-1 alpha L is a partial agonist for outer surface protein A-reactive T cells. *J. Immunol. 166:5286*.
58. Gross, D. M., T. Forsthuber, M. Tary-Lehmann, C. Etling, K. Ito, Z. A. Nagy, J. A. Field, A. C. Steere, and B. T. Huber. 1998. Identification

of LFA-1 as a candidate autoantigen in treatment-resistant Lyme arthritis [see comments]. *Science 281:703*.

59. Hartung, H. P., K. Heininger, B. Schafer, W. Fierz, and K. V. Toyka. 1988. Immune mechanisms in inflammatory polyneuropathy. *Ann. NY Acad. Sci. 540:122*.
60. Kristoferitsch, W. 1989. Lyme borreliosis in Europe. Neurologic disorders. *Rheum. Dis. Clin. N. Am. 15:767*.
61. Kristoferitsch, W. 1991. Neurological manifestations of Lyme borreliosis: clinical definition and differential diagnosis. *Scand. J. of Inf. Dis. - Supplementum 77:64*.
62. Rostami, A. M. 1993. Pathogenesis of immune-mediated neuropathies. *Pediatr. Res. 33:S90*.
63. Halperin, J. J. 2003. Lyme disease and the peripheral nervous system. *Muscle Nerve 28:133*.
64. Fraser, C. M., S. Casjens, W. M. Huang, G. G. Sutton, R. Clayton, R. Lathigra, O. White, K. A. Ketchum, R. Dodson, E. K. Hickey, M. Gwinn, B. Dougherty, J. F. Tomb, R. D. Fleischmann, D. Richardson, J. Peterson, A. R. Kerlavage, J. Quackenbush, S. Salzberg, M. Hanson, R. van Vugt, N. Palmer, M. D. Adams, J. Gocayne, J. C. Venter, and et al. 1997. Genomic sequence of a Lyme disease spirochaete, *Borrelia burgdorferi*. *Nature 390:580*.
65. Peterson, J. D., L. A. Umayam, T. Dickinson, E. K. Hickey, and O. White. 2001. The Comprehensive Microbial Resource. *Nucleic Acids Res. 29:123*.
66. Hefty, P. S., S. E. Jolliff, M. J. Caimano, S. K. Wikel, and D. R. Akins. 2002. Changes in temporal and spatial patterns of outer surface lipoprotein expression generate population heterogeneity and antigenic diversity in the Lyme disease spirochete, *Borrelia burgdorferi*. *Infect. Immun. 70:3468*.
67. Pugsley, A. P. 1993. The complete general secretory pathway in gram-negative bacteria. *Microbiol. Rev. 57:50*.
68. Zhao, Z., H. Chang, R. P. Trevino, K. Whren, J. Bhawan, and M. S. Klempner. 2003. Selective up-regulation of matrix metalloproteinase-9 expression in human *erythema migrans* skin lesions of acute lyme disease. *J. Infect. Dis. 188:1098*.
69. Gebbia, J. A., J. L. Coleman, and J. L. Benach. 2001. Borrelia spirochetes upregulate release and activation of matrix metalloproteinase gelatinase B (MMP-9) and collagenase 1 (MMP-1) in human cells. *Infect. Immun. 69:456*.
70. Coleman, J. L., and J. L. Benach. 2000. The generation of enzymatically active plasmin on the surface of spirochetes. *Methods 21:133*.

71. Coleman, J. L., E. J. Roemer, and J. L. Benach. 1999. Plasmin-coated *Borrelia burgdorferi* degrades soluble and insoluble components of the mammalian extracellular matrix. *Infect. Immun. 67:3929*.
72. Coleman, J. L., T. J. Sellati, J. E. Testa, R. R. Kew, M. B. Furie, and J. L. Benach. 1995. *Borrelia burgdorferi* binds plasminogen, resulting in enhanced penetration of endothelial monolayers. *Infect. Immun. 63:2478*.
73. Fuchs, H., R. Wallich, M. M. Simon, and M. D. Kramer. 1994. The outer surface protein A of the spirochete *Borrelia burgdorferi* is a plasmin(ogen) receptor. *Proc. Natl. Acad. Sci. U. S. A. 91:12594*.
74. Hu, L. T., G. Perides, R. Noring, and M. S. Klempner. 1995. Binding of human plasminogen to *Borrelia burgdorferi*. *Infect. Immun. 63:3491*.
75. Hu, L. T., M. A. Eskildsen, C. Masgala, A. C. Steere, E. C. Arner, M. A. Pratta, A. J. Grodzinsky, A. Loening, and G. Perides. 2001. Host metalloproteinases in Lyme arthritis. *Arthritis Rheum. 44:1401*.
76. Embers, M. E., R. Ramamoorthy, and M. T. Philipp. 2004. Survival strategies of *Borrelia burgdorferi*, the etiologic agent of Lyme disease. *Microbes Infect. 6:312*.
77. Robertson, J. N., J. S. Gray, S. MacDonald, and H. Johnson. 1998. Seroprevalence of *Borrelia burgdorferi* sensu lato infection in blood donors and park rangers in relation to local habitat. *Zentralbl. Bakteriol. 288:293*.
78. Carlsson, S. A., H. Granlund, D. Nyman, and P. Wahlberg. 1998. IgG seroprevalence of Lyme borreliosis in the population of the Åland Islands in Finland. *Scand. J. Infect. Dis. 30:501*.
79. McKisic, M. D., and S. W. Barthold. 2000. T-cell-independent responses to *Borrelia burgdorferi* are critical for protective immunity and resolution of lyme disease. *Infect. Immun. 68:5190*.
80. McKisic, M. D., W. L. Redmond, and S. W. Barthold. 2000. Cutting edge: T cell-mediated pathology in murine Lyme borreliosis. *J. Immunol. 164:6096*.
81. Breitner-Ruddock, S., R. Würzner, J. Schulze, and V. Brade. 1997. Heterogeneity in the complement-dependent bacteriolysis within the species of *Borrelia burgdorferi*. *Med. Microb. Immunol. 185:253*.
82. Patarakul, K., M. F. Cole, and C. A. Hughes. 1999. Complement resistance in *Borrelia burgdorferi* strain 297: outer membrane proteins prevent MAC formation at lysis susceptible sites. *Microbial Pathogen. 27:25*.
83. Pausa, M., V. Pellis, M. Cinco, P. G. Giulianini, G. Presani, S. Perticarari, R. Murgia, and F. Tedesco. 2003. Serum-Resistant

Strains of *Borrelia burgdorferi* Evade Complement-Mediated Killing by Expressing a CD59-Like Complement Inhibitory Molecule. *J. Immunol. 170:3214*.

84. Rosa, P. 2003. Molecular Genetic Basis of the Infectious Cycle of the Lyme Disease Spirochete *Borrelia burgdorferi*, Vol. 2004. NIAID, Atlanta, GA, p. Informative website on *B. burgdorferi* genetics.
85. Koistinen, V., S. Wessberg, and J. Leikola. 1989. Common binding region of complement factors B, H and CR1 on C3b revealed by monoclonal anti-C3d. *Compl. Inflamm. 6:270*.
86. Pangburn, M. K., and H. J. Muller-Eberhard. 1978. Complement C3 convertase: cell surface restriction of beta1H control and generation of restriction on neuraminidase-treated cells. *Proc. Natl. Acad. Sci. U. S. A. 75:2416*.
87. Hellwage, J., S. Kuhn, and P. F. Zipfel. 1997. The human complement regulatory factor-H-like protein 1, which represents a truncated form of factor H, displays cell-attachment activity. *Biochem. J. 326:321*.
88. Kuhn, S., C. Skerka, and P. F. Zipfel. 1995. Mapping of the complement regulatory domains in the human factor H-like protein 1 and in factor H. *J. Immunol. 155:5663*.
89. Prodinger, W. M., J. Hellwage, M. Spruth, M. P. Dierich, and P. F. Zipfel. 1998. The C-terminus of factor H: monoclonal antibodies inhibit heparin binding and identify epitopes common to factor H and factor H-related proteins. *Biochem. J. 331:41*.
90. Hellwage, J., T. S. Jokiranta, V. Koistinen, O. Vaarala, S. Meri, and P. F. Zipfel. 1999. Functional properties of complement factor H-related proteins FHR-3 and FHR-4: binding to the C3d region of C3b and differential regulation by heparin. *FEBS Lett. 462:345*.
91. Jokiranta, S., J. Hellwage, V. Koistinen, P. F. Zipfel, and S. Meri. 2000. Each of the three binding sites on complement factor H interacts with a distinct site on C3b. *J. Biol. Chem. 275:27657*.
92. Kuhn, S., and P. F. Zipfel. 1995. The baculovirus expression vector pBSV-8His directs secretion of histidine-tagged proteins. *Gene 162:225*.
93. Meri, S., and M. K. Pangburn. 1990. Discrimination between activators and nonactivators of the alternative pathway of complement: regulation via a sialic acid/polyanion binding site on factor H. *Proc. Natl. Acad. Sci. U. S. A. 87:3982*.
94. Koistinen, V. 1991. Effect of complement-protein-C3b density on the binding of complement factor H to surface-bound C3b. *Biochem. J. 280:255*.

95. Salacinski, P. R., C. McLean, J. E. Sykes, V. V. Clement-Jones, and P. J. Lowry. 1981. Iodination of proteins, glycoproteins, and peptides using a solid-phase oxidizing agent, 1,3,4,6-tetrachloro-3 alpha,6 alpha-diphenyl glycoluril (Iodogen). *Analytical Biochem. 117:136*.

96. Junttila, J., M. Peltomaa, H. Soini, M. Marjamaki, and M. K. Viljanen. 1999. Prevalence of *Borrelia burgdorferi* in *Ixodes ricinus* ticks in urban recreational areas of Helsinki. *J. Clin. Microbiol. 37:1361*.

97. Schmidt, B., R. R. Muellegger, C. Stockenhuber, H. P. Soyer, S. Hoedl, A. Luger, and H. Kerl. 1996. Detection of *Borrelia burgdorferi*-specific DNA in urine specimens from patients with *erythema migrans* before and after antibiotic therapy. *J. Clin. Microbiol. 34:1359*.

98. Viljanen, M. K., J. Oksi, P. Salomaa, M. Skurnik, R. Peltonen, and H. Kalimo. 1992. Cultivation of *Borrelia burgdorferi* from the blood and a subcutaneous lesion of a patient with relapsing febrile nodular nonsuppurative panniculitis [letter]. *J. Inf. Dis. 165:596*.

99. Wahlberg, P., H. Granlund, D. Nyman, J. Panelius, and I. Seppala. 1993. Late Lyme borreliosis: epidemiology, diagnosis and clinical features. *Ann. Med. 25:349*.

100. Heimer, G. V., and C. E. Taylor. 1974. Improved mountant for immunofluorescence preparations. *J. Clin. Pathol. 27:254*.

101. Hefty, P. S., S. E. Jolliff, M. J. Caimano, S. K. Wikel, J. D. Radolf, and D. R. Akins. 2001. Regulation of OspE-related, OspF-related, and Elp lipoproteins of *Borrelia burgdorferi* strain 297 by mammalian host-specific signals. *Infect. Immun. 69:3618*.

102. Soini, H., E. C. Bottger, and M. K. Viljanen. 1994. Identification of mycobacteria by PCR-based sequence determination of the 32-kilodalton protein gene. *J. Clin. Microbiol. 32:2944*.

103. Akins, D. R., K. W. Bourell, M. J. Caimano, M. V. Norgard, and J. D. Radolf. 1998. A new animal model for studying Lyme disease spirochetes in a mammalian host-adapted state. *J. Clin. Invest. 101:2240*.

104. Akins, D. R., M. J. Caimano, X. Yang, F. Cerna, M. V. Norgard, and J. D. Radolf. 1999. Molecular and evolutionary analysis of *Borrelia burgdorferi* 297 circular plasmid-encoded lipoproteins with OspE- and OspF-like leader peptides. *Infect. Immun. 67:1526*.

105. Akins, D. R., B. K. Purcell, M. M. Mitra, M. V. Norgard, and J. D. Radolf. 1993. Lipid modification of the 17-kilodalton membrane immunogen of *Treponema pallidum* determines macrophage activation as well as amphiphilicity. *Infect. Immun. 61:1202*.

106. Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL_X windows interface: flexible

strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Ac. Res. 25:4876*.

107. Felsenstein, J. 1995. Phylip - Phylogeny inference package (Version 3.57c). Distributed by the author, Seattle, p. Phylogeny software.
108. Entrez Proteins. National Center for Biotechnology Information, National Library on Medicine, Bethesda, MD, p. Protein sequences database containing GenPept.
109. Casjens, S., N. Palmer, R. van Vugt, W. M. Huang, B. Stevenson, P. Rosa, R. Lathigra, G. Sutton, J. Peterson, R. J. Dodson, D. Haft, E. Hickey, M. Gwinn, O. White, and C. M. Fraser. 2000. A bacterial genome in flux: the twelve linear and nine circular extrachromosomal DNAs in an infectious isolate of the Lyme disease spirochete *Borrelia burgdorferi*. *Mol. Microbiol. 35:490*.
110. Lam, T. T., T. P. Nguyen, E. Fikrig, and R. A. Flavell. 1994. A chromosomal *Borrelia burgdorferi* gene encodes a 22-kilodalton lipoprotein, P22, that is serologically recognized in Lyme disease. *J. Clin. Microbiol. 32:876*.
111. Kaelin, W. G., Jr., D. C. Pallas, J. A. DeCaprio, F. J. Kaye, and D. M. Livingston. 1991. Identification of cellular proteins that can interact specifically with the T/E1A-binding region of the retinoblastoma gene product. *Cell 64:521*.
112. Jeffery, L. 2003. GoCore: Better prediction of function from sequence, Helsinki.
113. Jokiranta, T. S., P. F. Zipfel, J. Hakulinen, S. Kuhn, M. K. Pangburn, J. D. Tamerius, and S. Meri. 1996. Analysis of the recognition mechanism of the alternative pathway of complement by monoclonal anti-factor H antibodies: evidence for multiple interactions between H and surface bound C3b. *FEBS Lett. 393:297*.
114. Barbour, A. G. 1984. Isolation and cultivation of Lyme disease spirochetes. *Yale J. Biol. Med. 57:521*.
115. Stevenson, B., N. El-Hage, M. A. Hines, J. C. Miller, and K. Babb. 2002. Differential binding of host complement inhibitor factor H by *Borrelia burgdorferi* Erp surface proteins: a possible mechanism underlying the expansive host range of Lyme disease spirochetes. *Infect. Immun. 70:491*.
116. Kurtenbach, K., S. De Michelis, S. Etti, S. M. Schafer, H. S. Sewell, V. Brade, and P. Kraiczy. 2002. Host association of *Borrelia burgdorferi* sensu lato--the key role of host complement. *Trends Microbiol. 10:74*.
117. Steere, A. C., S. E. Malawista, D. R. Snydman, R. E. Shope, W. A. Andiman, M. R. Ross, and F. M. Steele. 1977. Lyme arthritis: an

epidemic of oligoarticular arthritis in children and adults in three connecticut communities. *Arthritis Rheum. 20:7*.

118. Burgdorfer, W., A. G. Barbour, S. F. Hayes, J. L. Benach, E. Grunwaldt, and J. P. Davis. 1982. Lyme disease-a tick-borne spirochetosis? *Science 216:1317*.
119. van Dam, A. P., H. Kuiper, K. Vos, A. Widjojokusumo, B. M. de Jongh, L. Spanjaard, A. C. Ramselaar, M. D. Kramer, and J. Dankert. 1993. Different genospecies of *Borrelia burgdorferi* are associated with distinct clinical manifestations of Lyme borreliosis. *Clin. Inf. Dis. 17:708*.
120. Sung, S. Y., C. P. Lavoie, J. A. Carlyon, and R. T. Marconi. 1998. Genetic divergence and evolutionary instability in ospE-related members of the upstream homology box gene family in *Borrelia burgdorferi* sensu lato complex isolates. *Infect. Immun. 66:4656*.
121. Alberts, B., D. Bray, J. Lewis, M. Raff, K. Roberts, and J. D. Watson. 1989. *Molecular Biology of the Cell*. Garland Publishing Inc., New York.
122. Assous, M. V., D. Postic, G. Paul, P. Nevot, and G. Baranton. 1993. Western blot analysis of sera from Lyme borreliosis patients according to the genomic species of the *Borrelia* strains used as antigens. *Eur. J. Clin. Microb. Inf. Dis. 12:261*.
123. Eggers, C. H., B. J. Kimmel, J. L. Bono, A. F. Elias, P. Rosa, and D. S. Samuels. 2001. Transduction by phiBB-1, a bacteriophage of *Borrelia burgdorferi*. *J. Bacteriol. 183:4771*.
124. Lindsberg, P. J., J. Ohman, T. Lehto, M. L. Karjalainen-Lindsberg, A. Paetau, T. Wuorimaa, O. Carpen, M. Kaste, and S. Meri. 1996. Complement activation in the central nervous system following blood-brain barrier damage in man. *Ann. Neurol. 40:587*.
125. Kraiczy, P., C. Skerka, V. Brade, and P. F. Zipfel. 2001. Further characterization of complement regulator-acquiring surface proteins of *Borrelia burgdorferi*. *Infect. Immun. 69:7800*.
126. Kraiczy, P., J. Hellwage, C. Skerka, H. Becker, M. Kirschfink, M. M. Simon, V. Brade, P. F. Zipfel, and R. Wallich. 2004. Complement resistance of *Borrelia burgdorferi* correlates with the expression of BbCRASP-1, a novel linear plasmid-encoded surface protein that interacts with human factor H and FHL-1 and is unrelated to Erp proteins. *J. Biol. Chem. 279:2421*.
127. Miller, J. C., K. von Lackum, K. Babb, J. D. McAlister, and B. Stevenson. 2003. Temporal analysis of *Borrelia burgdorferi* Erp protein expression throughout the mammal-tick infectious cycle. *Infect. Immun. 71:6943*.

128. Hakulinen, J. 2003. Complement-mediated killing of microtumors. In *Department of Bacteriology and Immunology*. University of Helsinki, Helsinki, p. 74.
129. Lin, B., J. M. Kidder, R. Noring, A. C. Steere, M. S. Klempner, and L. T. Hu. 2001. Differences in synovial fluid levels of matrix metalloproteinases suggest separate mechanisms of pathogenesis in lyme arthritis before and after antibiotic treatment. *J. Inf. Dis. 184:174*.
130. Kramer, M. D., R. Wallich, and M. M. Simon. 1996. The outer surface protein A (OspA) of *Borrelia burgdorferi*: a vaccine candidate and bioactive mediator. *Infection 24:190*.
131. Wilske, B., V. Preac-Mursic, S. Jauris, A. Hofmann, I. Pradel, E. Soutschek, E. Schwab, G. Will, and G. Wanner. 1993. Immunological and molecular polymorphisms of OspC, an immunodominant major outer surface protein of *Borrelia burgdorferi*. *Infect. Immun. 61:2182*.
132. Rose, C. D., P. T. Fawcett, and K. M. Gibney. 2001. Arthritis following recombinant outer surface protein A vaccination for Lyme disease. *J. Rheumatol. 28:2555*.
133. Willett, T. A., A. L. Meyer, E. L. Brown, and B. T. Huber. 2004. An effective second-generation outer surface protein A-derived Lyme vaccine that eliminates a potentially autoreactive T cell epitope. *Proc. Natl. Acad. Sci. U. S. A. 101:1303*.
134. Wahlberg, P. 1999. Vaccination against Lyme borreliosis. *Ann. Med. 31:233*.
135. Scheiblhofer, S., R. Weiss, H. Durnberger, S. Mostbock, M. Breitenbach, I. Livey, and J. Thalhamer. 2003. A DNA vaccine encoding the outer surface protein C from *Borrelia burgdorferi* is able to induce protective immune responses. *Microbes Infect. 5:939*.
136. Horstmann, R. D., H. J. Sievertsen, J. Knobloch, and V. A. Fischetti. 1988. Antiphagocytic activity of streptococcal M protein: selective binding of complement control protein factor H. *Proc. Natl. Acad. Sci. U. S. A. 85:1657*.