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Interactions of Soluble Complement Regulators with Pathogenic Bacteria

Hanna Jarva

Department of Bacteriology and Immunology

Haartman Institute

University of Helsinki

Finland

Academic dissertation

To be publicly discussed, with permission of the Medical Faculty of the University of Helsinki, in the small auditorium of the Haartman Institute, Haartmaninkatu 3, on Saturday, June 5th, 2004, at 12 o'clock noon

SUPERVISOR

Seppo Meri
MD, PhD, Professor
Haartman Institute
Department of Bacteriology and Immunology
University of Helsinki, Finland

REVIEWERS

Markku Heikinheimo
MD, PhD, Professor
Hospital for Children and Adolescents, and
Program for Developmental and Reproductive Biology
Biomedicum Helsinki
University of Helsinki, Finland

and

Markku Viander
MD, PhD, Docent
Department of Medical Microbiology
University of Turku, Finland

OPPONENT

Sir Peter Lachmann
FRS, FMedSci, Professor
Microbial Immunology Group
Centre for Veterinary Science
Cambridge, UK

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My mind and my body may grow weak,
but God is my strength;
he is all I ever need

How wonderful to be near God,
to find protection with the Sovereign Lord!

Psalm 73: 26, 28

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Abstract

The complement (C) system is part of the innate immune system in the first line of defense. It consists of over 30 soluble or membrane-bound components. The complement system can become activated through three different (the lectin, the classical and the alternative) pathways, and the activation is tightly regulated. The main functions of complement are to defend us against invaders, e. g. microbes, and to remove immune complexes, cell debris and apoptotic cells from the body. In the antimicrobial defense, C activation may lead to opsonization or lysis of the target, enhanced inflammatory reaction and recruitment of inflammatory cells.

Despite the antimicrobial activities of complement, pathogenic microbes have developed mechanisms whereby they can evade C attack. How do they do this? This question has been addressed in this work by analyzing the C evasion mechanisms of the important pathogens *Streptococcus pneumoniae* (pneumococcus), *Streptococcus agalactiae* (group B streptococci, GBS) and *Neisseria meningitidis* (meningococcus). The focus was on factors that regulate the activities of complement C3 convertases on the surface of bacteria. C3 convertases are central elements in C activation. Thus, their inhibition is a key feature that makes these bacteria virulent.

C-reactive protein (CRP) was first identified by its ability to bind to the C-polysaccharide of pneumococci. It is the prototype acute phase protein, whose level increases during infection and tissue damage. In the present study it was found that CRP binds factor H, a fluid-phase inhibitor of the alternative pathway C3 convertase (I). CRP binds to two sites in the middle region of factor H, thereby leaving the functional activity of factor H intact. The results suggest that CRP can target factor H to areas of tissue damage to suppress unnecessary inflammatory reaction (I, II). On the other hand, CRP-mediated binding of factor H could help pneumococci to evade C attack.

Pneumococci cause respiratory infections, meningitis and sepsis. GBS cause neonatal sepsis and skin infections in adults. Some strains of pneumococci and GBS express surface proteins that bind factor H. These proteins, pneumococcal Hic and B streptococcal β protein, were both found to bind to

two similar sites in the middle region of factor H (II, III). The middle part of factor H, by binding to CRP, pneumococci and GBS, thus turned out to be a new functional region on factor H (I, II, III). Previously, there were no known ligands for this area of factor H. Pneumococci and GBS, via Hic and β, can directly acquire factor H to their surfaces. The results showed that surface-bound factor H remained functionally active and inhibited the alternative pathway C3 convertase. Hic and β protein, although from two different bacterial species, showed remarkable structural similarity and their binding sites on factor H overlapped. This suggests that the expression of these proteins is an analogous virulence mechanism that has been conserved through evolution of these two bacterial species, pneumococci and group B streptococci.

Serogroup B meningococci are gram-negative bacteria that cause meningitis and sepsis. They were found to regulate the classical pathway C3 convertase activity by binding another soluble C3 convertase regulator, the C4b-binding protein (C4bp) (IV). Binding was dependent on the expression of the PorA surface protein. By binding C4bp the meningococci were able to promote inactivation of C4b and inhibit the classical C pathway. Thus, while pneumococci and GBS evade the alternative pathway of C by binding factor H, meningococci protect themselves against C attack by acquiring the classical pathway inhibitor C4bp to their surface. Since C4bp is usually in tight complex with the anticoagulant protein S, meningococci may also indirectly influence blood coagulation via C4bp binding.

In summary, in this study the mechanisms whereby three important pathogenic bacteria evade C3 convertase activity and complement attack have been analyzed. Pneumococci, GBS and meningococci are pathogens that cause pneumonia, meningitis and sepsis and are responsible for significant morbidity and mortality worldwide. They express specific proteins with which they bind host complement regulators to their surfaces. These proteins (Hic, β and PorA) apparently are important virulence factors and could be exploited in the development of vaccines. An ideal vaccine could raise antibodies that not only bind to the microbes but also neutralize their complement evasion molecules.

Publications

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

I H. Jarva, T.S. Jokiranta, J. Hellwage, P.F. Zipfel and S. Meri. 1999. Regulation of complement activation by C-reactive protein: targeting the complement inhibitory activity of factor H by an interaction with short consensus repeat domains 7 and 8-11. *J. Immunol*. 163: 3957-62.

II H. Jarva, R. Janulczyk, J. Hellwage, P.F. Zipfel, L. Björck 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*. 168:1886-94.

III H. Jarva, J. Hellwage, T.S. Jokiranta, M.J. Lehtinen, P.F. Zipfel and S. Meri. 2004. The group B streptococcal β and pneumococcal Hic proteins are structurally related immune evasion molecules that bind the complement inhibitor factor H in an analogous fashion. *J. Immunol*. 172: 3111-8.

IV H. Jarva, S. Ram, U. Vogel, A.M. Blom and S. Meri. 2004. Binding of the complement inhibitor C4bp to serogroup B *Neisseria meningitidis*. *Submitted.*

Abbreviations

AP	Alternative pathway of complement
BSA	Bovine serum albumin
C	Complement
C1-INH	C1-inhibitor
C4bp	C4b-binding protein
CCP	Complement control protein unit
CP	Classical pathway of complement
CR1	Complement receptor 1
CR3	Complement receptor 3
CRP	C-reactive protein
DAF	Decay-accelerating factor
ELISA	Enzyme-linked immunoadsorbent assay
FACS	Fluorescence-activated cell sorter
Fc	Crystallizable part of immunoglobulins
FH	Factor H
FHL-1	Factor H-like protein 1
FHR	Factor H-related protein
GAS	Group A streptococcus, *S. pyogenes*
GBS	Group B streptococcus, *S. agalactiae*
GPI	Glycosyl phosphatidyl inositol
Ig	Immunoglobulin
IL	Interleukin
Hic	Factor H-binding inhibitor of complement
kDa	kilodalton
LNnT	Lacto-N-neotetraose
LOS	Lipo-oligosaccharide
MAC	Membrane attack complex
MASP	MBL-associated serine protease
MBL	Mannose binding lectin
MCP	Membrane cofactor protein
OMV	Outer membrane vesicle
Opa	Opacity protein
PorA	Porin A
PorB	Porin B
Psp	Pneumococcal surface protein
PTX3	Pentraxin 3
SAP	Serum amyloid P component
SCR	Short consensus repeat
SDS-PAGE	Sodium-dodecyl sulphate polyacrylamide gel electrophoresis
TNF	Tumor necrosis factor
VR	Variable region

Introduction

The complement (C) system is an essential part of the innate immune system in the first line of defense. Its functions include opsonization and lysis of microbes, attraction and activation of leukocytes and enhancement of the inflammatory response. In addition to acting independently, it also is an important effector arm for the adaptive immune system. With all its activities, complement also serves as a clean-up or waste-disposal system in the removal of immune complexes, chromatin, apoptotic cells and cell debris.

Microbes have survived on earth for over 3 billion years. Since the emergence of animal hosts, the microbes have had a long time to develop ways to evade the immune defense mechanisms of the potential hosts. Pathogenic bacteria have also developed specific mechanisms to evade complement. Although bacteria and the C system have been known for over a hundred years, it is only relatively recently that the roles of C evasion mechanisms in bacterial virulence have been addressed.

Some microbes are disguised with a capsule or lipopolysaccharide structures resembling host surfaces, and some express proteases that cleave C components. For many bacteria the C evasion mechanisms are still unknown.

The purpose of this study was to examine the complement evasion mechanisms of three pathogenic bacterial species, *Streptococcus pneumoniae*, *Streptococcus agalactiae* and *Neisseria meningitidis*. These bacteria cause some of the most severe infections like pneumonia, sepsis and meningitis. Complement evasion mechanisms affect directly the virulence of the microbe. Thus, by identifying factors whereby a particular microbe evades complement, it is possible to understand better how the microbe causes disease and how this could be prevented.

Literature review

The complement system

The complement system (C) is part of the innate immune system and consists of over 30 soluble or membrane-bound proteins (Table 1). Complement acts in the defense against microbes and other invaders and, on the other hand, in the removal of immune complexes and apoptotic or damaged cells, i.e. in the clean-up of the body.

Complement is activated through three different cascade-like pathways, (i) the classical pathway (CP), (ii) the alternative pathway (AP) and (iii) the lectin pathway. All three pathways converge at the C3 level and activation continues through the terminal pathway.

The classical pathway

When antibodies bind to their target with a sufficient density they can initiate activation of the CP. The first component in the CP, C1q is a complex 460 kDa molecule consisting of six subunits, each of which is comprised of 3 structurally related polypeptide chains (A, B and C) (53). C1q circulates in complex with two C1r and two C1s molecules ($C1qr_2s_2$) (193). The globular heads of the six subunits bind to the Fc part of IgG or IgM. Other activators of CP include C-reactive protein (CRP), nucleic acids and damaged cell membranes (61, 159, 227, 325). The binding of C1q to multiple IgG molecules or to surface-bound IgM results in a conformational change in C1q. This leads to proteolytic activation of C1r, which then, also proteolytically, activates C1s. C1s is the active enzyme in the $C1qr_2s_2$ complex, which cleaves C4 and C2 (Fig. 1).

C4 consists of three chains which are held together by disulphide bonds (280). C1s in the activated C1 complex cleaves C4 at a single site in the α-chain. This leads to the release of a small C4a fragment and the disruption of an internal thioester in the C4b polypeptide. Subsequently, C4b can bind covalently to amino or hydroxyl groups on nearby surfaces (78, 187). However, the exposed thioester site is labile and quickly inactivated by hydrolysis if no target is available. Thus, C4b can only be deposited in the

immediate vicinity of the C1 complex. Membrane-bound C4b is a receptor for C2, a single-chain plasma molecule. C2 is bound to C4b and cleaved by C1s to C2a, which remains bound to C4b, and to a smaller fragment C2b which is released from the complex (222, 223). C4b2a constitutes the CP C3 convertase (63).

The next component in the cascade is C3, which is composed of two chains, α and β, held together by disulphide bonds (221, 296). C3 is the most abundant C protein in plasma. Its concentration is 1-2 mg/ml and half of it is turned over daily. C3 is cleaved by C2a of the C4b2a complex at a single site on the α-chain. This cleavage leads to the release of the C3a fragment and the exposure of a thioester on the remaining C3b fragment. Upon disruption of the thioester, C3b can bind covalently to a target surface. When C3b binds to the C4b2a complex, the CP C5 convertase C3b4b2a is formed (168, 299).

Figure 1. Complement activation pathways. The regulators of complement are boxed. Enzymatic activity is indicated by broken arrows. Abbreviations: C1-INH, C1-inhibitor; MBL, mannose binding lectin; DAF, decay accelerating factor (CD55); C4bp, C4b binding protein; P, properdin; CR1, complement receptor 1 (CD35); FH, factor H; I, factor I; D, factor D; B, factor B; MCP, membrane cofactor protein (CD46); MAC, membrane attack complex; SC5b-9, soluble terminal complement complex.

Table 1. Components of the complement activation pathways.

Component	MW (kDa)	Serum conc. (µg/ml)	Function
Classical pathway			
C1q	460	80	Binds to IgG and IgM, initiates CP activation
C1r	83	50	Cleaves and activates C1s
C1s	83	50	Cleaves and activates C4 and C2
C4	205	600	C4b binds C2 and forms CP C3 convertase with C2a
C2	102	20	C2a forms CP C3 convertase with C4b, cleaves and activates C3 and C5
Alternative pathway			
Factor B	93	210	Bb forms AP C3 convertase with C3b, cleaves and activates C3 and C5
Factor D	24	2	Cleaves and activates factor B
Lectin pathway			
MBL	96 x 2-6	0-5	Binds to carbohydrates on microbial surfaces, initiates the lectin pathway
MASP-1	83		Cleaves and activates C3
MASP-2	76		Cleaves and activates C4 and C2
Common			
C3	185	1300	C3b forms the AP C3 convertase with Bb, forms the CP and AP C5 convertases with C4b2a and C3bBb
Terminal pathway			
C5	190	70	C5b is part of MAC C5a is chemotactic and anaphylatoxic
C6	120	65	Part of MAC
C7	110	55	Part of MAC
C8	150	55	Part of MAC
C9	69	60	Part of MAC

Abbreviations: MW, molecular weight; kDa, kilodalton; conc., concentration; CP, classical pathway; AP, alternative pathway; MBL, mannose binding lectin; MASP, mannose binding lectin-associated serine protease; MAC, membrane attack complex.

The alternative pathway

C3 is a central component also in AP activation. C3 is constantly hydrolyzed in the fluid-phase at a slow rate to form C3(H_2O) (181). C3(H_2O) binds factor B, a single-chain AP analogue of C2. Factor B, once bound to C3(H_2O) is cleaved by factor D (189, 190). Factor D is a serine protease, which cleaves factor B to a larger Bb fragment and a smaller Ba fragment that is released into the fluid phase. The formed C3(H_2O)Bb complex is a fluid phase C3 convertase, where the serine protease Bb cleaves C3 to C3b which can deposit on nearby surfaces. This constant low-level deposition of C3b on surfaces is called the tick-over phenomenon of the AP (181, 231). Subsequently, factor B binds to the deposited C3b on the surfaces. After cleavage by factor D, the resulting AP C3/C5 convertase C3bBb cleaves further C3 molecules.

The activation of AP augments also activation initiated by CP. This occurs after CP activation leads to the deposition of C3b on surfaces. C3b molecules are capable of binding factor B, which is then susceptible to cleavage by factor D. Thus, CP activation is amplified by the activation of AP. Therefore, the C3b-Bb-factor D circle is called the amplification loop. Even when activation is initiated through CP, the AP amplification loop needs to be recruited to ensure efficient C activation.

The lectin pathway

Mannose binding lectin (MBL) is a large molecule resembling C1q in structure. Its serum levels (≈ 1 µg/ml), however, are only 1/100th of those of C1q (108). MBL binds to polysaccharides rich in mannose and N-acetylglucosamine residues, which are present on some microbial cells. MBL is associated with two serine proteases, MBL-associated serine protease –1 and –2 (MASP-1 and -2) (275, 303). The surface-bound MBL-MASP-complex cleaves C4 and C2 and the activation of C continues as described above for CP.

The terminal pathway

C5 is a two-chain plasma protein which is cleaved by the C3b4b2a complex to a larger C5b fragment and and a smaller C5a, a powerful chemotactic and

anaphylatoxic agent (234, 324, 332). Analogously, C5 can be cleaved by the AP C5 convertase complex, C3bBbC3b. Regardless of whether C5 becomes cleaved by the CP or AP C5 convertase, the cleavage results in the exposure of a C6-binding site on C5b and initiation of membrane attack complex (MAC) formation.

C5b-6 complexes bind C7, the next C component. The binding of C7 results in a conformational change in the C5b67 complex, which is capable of inserting into nearby cell membranes (77, 183). The C8 component binds C7 in the complex, which then becomes more deeply buried in the target cell membrane (300).

C9 binds to the C5b-8 complex. This results in a conformational change in C9, which will enable it to traverse the membrane. At the same time, binding sites for additional C9 molecules are exposed on the C5b-9 complex. As more C9 molecules bind to the C5b-9 complex, a pore causing membrane leakiness is formed (311, 312). The MAC is the endpoint of C activation. It can cause osmotic lysis of the target cell or lead to various other effects, like cell activation because of calcium influx.

Regulators of complement

As complement can cause significant tissue damage, its activation must be kept in tight control. Both soluble and membrane-bound regulators keep C activation under control and protect self cells from unwanted consequences of C activation (Table 2).

Soluble regulators

C1-inhibitor

C1-inhibitor (C1-INH) is a soluble regulator of the CP. It is a single-chain, 105 kDa glycoprotein that belongs to the family of serine protease inhibitors (serpins), which share structural and functional properties (55, 128). The target specificities of these inhibitors are determined by amino acids at and close to the reactive center, which binds to the target proteases, i.e. C1r and C1s in the case of C1-INH. C1-INH is cleaved by the protease activities of C1r and C1s but remains tightly bound to the complex, thereby rendering the complex inactive (Fig. 1, Table 2).

C1-INH is also a biologically significant inhibitor of kallikrein and coagulation factor XII (71, 112). In addition, C1-INH inhibits plasmin and coagulation factor XIa (101, 127). C1-INH can also inhibit the MBL-associated serine proteases MASP-1 and MASP-2 (200). The inherited deficiency of functional C1-INH causes hereditary angioedema, a rare disease characterized by transient, recurrent attacks of cutaneous and mucosal edema. Even though the disease usually has a mild course, it is potentially life-threatening in case of laryngeal edema and consequent suffocation.

Table 2. Soluble and membrane-bound regulators of complement

Component	MW (kDa)	Serum conc. (μg/ml)	Function
Soluble regulators			
C1-INH	105	200	Binds and inactivates C1r and C1s
C4bp	540-590	250	Accelerates decay and inhibits formation of CP C3 convertase, cofactor for factor I
Factor H	150	500	Accelerates decay and inhibits formation of AP C3 convertase, cofactor for factor I
Factor I	90	35	Cleaves and inactivates C3b and C4b in the presence of a cofactor
Properdin	220	26	Stabilizes AP C3-convertase
S protein	80	500	Prevents MAC formation
Clusterin	70-80	50	Prevents MAC formation
Membrane-bound regulators			
DAF (CD55)	70		Accelerates decay of AP and CP C3- and C5-convertases
MCP (CD46)	51-68		Cofactor for factor I
CR1 (CD35)	190-220		Accelerates decay of AP and CP C3- and C5-convertases, cofactor for factor I
Protectin (CD59)	18-23		Prevents MAC formation

Abbreviations: MW, molecular weight; kDa, kilodalton; conc., concentration; C1-INH, C1-inhibitor; C4bp, C4b-binding protein; CP, classical pathway; AP, alternative pathway; MAC, membrane attack complex.

C4b-binding protein

C4b-binding protein (C4bp) is a 540-590 kDa multi-chain regulator of the CP. C4bp usually consists of 7 α-chains (70 kDa) and one β-chain (45 kDa) (59, 140, 277). The chains are linked together by a central core (Fig. 2). The α-chains are composed of 8 and β-chains of 3 short consensus repeat (SCR; also called complement control protein repeat, CCP) units. Each SCR has appr. 60 amino acids held together in a domain structure by two internal disulphide bridges (149, 169).

C4bp regulates the activation of CP by preventing the assembly of the CP C3-convertase C4b2a and accelerating the decay of this complex by promoting dissociation of C2a from C4b (decay acceleration activity). It also acts as a cofactor for factor I in the cleavage of C4b (cofactor activity) (Fig. 1, Table 2) (104, 111, 224). The C4b binding sites on C4bp have been located to the N-terminal SCRs 1-3 of the α-chains (Fig. 2) (37). C4bp also binds heparin with a relatively high affinity, and the heparin binding sites have been mapped to the same SCRs 1-3 of the α-chains (37, 138). The C4bp-C4b interaction can be inhibited by heparin (138). Serum amyloid P component (SAP) binds to the central core (282). Some reports state that this interaction inhibits the C regulatory activities of C4bp, while other investigators have found no interference (107, 282).

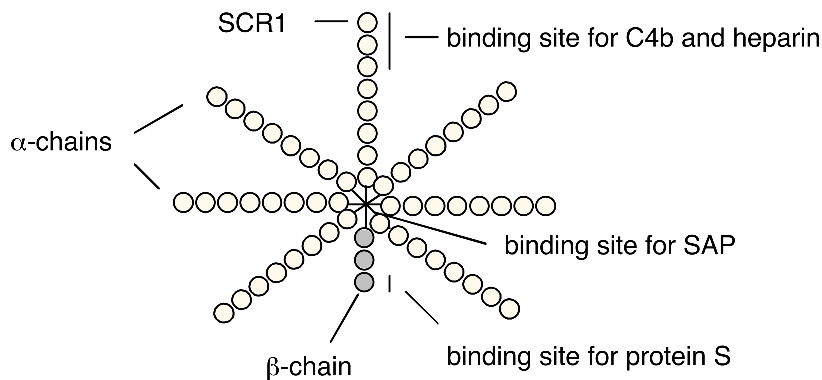

Figure 2. Schematic structure of C4bp. The binding sites (SCR1-3) for C4b and heparin are marked on one α-chain. SAP, serum amyloid P component; SCR, short consensus repeat.

In circulation, 50% of C4bp is in complex with protein S, an anticoagulant protein. In regulating the coagulation cascade, activated protein C requires

protein S as a cofactor for its anticoagulant functions (330, 331). Protein S binds very strongly to CCP1 of the C4bp β-chain (131, 132). C4bp-bound protein S is functionally inactive (66). Appr. 70% of protein S is in complex with C4bp and only 30% remains free (and active) (67-69). On the other hand, protein S does not interfere with the C regulatory functions of C4bp.

Factor H

Factor H (FH) is a fluid-phase regulator of the AP amplification loop. It is a soluble 150 kDa protein composed of 20 SCR domains (Fig. 3) (233, 359). FH regulates the AP by inhibiting the binding of factor B to C3b, acting as a cofactor for factor I-mediated cleavage of C3b (cofactor activity) and accelerating the decay of the AP convertase C3bBb (decay-accelerating activity) (243, 335, 343). All these steps are essential in keeping the AP amplification loop under control. By controlling the key steps of the amplification loop FH inhibits also activation that has been initiated via the classical or the lectin pathway.

The cofactor and decay-accelerating activities of FH have been located to the SCR domains 1-4 (117, 178, 179). FH has three binding sites for C3b, the first at the N-terminal SCRs 1-4, the second in the middle part of FH at SCRs 12-14 and the third at the most C-terminal SCRs 19-20 (160, 162, 284). FH has also three binding sites for heparin, at SCR7, around SCR13 and at SCR20 (Fig.3) (34, 35, 211, 241). These heparin binding sites correlate with the binding of FH to polyanions, like glycosaminoglycans and sialic acids.

In addition to controlling the AP activation in the fluid phase, FH has an important function in discrimination between AP activating and nonactivating surfaces. As C3 undergoes spontaneously low level hydrolysis to produce C3(H_2O) that can bind factor B (tick-over), the AP is in a continuous state of alertness to react with target structures (181). Upon contact with a surface the default for AP is to become activated, unless inhibited. As a consequence, C3b molecules get constantly deposited on nearby surfaces. Surfaces of intact human cells ("nonactivators") are abundant in terminal sialic acids and glycosaminoglycans. As FH has a high affinity for C3b when the surface around C3b is coated with these polyanions, AP activation is kept under control (91, 166, 211, 242).

However, if the surface is devoid of polyanions, the affinity of FH for surface-associated C3b is reduced and C activation proceeds.

Following tissue damage because e.g. of infection, inflammation or ischemia, the structural integrity of cell membranes breaks down and different types of structures like certain phospholipids, cytoskeletal components and nuclear chromatin become exposed. Many of these structures activate the CP. As the emerging structures may lead to unrestricted activation of C, there is a need to suppress excessive AP amplification. At the same time, however, the clearance of nonviable structures should occur in a well-regulated and focused manner. At present, we only partially understand how this important process is orchestrated.

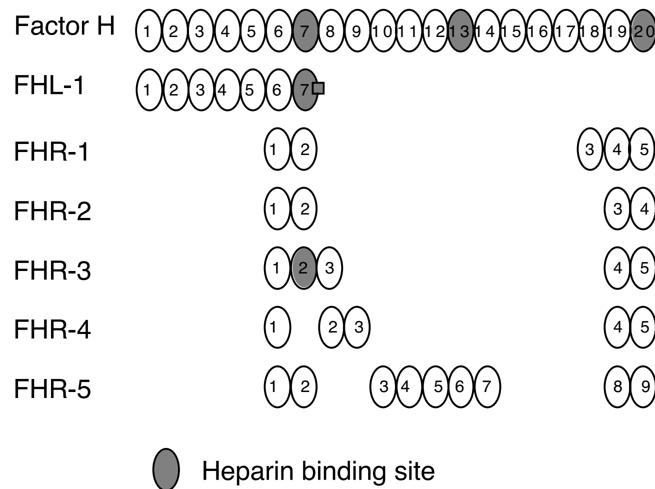

Figure 3. Factor H protein family. All members consist exclusively of SCR domains. The individual domains are aligned according to the highest homology. Heparin binding sites are marked on FH, FHL-1 and FHR-3. FHR-1 and -5 also bind heparin but the heparin-binding domain has not been established yet. FHL-1, factor H-like protein 1; FHR, factor H-related protein.

Factor H protein family

Factor H is a member of a family of proteins that also contains factor H-like protein 1 (FHL-1) and factor H-related proteins 1-5 (FHR-1-5) (Fig. 3) (205, 359). FHL-1 is an alternatively spliced product of the FH gene. FHL-1 consists of the seven most N-terminal SCR-domains of FH and four unique amino acids at the C-terminal end (360). The plasma concentration of FHL-1

is only 10-50 μg/ml, i.e. about 2-10 % of that of FH. FHL-1 has decay-accelerating and cofactor activities, which suggests that FHL-1 has C regulatory activity *in vivo* (178, 179). FHRs are encoded for by separate genes located in the "regulators of complement activation" (RCA) gene cluster on chromosome 1, band 1q32. To date, 5 FHRs have been identified. All of these are exclusively composed of SCR domains and bear striking structural similarity to each other and FH (359). Despite the fact that FHRs all are known to bind C3d part of C3b with their two most C-terminal domains, their functions are still unknown (134, 135).

Factor I

Factor I is a soluble 90 kDa protein that consists of two chains, the heavy (50 kDa) and the light (38 kDa) chain (182, 270). Factor I cleaves C3b and C4b in the presence of a cofactor. The cofactor can be either C4bp, factor H, membrane cofactor protein (MCP) or complement receptor type 1 (CR1). The function of factor I is essential in the regulation of C activation. The deficiency of factor I is very rare, only a few dozen cases have been described in the literature (328). Analysis of patients with factor I deficiency has provided important insight into the activities of the AP (2, 358). Factor I deficiency leads to a secondary deficiency of C3 and susceptiblity to recurrent pyogenic infections.

Other soluble regulators

Properdin is the only known physiological positive regulator of C. Properdin stabilizes the C3bBb complex of the AP. Binding of properdin to the complex extends its half-life 3-4 –fold (93). Properdin circulates in plasma as oligomers of 2-4 53 kDa polypeptides (286).

Two soluble inhibitors of the terminal C pathway have been described. Clusterin (SP40,40, apolipoprotein J) is a multifunctional 70-80 kDa glycoprotein, which consists of disulphide-linked α- and β-chains (156). Clusterin was first recognized by its ability to aggregate Sertoli cells (36). Later, it was recognized as a C inhibitor. It binds to the terminal C5b67 and C5b678 complexes and prevents their insertion into cell membranes (310). S protein (vitronectin, cell-spreading factor) is an 80 kDa protein composed of two disulphide-linked chains (249). One form of S protein mediates cell attachment in tissues (19). In the C system, soluble S protein binds to the

fluid-phase C5b67 complex, and keeps it in the fluid phase similarly as clusterin (249).

Membrane-bound regulators

To date, four membrane-bound regulators of C have been identified in man (Table 2). Three of these inhibit the C3 and C5 convertases and one (protectin) inhibits formation of the MAC.

Membrane cofactor protein (MCP, CD46)

Membrane cofactor protein is a 51-68 kDa integral membrane glycoprotein. It is present on nearly all cell types except erythrocytes. MCP contains 4 SCR domains, a transmembrane region and a cytoplasmic tail (194). MCP binds to C3b and C4b and acts as a cofactor for factor I-mediated cleavage of both proteins (283).

Decay accelerating factor (DAF, CD55)

Decay accelerating factor is a 70 kDa membrane glycoprotein. It consists of 4 SCR domains and is anchored to the cell-membrane via a glycophosphoinositol (GPI) anchor (207, 208). DAF binds to and dissociates both the CP (C4b2a) and AP (C3bBb) C3/C5 convertases (230).

Complement receptor 1 (CR1, CD35)

Complement receptor 1 is a receptor for C3b and C4b (92). It is a long membrane glycoprotein that exists in two major allelic froms of 190 and 220 kDa. CR1 consists of 30 SCR units and has three binding sites for C3b and/or C4b (172, 173). Multiple binding sites allow multivalent interactions with immune complexes containing several C3b and C4b molecules. CR1 acts as a cofactor for the factor I-mediated cleavage of C3b and C4b (148, 206). It also accelerates the decay of the AP and CP C3/C5 convertases, C3bBb and C4b2a, respectively.

CD59 (protectin)

Protectin is another GPI-anchored C regulator (293). It is a glycosylated membrane protein with an apparent mass of 18-23 kDa. Protectin is present on practically all cell surfaces. It inhibits the assembly of MAC by binding to

terminal C complex-associated C8 and C9 and preventing C9 incorporation and polymerization (210, 263). Protectin can become released from the cell surface. When detached from its lipid anchor by a phospholipase, protectin is converted into a soluble form (209).

Functions of complement

Complement has functions both in the defense against microbes and, on the other hand, in the clean-up of tissues. Complement activation leads to deposition of C4b and C3b and their inactivation products on the target surface. Phagocytes have receptors for C3b and C4b (CR1) and iC3b (CR3 and CR4) (94, 269). The recognition of these opsonins by the phagocytic cells enhances phagocytosis. The soluble cleavage products C3a and C5a released during C activation are powerful chemotactic agents and anaphylatoxins (332). Thus, an inflammatory response is generated at the site of C activation. This also promotes removal of the intruding microbes. Terminal pathway activation leads to the formation of MAC on the target surface. This is important in the defense against gram-negative microbes. In gram-positive organisms the peptidoglycan effectively inhibits the formation of MAC. The formation of MAC is especially important in the killing of *Neisseriae*. This is underlined by the fact that individuals deficient in the late components of C are susceptible to recurrent neisserial infections.

Recently, it was found that C3d acts as a powerful natural adjuvant in the antigen presentation to B cells (73). Antigens become coated with C3b which is eventually cleaved into iC3b and C3d. Follicular dendritic cells and B cells have receptors for C3b and C3d (CR1/CD35 and CR2/CD21). A contact between the follicular dendritic cell-bound antigen-C3d complex and the B cell coreceptor complex of CD21/CD19/CD81 leads to an enhanced B cell response (232). Without the presence of C components, the antibody response and the development of memory cells would be less efficient.

A very important function of complement is the solubilization and transport of immune complexes (278). The CP gets activated on immune complexes and this leads to the deposition of C4b and C3b on the complexes. The binding of C proteins inhibits the formation of large complexes and also makes them more soluble. C4b and C3b are recognized and bound by CR1

on erythrocytes, and the complexes get transported to the liver and spleen for disposal.

Complement also has a role in the clearance of apoptotic cells. C1q binds directly to surface blebs of apoptotic cells and CP gets activated until the C3b level (176, 227, 228). C3b is inactivated to iC3b, which is bound by the CR3 (CD11b/CD18) receptor on macrophages. The apoptotic cell becomes phagocytosed. As C activation does not proceed to the C5 level, C5a, the powerful chemotaxin and anaphylatoxin, is not released. Thus, the inflammatory reaction remains controlled. Recent results also suggest a role for C in the development of B cell tolerance against autoantigens. C4 and the CR1 and CR2 receptors are thought to be involved in the negative selection of self-reactive B cells (250).

Pentraxins – complement-interacting proteins

The complement system interacts with several plasma proteins, e.g. the pentraxins. Pentraxins are a family of proteins with a characteristic pentameric organization of identical subunits (110). The classical pentraxins are C-reactive protein (CRP) and serum amyloid P component (SAP). CRP consists of 5 monomers in a β-jelly roll topology while SAP has 10 monomers arranged in two layers (110). SAP has 60% amino acid homology with CRP (175). The genes for both SAP and CRP are located in chromosome 1 (288). CRP and SAP have been found in all mammalian species studied so far and also in many invertebrates. Their structures have remained conserved during evolution and no deficiencies are known (248). In humans, monkeys, dogs and rabbits CRP is an acute phase protein whereas SAP is constitutively expressed. In contrast, in mice SAP is an acute phase reactant and CRP is constitutively expressed (175, 248).

In recent years, new, so-called long pentraxins have been identified. The best known of these is pentraxin 3 (PTX3) (43, 188). Long pentraxins are structurally related to the short pentraxins SAP and CRP. PTX3 has a C-terminal pentraxin-like domain and an N-terminal portion which is not related to CRP or SAP (43). Common functional features of all three pentraxins include the binding of C1q and the ability to activate the CP (61,

225, 353). It has also been postulated that pentraxins have a role in the removal of apoptotic cells (87, 109, 226).

C-reactive protein (CRP)

C-reactive protein is the prototype acute phase protein. Its serum level is usually below 1 µg/ml but during inflammation or extensive tissue damage it may rise up to 500 µg/ml within 24 hours due to increased synthesis in liver (60, 248). CRP production is stimulated by IL-1 and IL-6 and quickly subsides once the triggering factor has been eliminated (21). Measurement of CRP is widely used in clinical practice to assess the severity and extent of infection, inflammation and tissue damage (105, 154).

CRP was initially identified and named by its capacity to bind to and precipitate the C-polysaccharide of *Streptococcus pneumoniae* (pneumococcus) (1, 304). Later, the binding ligand on the pneumococcal C-polysaccharide was found to be phosphorylcholine (326). Several other ligands for CRP have been recognized during the last decades. The binding of CRP to its ligands is dependent on the presence of calcium (1, 175). CRP has two calcium-binding sites on each of its monomers. CRP has been shown to bind to e.g. chromatin, DNA, nucleosomes, histones, cell debris and FcγRII receptors on macrophages (30, 80, 260, 261, 289, 351).

One of the first CRP ligands identified was phosphorylcholine present on damaged or apoptotic cell membranes (261, 327). This ligand is normally not accessible on the cell surface but becomes exposed after tissue injury. CRP binds C1q and activates CP (61, 325, 344). However, C activation by CRP does not lead to the formation of MAC. Instead C activation seems to be stopped at the C3b level but the mechanism of this has not been known (24).

Despite the fact that CRP-measurement is one of the most common laboratory tests in medicine, the physiological function of CRP has remained unknown. CRP binds to pneumococci and some other microbes, e.g. *Haemophilus influenzae* and *Leishmania donovani* (65, 337). In animal experiments CRP protects mice from pneumococcal infection (220, 295, 355). However, this can hardly be the main physiological role of CRP. A more important function for CRP could be in the removal of apoptotic cells

and cell debris (109). CRP binds to apoptotic cells and to cell material exposed during cellular damage (e.g. chromatin and phospholipids) (261).

Complement and microbes

Microbes in general are susceptible to C attack. However, in order to be pathogenic, an organism must survive in the host and evade the immune system. Thus, many pathogenic microbes have evolved mechanisms to evade C attack (90, 161, 256, 348).

The thick peptidoglycan layer of gram-positive bacteria is in general protective against MAC formation and lysis. Therefore, activation of the terminal pathway has a minor role in the defense against gram-positive bacteria. Also the bacterial capsule and, and especially in gram-negative organisms, the *O*-polysaccharide side chains of the lipopolysaccharides offer steric hindrance against MAC formation.

Some microbes have been found to bind C regulators, e.g. factor H. Microbes do not naturally produce glycosaminoglycans for FH to bind, but they can have e.g. hyaluronic or sialic acid moieties. For example, serotype III group B streptococci, group B meningococci and *Escherichia coli* K1 produce capsules that are composed of polysialic acid (17, 31, 164). Although polymeric sialic acid does not seem to bind FH efficiently, the capsules of these bacteria are thought to mediate C resistance via FH binding. Specific resistance to the AP can also be mediated by surface proteins that bind FH. Once FH is bound to the surface through these molecules, C activation is restricted. E.g. the M-protein of group A streptococcus (GAS, *Streptococcus pyogenes*) and the OspE-protein family of serum-resistant strains of *Borrelia burgdorferi* bind FH from serum (136, 143). The FH-binding capacity is not restricted to bacteria as the yeast *Candida albicans* and the nematode parasite *Onchocerca volvulus* have also been shown to acquire FH to their surfaces (212, 213).

Microbes have also been shown to acquire the CP regulator C4bp to their surfaces. At least group A streptococcal M-protein, *N. gonorrhoeae* and *Bordetella pertussis* bind C4bp (23, 252, 302). In general, the binding of FH

or C4bp may confer serum resistance to bacteria and prevent their opsonophagocytosis.

Also the membrane-bound regulators of C are utilized by several microbes. *Helicobacter pylori* and *E. coli* have been shown to acquire GPI-anchored protectin (CD59) from human cells at the site of infection (255, 257). The hijacked CD59 is incorporated into the bacterial cell membrane in a functionally active form. Thus, the microbes are protected against C lysis. As an example of C inhibitors that microbes produce themselves, trypanosomes are capable of synthesizing a DAF-like inhibitor (T-DAF) of the CP or AP C3/C5 convertases (235).

Some pox- and herpesviruses encode proteins with functional similarities to human C regulators. E.g Herpes simplex –virus HSV-1 encodes the gC-1 protein that is not structurally homologous to human C inhibitors but accelerates the decay of the AP C3 convertase and inhibits the binding of properdin to C3b (103, 177). It thus resembles functionally CR1 and FH.

In addition to acquisition of CD59, *H. pylori* can avoid C activation by expressing the urease enzyme which cleaves C3 (262). Also *Pseudomonas aeruginosa* expresses proteases that cleave C1q and C3 (142). By cleaving the chemotactic and anaphylatoxic C5a, microbes can restrict the developing inflammatory reaction and the recruitment of inflammatory cells to the site of infection. At least GAS, *Entamoeba histolytica*, *Serratia marcescens* and group B streptococci (see below) have been shown to express a C5a peptidase (38, 237, 258, 342).

Only some of the various microbial C evasion mechanisms have been described above. The multiplicity of mechanisms becomes evident even from these examples. However, despite the constantly accumulating knowledge, the complement evasion mechanisms of pathogenic microbes are still only partially known. Although some mechanisms how microbes avoid C activation *in vitro* have been recognized, it is not known whether these mechanisms have any functional significance for the microbial survival in the human host. The C evasion molecules often represent microbial virulence factors. Also, these factors represent putative vaccine targets. Therefore, their recognition is important for attempts to prevent infections.

Streptococcus pneumoniae

Streptococcus pneumoniae is a frequent colonizer of the nasopharynx both in children and in adults. In small amounts, it is considered as part of the normal upper respiratory tract flora. However, pneumococci cause upper and lower respiratory tract infections, as well as invasive infections like sepsis, meningitis and suppurative arthritis. Pneumococcus causes more deaths than any other bacterium, and pneumococcal infections are the fifth leading cause of mortality worldwide (163). The disease burden lies mostly on infants, elderly people over 60 years of age and on the immunocompromised.

Pneumococci are divided into over 90 serotypes according to the structure of their capsular polysaccharide (137). However, only about 20 serotypes are frequently isolated in clinical samples (52, 119). There is considerable variation in virulence among pneumococcal strains even with identical capsular serotypes.

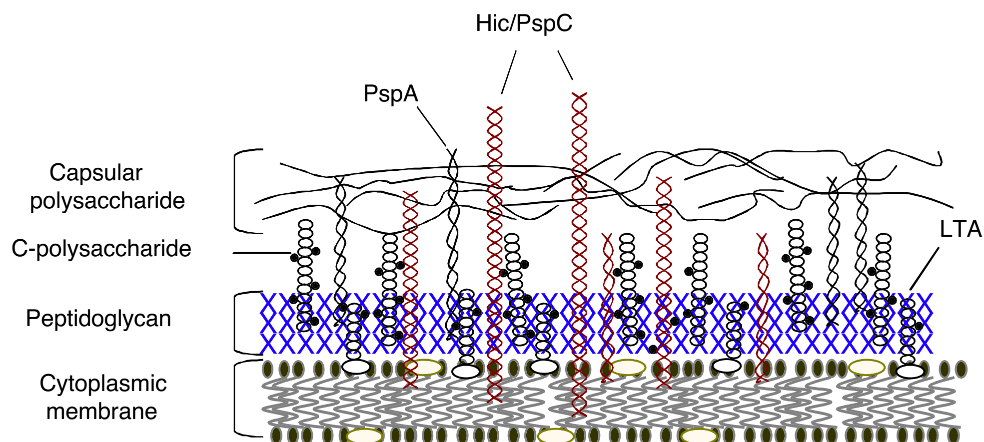

Figure 4. Schematic structure of the pneumococcal cell surface. Hic and several other PspC (pneumococcal surface protein C) family proteins are anchored to the cell wall via an LPXTG motif. PspA and several other choline binding proteins are attached to the choline residues of lipoteichoic acid (LTA) and C-polysaccharide (teichoic acid). The structure of the capsular polysaccharide is the basis of pneumococcal serotype division.

The pneumococcal surface consists of several layers (Fig. 4). Innermost is the cell membrane, followed by a few layers of peptidoglycan and the cell wall polysaccharide (C-polysaccharide or teichoic acid) (305). The polysaccharide capsule of virtually all virulent streptococci has a negative

charge (155, 294). The C-polysaccharide and the lipoteichoic acid, that is attached to the cell membrane, have an unusual structure including choline (48, 307). Several surface proteins of pneumococci are attached noncovalently via a choline binding domain to the choline moiety (124, 264, 356). Some surface proteins are anchored to the cell wall through the common gram-positive bacterial cell wall peptidoglycan attachment motif (LPXTG) (147, 152). Many surface proteins have positively charged regions that interact with the negatively charged capsule (155, 294). The capsule itself is a loose structure and following complement activation C3b becomes deposited both on the capsular surface and on the cell wall (146).

The virulence factors of pneumococci have been extensively studied for decades and several factors have been shown to be involved. The capsule is a prerequisite for virulence. Non-capsulated mutants have low virulence and are quickly cleared from the circulation (333, 334). However, the main role of the capsule in virulence is probably to protect pneumococci against phagocytosis. Other factors significant for virulence or evasion of the host defense include pneumococcal surface proteins A and C (PspA, PspC), pneumococcal surface adhesin A (PsaA), pneumolysin and autolysins (e.g. LytA) (25, 26, 28, 47, 49, 54).

Pneumococcal vaccines

Currently, the focus in pneumococcal vaccines is in capsular conjugate vaccines with a protein carrier. They have proven out to be more immunogenic than the polysaccharide vaccines (180, 246, 347). At present, the first 7-valent conjugate vaccine with diphteria toxin as a carrier is in clinical use in many countries and several conjugate vaccines are in clinical trials (347). Conjugate vaccines are immunogenic in infants as well as in adults but they are more expensive which limits the use in the developing countries. Furthermore, the valency is limited and there have been signs that the serotypes against which the vaccine is not directed become more prevalent (236, 240). Another possibility is to use pneumococcal proteins as vaccines or, more attractively, combinations of proteins and polysaccharides. The latter could confer cross-protection against several serotypes. Pneumococcal proteins that have been or are being tested in animal models or in humans include pneumolysin, PspA, choline binding protein A (CbpA), pneumococcal surface adhesin A (PsaA) and the PhpA protein (44-46, 238, 357). The hope is that antibodies developing against important virulence

factors would neutralize them and promote immune clearance of the pathogenic bacterium.

Complement and pneumococci

Both the classical and alternative pathway of C are important in defense against pneumococcal infections (130, 144, 151). Terminal pathway activation and the formation of the membrane attack complex are ineffective against pneumococci because as gram-positive bacteria pneumococci lack an outer membrane to which the MAC could insert. Individuals deficient in the early components of CP or AP are susceptible to pneumococcal infections, even to recurrent infections (96, 151). Antibody production against pneumococci is compromised in patients lacking C3 (130). IgG2 appears to be the most important antibody subclass in the humoral response to pneumococcus. Patients deficient in IgG2, and IgA-deficient individuals who have concomitantly impaired IgG2 responses, are at an increased risk for pneumococcal infection (185, 272). This is probably based on the ability of IgG2 to preferentially recognize carbohydrates.

The activation of AP is crucial for opsonophagocytosis and the clearance of pneumococci. Tu et al. demonstrated the significance of AP by using mice deficient in factor B, C3 or C5 (313). Natural infection and immunization elicit a capsule-specific IgA-response. In the absence of complement, this specific IgA could induce minimal bacterial uptake and killing by phagocytes. AP activation was required for efficient phagocytosis. Interestingly, polymeric (dimeric) IgA seems to be capable of activating complement on the surface of pneumococci and participate in the opsonophagocytosis (150). It has also been shown that the efficiency of opsonization with C3 fragments depended on intact AP even though it was initiated by the CP (350). In contrast, Brown et al. have demonstrated, using knock-out mice, that both CP and AP are needed for the clearance of pneumococci in mice but the CP is the dominant one (50). Studies in mice have demonstrated that natural IgM-class antibodies play an important role in activating the CP on pneumococci. Thus, it appears that in the defense against pneumococci the CP is important in initiating complement activation, whereas the AP determines the final amount of C3 deposition on the bacteria.

Complement evasion by pneumococci

As pathogens pneumococci need to protect themselves against C attack. The capsule efficiently prevents MAC attack but multiple mechanisms are needed to prevent opsonophagocytosis. In the following, some factors that are presumed to influence C attack on pneumococci are descibed.

Pneumolysin

Pneumolysin is a 53 kDa intracellular protein expressed by all virulent strains of pneumococci (62). It is cytotoxic to several cell types, e.g. erythrocytes, leukocytes, endothelial cells and alveolar epithelial cells (62). Pneumolysin also activates the CP even at sites distant from the organism (247). This may lead to complement depletion and reduced serum opsonic activity. CP activation has been suggested to be mediated through pneumolysin's capacity to bind to the Fc part of IgG (217). Pneumolysin is a virulence factor for pneumococci, as mutant strains not expressing pneumolysin are avirulent (27, 28). However, the virulence differences between strains can not be attributed to differences in pneumolysin production alone (22).

Pneumococcal factors affecting C3b degradation

Early studies have shown that pneumococcal strains with different capsular types differ in the amounts and sites of bound C3b, as well as in the types C3b degradation products generated (146). Cheng et al. showed that CbpA, a choline-binding protein of the PspC family binds directly C3 (58). Later it has been suggested that pneumococci can degrade both α and β chains of C3 in the absence of serum components (8). Hostetter has reported that two pneumococcal proteins appear to have proteolytic activity against C3 (145). Using sequences of these proteins it was recognized that one of them is a fragment of a larger protein, which was cloned and named PhpA (357). A 79 kDa fragment of PhpA was used for immunization in mice and found to be protective against several pneumococcal strains. However, the ultimate function of this protein remains unclear, as it has not yet been confirmed that this fragment of PhpA has proteolytic activity for C3.

Pneumococcal surface protein A

Pneumococcal surface protein A (PspA) is a surface exposed protein that is one of the most extensively studied pneumococcal surface antigens. PspA is expressed by all clinically important pneumococcal serotypes (64). It consists of a highly charged N-terminal α-helical coiled-coil domain, a proline-rich region, a choline-binding domain and a 17 amino acid hydrophobic C-terminal tail (354).

Although the specific mechanism of action of PspA is not known, it is required for full virulence in mouse models of pneumococcal infection (202). It has been suggested that PspA functions as a complement inhibitor. Tu et al. showed that in mice deficient in C3 or factor B, PspA-negative strains became fully virulent (313). In wild type or C5-deficient mice PspA-negative strains were avirulent. PspA reduced the amount of C3b deposited on pneumococci. This suggests that PspA affects the activation of the AP and inhibits the formation and/or function of the AP C3 convertase (313). This in turn would reduce the efficiency of complement receptor-mediated clearance. The precise mechanism how PspA acts in this respect is not yet known. Based on the results of Tu et al. it can not be excluded that PspA acts through binding FH (313). However, Neeleman et al. have shown that pneumococcal binding of FH is not dependent on the expression of PspA, and at least on serotype 3 pneumococci the resistance to phagocytosis is not dependent on PspA expression (229). It has also later been observed both by FACS analysis and by surface plasmon resonance assays that PspA does not bind FH (70, Hellwage et al., unpublished). Thus, the complement inhibitory mechanism of PspA remains open.

Pneumococcal surface protein C

The pneumococcal surface protein C (PspC) -family is a group of relatively polymorphic proteins. Originally, PspC was identified by its similarity to PspA (49, 201). Later, Hammerschmidt et al. found an IgA-binding protein SpsA, and Rosenow et al. isolated a choline-binding protein which they named CbpA (choline-binding protein A) (124, 264). Sequence analyses showed that these proteins are encoded by alleles of the same locus (49). Other allelic forms have been named after their binding characteristics, e.g. C3-binding protein and FH-binding inhibitor of complement (Hic) (58, 152).

The *pspC* gene is present in 75-100% of pneumococcal strains (49, 147). The lower estimate is at least partly due to the fact that the PspC proteins are so polymorphic that they are not always recognized as part of the group. Iannelli et al. sequenced the *pspC* locus of 43 pneumococcal strains (147). According to their results each pneumococcal strain contains a *pspC* gene at the same chromosomal location and each pneumococcal strain has a unique sequence at the *pspC* locus (147). PspC proteins can be divided into 11 groups. Common features for all groups are an N-terminal signal peptide followed by an α–helical region, a proline-rich region and a C-terminal anchor. In groups 1-6, the protein is anchored to the cell wall by a choline binding motif. Group 7-11 PspC proteins are not anchored via choline, instead they have the LPXTG motif, the typical cell wall sorting signal of gram-positive bacteria. The main N-terminal region is predicted to have an α-helical structure and its size varies between 118-589 amino acids (147). Eight structurally conserved domains could be recognized by amino acid sequence comparison. It seems that these eight domains are building blocks for the N-terminal variable region in all PspC-proteins. The proline-rich region is similar within the groups and also resembles the proline-rich region of PspA (147).

PspCs are the only pneumococcal proteins that have been shown to bind FH directly. First, Janulczyk et al. used streptococcal M protein and yersinial YadA protein nucleotide and amino acid sequences to find putative FH-binding proteins in the pneumococcal genome (152). The highest scoring homology was identified to an allele of the *pspC* locus. The protein encoded by this gene was found to be the FH-binding protein on a serotype 3 pneumococcal strain and named as the factor H-binding inhibitor of complement or Hic (152). Structurally, Hic differs from the previously recognized PspC-proteins, which might explain why serotype 3 was originally considered to be PspC-negative. In the new nomenclature suggested by Iannelli Hic belongs to group 11 with two other serotype 3 PspC-proteins (147).

Hic is an ≈ 70 kDa protein that is anchored to the pneumococcal cell wall via the LPXTG motif which resembles the cell wall binding motif of e.g. group A streptococcal M-proteins (152). The FH-binding region on Hic has been mapped to the N-terminal amino acids 39-261 (or 1-223 when the signal peptide is omitted) (Fig. 5). There is no 3-dimensional model of Hic but

computer predictions based on amino acid sequence suggest that the N-terminal signal peptide is followed by an α-helical region, also responsible for FH binding (152). The C-terminal part of Hic has the typical proline-rich repeats of PspC proteins. In the case of Hic, there are 24 repeats of 11 amino acids (152). The function of this region remains is unknown. In the C-terminus, the cell-wall spanning part is followed by the LPSTGS sequence by which Hic is anchored to the bacterial cell wall.

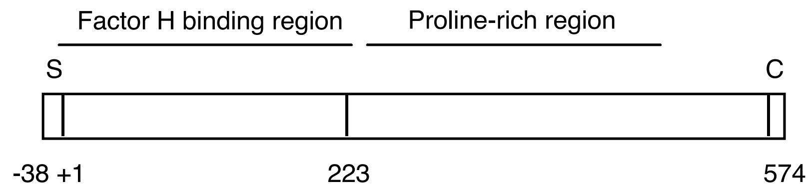

Figure 5. A schematic representation of the Hic protein. Factor H binding region is located at the N-terminal half of the protein and followed by a proline-rich region. S, signal peptide; C, C-terminal region.

The PspC-proteins have also other ligands than factor H. Originally, the PspC protein named SpsA was found to bind secretory IgA (124). The IgA-binding motif is a hexapeptide YRNYPT, present in the N-terminal part of several PspCs (125, 147). As an exception, the motif was absent from PspCs expressed by serotype 3 strains (147). Therefore, it seems that the ability to bind IgA is a widespread and conserved characteristic within the PspC family. Whether this type of IgA binding has anything to do with the ability of pneumococcus-bound polymeric IgA to activate complement, remains to be solved (150).

Streptococcus agalactiae

Group B beta-hemolytic streptococci (GBS, *Streptococcus agalactiae*) are the leading cause of severe neonatal infections. GBS are currently divided into 9 serotypes (Ia, Ib, II-VIII) according to the structure of their capsular polysaccharide. Protective antibodies are directed against the polysaccharide capsule but they are not cross-protective against other serotypes (57).

GBS are part of the normal vaginal flora in 15-35 % of women (14). The neonate usually acquires the infection through perinatal transmission from the mother's genital tract. If the mother has serotype-specific IgG-antibodies, these are transmitted via placenta to the fetus and have been shown to protect the newborn from invasive disease (16). In the neonate the infection usually presents with sepsis, pneumonia or meningitis (14). GBS also cause skin infections, sepsis, meningitis and pneumonia in adults (79, 88).

GBS vaccines

Efficient GBS vaccines are needed to prevent neonatal morbidity and mortality. As the protective capsular polysaccharide antibodies are transmitted through the placenta, vaccination of the mother confers immunity also to the newborn. Levels of maternal antibodies against GBS correlate with the neonate's immunity to infection (16). The first vaccines in clinical trials were composed of capsular polysaccharides. However, polysaccharide vaccines are not immunogenic enough, furthermore, they are not cross-protective. To prevent invasive disease in the Western world, at least a pentavalent (Ia, Ib, II, III and V) polysaccharide vaccine is needed (244). Furthermore, the serotype prevalence varies between regions. Several carrier proteins have been tried, including tetanus toxoid, the β protein and C5a-ase (57, 184, 196, 339). Some of the conjugate vaccines are in clinical trials but none is yet widely available. Also bivalent protein vaccines have been tested in animal models (186).

Complement and GBS

The polysaccharide capsule of GBS prevents opsonophagocytosis. Type III GBS are one of the most common isolates in invasive infections (14, 281). The type III strains expressing an unusually large amount of capsular polysaccharide have been associated with higher invasiveness (199). Already over twenty years ago it was shown that the sialic-acid rich capsule of type III GBS inhibits AP activation in adult sera deficient in type-specific anticapsular antibodies (85). Later, it was observed that the sialic acid moiety of the type III capsular polysaccharide was crucial in inhibiting AP activation (84). Asialylation of the capsular polysaccharide led to the loss of virulence of the mutant strain (340). Therefore, sialic acid in the capsule is a critical factor in the pathogenicity of type III GBS. The sialic acid-rich capsule

prevents C3b deposition, presumably through the action of factor H (199). Inhibition of AP activation can be overcome by specific antibodies, thus resulting in the deposition of C3b on the bacterial surface and opsonophagocytosis by neutrophils (84, 85, 199).

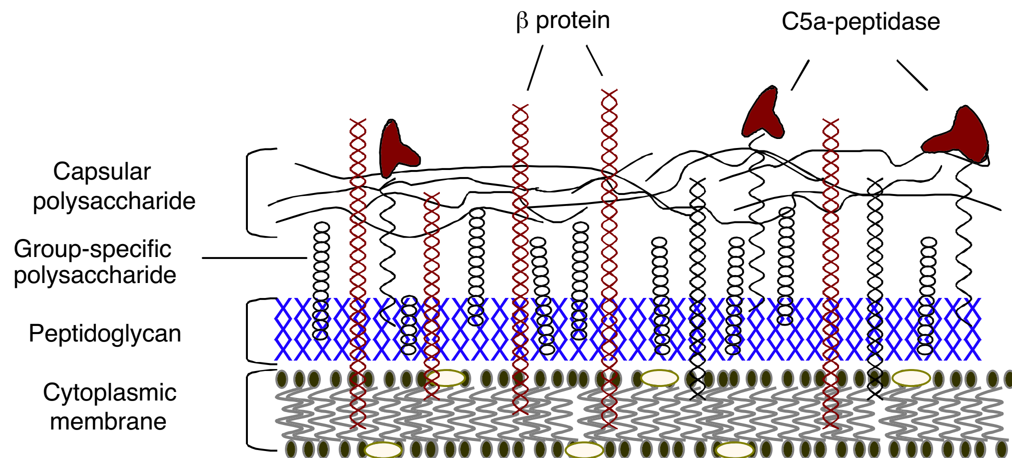

Figure 6. Schematic structure of the group B streptococcal cell surface. The β protein is anchored to the cell wall via an LPXTG motif common to many gram-positive bacterial surface proteins. The group-specific cell wall polysaccharide is attached to the peptidoglycan layer.

While the sialic acid content of the capsule correlates with the inhibition of the AP activation and resistance to phagocytosis, the CP has been implicated in the antibody-independent humoral defense against GBS. Both C4-deficient and C3-deficient mice were susceptible to GBS infection suggesting that the CP also has a role in opsonization even in the absence of specific antibodies (338). Other studies have shown that GBS can directly activate the CP (15, 82). Butko et al. showed that C1q directly binds to GBS through the globular head region and this binding is not dependent on the polysaccharide capsule (51). Binding of C1q by itself was not sufficient to promote opsonophagocytosis but the binding of C1q together with IgG promoted the binding of GBS to phagocytes (51).

Complement evasion by GBS

In addition to the capsule, several strains of GBS have been shown to express other factors useful in avoiding of C activation on or near their surface (Fig. 6).

C5a-ase

The AP and CP C5 convertases cleave C5 into C5b and C5a, which is a powerful chemotactic agent. In 1988 it was shown that a majority of GBS strains were capable of directly inactivating C5a (139). The responsible enzyme was called C5a-ase and it cleaves C5a and $C5a_{desArg}$ molecules between the His and Lys present at amino acid positions 67 and 68 (38). C5a-ase is believed to contribute to the pathogenicity of GBS. However, not all invasive strains express C5a-ase so the production of this enzyme is not necessary for virulence (5, 297). C5a-ase reduces the recruitment of leukocytes to sites of infection and probably also contributes to GBS virulence by preventing C5a from stimulating enhanced killing of the bacteria by phagocytes (39, 139, 298).

Binding of factor H by GBS

Several strains of GBS serotypes Ia, Ib, II and V express the β protein (also called Bac), which was originally identified by its capacity to bind IgA (271). The β protein binds to the Fc part of serum IgA but not to secretory IgA (191). The physiological basis for this is not clear. Recently, the β protein was also shown to bind FH (11). The expression of β elicits the production of protective antibodies (29).

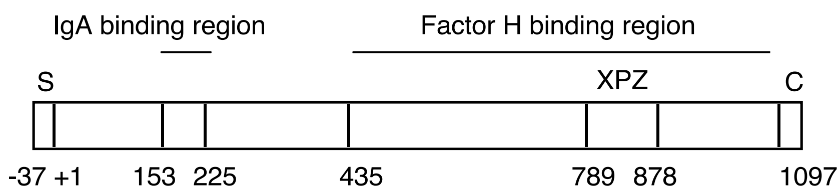

Figure 7. A schematic structure of the GBS β protein. The regions known to be needed for IgA and factor H binding are shown. The numbers indicate amino acids at the start and end of regions. Abbreviations: S, signal peptide; XPZ, the XPZ proline-rich region; C, C-terminal region.

The β protein is a 125 kDa surface protein (271). The N-terminal signal peptide is followed by a region containing the IgA-binding site, consisting of amino acids 153-225 (Fig. 7). The C-terminal half of the β protein contains a region called XPZ, which consists of 30-50 repeats of a unique periodic sequence in which every third amino acid is a proline (133). The size of the β protein varies slightly between strains depending on the number of repeating

XPZ motifs (10). The actual role of the XPZ region is not known. Similarly to pneumococcal Hic and M-proteins of GAS, the C-terminus of β is anchored to the cell wall peptidoglycan via an LPXTG motif (133). Sequence-based analysis of the secondary structure predicts that the β protein is mainly α-helical except in the XPZ region (133). There are no cysteines in the β protein which suggests a fibrillar structure. The β protein thus emerges as an additional filamentous FH-binding protein on the surface of a gram-positive coccus.

Neisseria meningitidis

Neisseria meningitidis (meningococcus) is a gram-negative aerobic diplococcus belonging to the family of *Neisseriaceae*. Human beings are the only natural hosts of meningococci. Meningococci cause sepsis and meningitis, and more rarely, conjunctivitis, sinusitis, arthritis and pneumonia (267). The annual incidence of meningococcal disease in the general population is 1-3 per 100 000 in Western Europe and in the USA (319). In Sub-Saharan Africa, in the so-called meningitis belt, the disease incidence may reach 1 000 per 100 000 (319). Meningococci are spread by direct mucosal contact and via aerosols for a distance of up to 1 meter (319).

Another pathogenic neisserial species is *Neisseria gonorrhoeae* (gonococcus), the causative agent of gonorrhea. As with meningococci, humans are the only natural hosts of gonococci. In addition to gonorrhea, gonococci can cause conjunctivits, pharyngitis, arthritis and, rarely, a systemic disease. Infection is transmitted by direct, usually sexual contact. Neonates can be infected during birth. Most other neisserial species, e.g. *N. lactamica*, *N. sicca*, *N. cinerea* and *N. subflava*, are commensal inhabitants of the nasopharynx and very seldom cause disease.

Polysaccharide capsule

Meningococci are divided into 13 serogroups (A, B, C, E-29, H, I, K, L, M, W135, X, Y, Z) according to the structure of their capsular polysaccharide. Of the 13 serogroups, 5 (A, B, C, W135 and Y) cause over 90% of clinical diseases (267, 319). Of these, serogroups B and C, and increasingly also Y,

are the most important ones in industrialized countries. Serogroups A and C dominate in Asia and Africa (319).

The expression of the polysaccharide capsule is a prerequisite for the survival of meningococci in the circulation. The capsule offers protection against C-mediated lysis and phagocytosis by neutrophils (170). The polysaccharide capsule of serogroup B and C meningococci consists of homopolymers of sialic acid (192). In group B meningococci, the sialic acids are linked in an α-(2→8) manner to the linear polysaccharide (31). Similar α-(2→8) linked oligomers of sialic acid have been identified in the gangliosides of human fetal brain tissue (100). Therefore, serogroup B polysaccharide is poorly immunogenic in humans (349).

The lipo-oligosaccharide of meningococci

The lipo-oligosaccharides (LOS) are major components of the meningococcal outer membrane (Fig. 8). They resemble in structure the lipopolysaccharide (LPS) of *Enterobacteriaceae* but lack the repeating *O*-antigen-bearing polysaccharides and thus present the rough variety (279). The LOS made by different species of *Neisseriae* are structurally similar. The LOS contains a terminal lactosamine structure, lacto-N-neotetraose (LNnT) (157). The α-2,3-sialyltransferase terminally links sialic acid to the LNnT residue of neisserial LOS (113, 197, 198). Serogroup B and C meningococci can sialylate their LOS endogenously, while gonococci require exogenous sialic acid e.g. from blood or genital secretions (9, 198).

Outer membrane proteins

The principal outer membrane proteins of meningococci are divided into 5 classes (class 1-5) according to their apparent molecular weights (309).

Class 1 proteins

Class 1 proteins, together with the class 2/3 proteins, are porins, which are the most abundant proteins present in the outer membrane of meningococci (Fig. 8). Porins do not undergo antigenic shift during infection. However, differences are seen between strains (20, 204). Porins are present in the outer membrane of meningococci as trimers and function as pores (126). They are

considered essential for bacterial survival as they modulate the exchange of ions between the bacterium and the surroundings (126).

Class 1 protein or porin A (PorA) is an approximately 42 kDa product of the *porA* gene (18). PorA has been proposed to consist of 16 transmembrane regions and 8 surface-exposed loops protruding from the bacterial surface (318). The transmembrane areas contain β-sheets which form a typical β-barrel porin structure (74, 318). PorA is highly selective for cations (287, 308). The antigenic diversity among PorA is the basis for serosubtyping of meningococci. However, the sequence variation is mainly confined to two variable regions called VR1 and VR2, and the rest of the protein is highly conserved (203). VR1 is situated on the first surface-exposed loop and VR2 on the fourth loop (318).

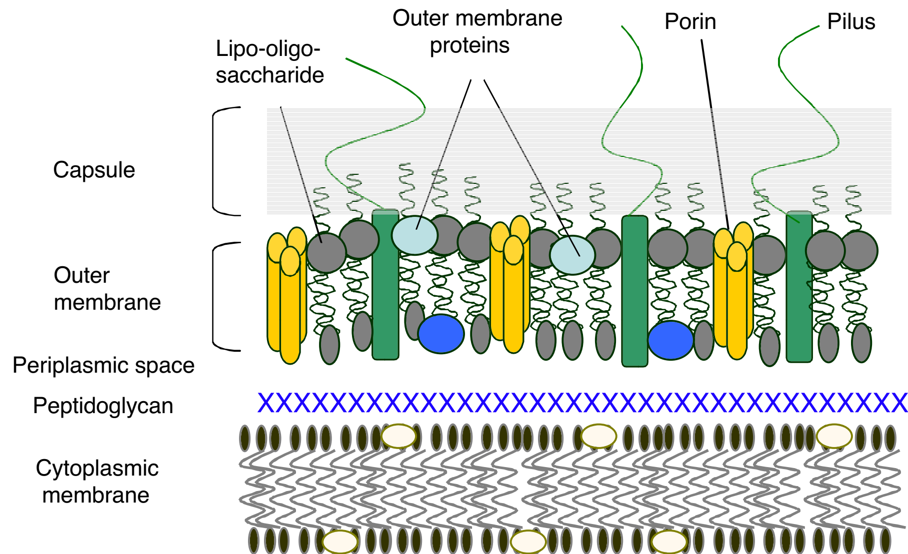

Figure 8. A schematic presentation of the meningococcal surface. Outer membrane proteins are divided in five classes of which porins (class 1-3) are the most abundant proteins.

Meningococcus is the only known neisserial species that expresses PorA. Gonococcus has a *porA* gene, which, however, is not expressed (95). PorA is expressed by most clinical isolates of meningococci but its level of expression varies (314, 315, 317). The variability in expression is due to e.g. point mutations in the coding region or even to the deletion of the gene (314, 315).

Class 2 and 3 proteins

Class 2 and 3 proteins are encoded for by different alleles of the same gene, *porB* (141). Their expression is thus mutually exclusive, i.e. one meningococcal strain expresses either class 2 (PorB2) or class 3 (PorB3) protein. The primary structure similarity between PorB2 and PorB3 is 60-70%. The average molecular weight for PorB2 is appr. 37 kDa and for PorB3 34 kDa. Structurally, PorB consists of a similar β-barrel with transmembrane regions and surface-exposed loops as PorA (215, 216, 318). Interestingly, PorB3 is totally resistant to a number of proteases and therefore it has been suggested that its surface-exposed loops are only minimally exposed (215). PorB2 and PorB3 are homologous to the gonococcal PorB1B and PorB1A, respectively (74). The interstrain antigenic variation of meningococcal PorB is used to define serotypes of meningococci.

Class 4 and 5 proteins

The class 4 protein is also called reduction modifiable protein (Rmp) because its mobility in SDS-PAGE changes after reduction (from 24 to 32 kDa) (174, 195). It is highly conserved (99% sequence identity) in all serogroups of meningococci and homologous to the gonococcal protein III (174). Antibodies formed against the class 4 protein do not seem to be bactericidal or opsonic against meningococci and structural studies indicate that no substantial portion of class 4 protein is exposed on the microbial surface (120, 265).

Class 5 proteins are trimeric in structure and their apparent molecular weight is 27 kDa. They are subdivided into Opc and opacity (Opa) proteins. Both Opa and Opc proteins are hypervariable and undergo antigenic shift even during infection (4, 165, 306, 345). A single strain may express no, one or several class 5 proteins (292). Opa proteins are expressed by both meningococci and gonococci. Instead, Opc proteins are expressed only by meningococci (3). Both Opa and Opc proteins are involved in the attachment of meningococci to epithelial cells in the early stages of disease (320, 321).

Pili

Gonococcal and meningococcal strains of *Neisseriae* express on their surface filamentous structures that are called pili (291). Pili protrude through the

capsule and mediate adhesion of the bacteria to epithelial and endothelial cells (251, 290). MCP (CD46) has been shown to be the cellular receptor for neisserial pili (167). Pili undergo phase variation and there are considerable antigenic intra- and interstrain differences (306). Pili can also undergo sequence variation during infection (273, 306).

Meningococcal vaccines

There is an efficient tetravalent polysaccharide vaccine against meningococcal serogroups A, C, W135 and Y in clinical use. However, the capsular polysaccharide of serogroup B consists of the $\alpha-(2\rightarrow8)$ sialic acid homopolymers, which are identical to the sialic acid on human fetal neural tissue (99, 100). Thus, polysaccharide vaccines against serogroup B meningococci are not immunogenic enough and vaccine research in this field is concentrating on developing a protein vaccine against group B meningococci.

Meningococcal disease

Meningococci frequently colonize the nasopharynx of healthy, asymptomatic persons (7, 56, 116). Up to 20 to 40% of young adults are transient carriers of meningococci (7). Small children are seldom meningococcal carriers. Even though most contacts with meningococci lead only to an asymptomatic carrier state, some people get a systemic, life-threatening infection. It is estimated that only 1 % of people carrying a virulent strain get a symptomatic meningococcal infection (56). The mortality rate of meningococcal meningitis is 1-12% and of meningococcal sepsis 20-40% (266, 319). About 20% of survivors suffer from permanent sequelae, e.g. sensorineural deafness, mental retardation or have to undergo limb amputation because of skin necrosis (83, 319). Half of the deaths occur in the acute phase of the infection, during the first 24 hours after the onset of symptoms (319). Meningococci can sometimes cause a persistent meningococcemia with mild symptoms (319).

During infection meningococci release endotoxin within outer membrane vesicles (OMV; also called blebs). OMVs consist of LOS, outer membrane proteins, lipids and capsular polysaccharides (75). LOS is one of the major factors that induces the host response during systemic infection. Endotoxins induce pro- and anti-inflammatory cytokine production and activate the

complement system, the contact system and the kallikrein-bradykinin system (319). High cytokine concentrations correlate with the severity of the developing shock (329). Both pro-inflammatory (e.g. TNF, IL-1β, IL-6) and anti-inflammatory cytokines (e.g. IL-10, IL-12) are released.

Meningococcal disease is characterized by petechiae, pin-head sized hemorrhages in the skin. In fulminant sepsis, the petechiae may coalesce into large necrotic areas. Microscopically, these lesions consist of endothelial damage, hemorrhages around small vessels and microthrombi in the vessels. There is a major change in the permeability of the vascular endothelium due to the inflammatory process induced by meningococci (171, 239, 245). This leads to loss of fluid in the tissues, diminished venous return and, consequently, the cardiac output falls. The compensatory vasoconstriction is a contributing factor to thrombosis within the microvasculature. The coagulation pathway is activated in meningococcal sepsis and the anticoagulant and fibrinolytic pathways are not functioning properly, for reasons not totally understood (42, 102). The plasma levels of the anticoagulant proteins antithrombin, protein C and protein S are all reduced (89). The dysfunction of the protein C activation pathway seems to be critical in the development of thrombosis in meningococcal sepsis (86, 89). The thrombotic events can ultimately result in tissue necrosis.

Complement and meningococci

The reasons why some individuals acquire a life-theatening meningococcal infection and others remain carriers are still obscure. The difference does not depend solely on the infecting strain, even though some strains are considered to be more virulent than others. The host immune defense system is one of the deciding factors why some individuals get an invasive meningococcal disease while others remain asymptomatic carriers of the pathogen.

The nasopharyngeal carriage of meningococci induces the production of protective antibodies, even with some cross-protection between strains (115, 116, 259). Small children are rarely colonized with meningococci. However, cross-protective antibodies can also be produced after contact with apathogenic neisseriae, common commensal organisms of the oro-

nasopharynx of children, or with antigenically cross-reacting enteric bacteria (114, 118, 164).

The complement system is essential in the defense against meningococci. Individuals deficient in the components of the alternative or terminal C pathway are at an increased risk of acquiring neisserial infections. Unusual meningococcal serogroups are often encountered in C deficient patients (96-98, 268). It is estimated that individuals with deficiencies of late C components have a 1000-10 000 –fold increased risk of meningococcal infection and the recurrency risk is 50-60% (96). However, the mortality rates for individuals deficient in late C components are lower than for the normal population (96, 268). The milder course of disease is possibly due to the fact that C activation could be responsible for many of the symptoms during fulminant meningococcal disease. In contrast, properdin-deficient individuals have a high fatality rate (30-50%) but only a low risk of recurrent infections (96, 285). This difference is probably due to the importance of MAC formation in the clearance of meningococci by the host immune system.

Complement, however, plays a dual role in meningococcal infections. An undisputed fact is that C is important in the defense against meningococci but, on the other hand, C activation is partially responsible for the symptoms during acute meningococcal infections (41, 129, 319). Complement activation results in an increase of the anaphylatoxin C3a and C5a levels, which in concert with activation products of the bradykinin system contribute to the endothelial damage, vasodilatation and capillary leakage. The level of C activation correlates with the severity of shock during meningococcal infection and also with the plasma levels of LOS (41, 129). C activation takes place not only on the meningococcal surface, but also on the released OMVs. Thus, C activation does not effectively lead to bacterial lysis. Lipopolysaccharides (and LOS) are potent activators of the C system (76, 106). However, the meningococcal outer membrane proteins may be even more potent C activators than meningococcal LOS (32). Both AP and CP are involved in C activation (40, 129). However, the relative importance of these pathways is not clear, nor do we understand how meningococci evade C attack and manage to cause disease.

Of the meningococcal C evasion factors, the capsule is thought to be the most important. The capsule offers protection against C-mediated lysis and phagocytosis by neutrophils (170). In group B meningococci, the capsule consists of sialic acid and also LOS is endogenously sialylated (192, 198). In gonococci, the sialylation of LOS correlates with serum resistance (341). In 1987, it was shown that both the sialic acid capsule and the sialylation of LOS in serogroup B meningococci inhibited the activation of AP *in vitro* (153). In 1994, Hammerschmidt et al. demonstrated that only LOS sialylation contributed to the protection of group B meningococci against AP activation (123). By comparing a group B meningococcus wild type strain, a nonencapsulated mutant and a *galE* mutant where LOS is truncated and not sialylated Vogel et al. argued that sialylation of both the capsule and LOS are indispensable for gonococcal serum resistance (323). However, when they used an *lst* mutant, which only lacks the terminal sialic acid of LOS, of the same parental strain, they found that this mutation did not affect the serum resistance of meningococci (322). Thus, in the presence of sialylated capsule, the lack of terminal sialic acid of LOS is not critical but mutation leading to the truncation of LOS (*galE*) is detrimental for the meningococcal virulence. These, somewhat conflicting, data indicate that although important factors have been identified on the surface of meningococci, the mechanisms how they mediate C resistance have remained open.

Aims

The general aim of this thesis work was to analyze factors that inhibit complement by regulating the activities of the complement C3 convertases on the surfaces of important pathogenic bacteria. The focus was on proteins that bind soluble C regulators and help the bacteria to avoid C-mediated opsonophagocytosis or direct lysis.

The specific aims were:

1. to examine the role of CRP as a regulator of complement activation (I, II)
2. to characterize the interactions of the C regulator factor H with the bacterial C evasion molecules: pneumococcal Hic and the group B streptococcal β (II, III)
3. to study the complement resistance mechanisms of *Neisseria meningitidis* (IV)

Materials and Methods

The materials and methods used in this study are listed in Table 3 and are described in more detail in the respective studies. The principal methods are briefly described below.

Table 3. Laboratory methods used in studies I-IV

Methods	**Used in**
Bichinonic acid (BCA) protein concentration determination assay	I, II, III
Cloning and expression of SCR constructs	III
Cofactor assay for C3b and C4b inactivation	I, II, IV
Direct binding of radiolabeled proteins to microbes	II, III, IV
ELISA for C activation and opsonization	II
Flow cytometry analysis of opsonophagocytosis	II
Iodine (^{125}I) labeling of proteins	I, II, III, IV
Microtiter plate binding assay	I, II
Peptide mapping of binding sites	III, IV
Opsonophagocytosis assay	II
Radioligand binding assay	II
Serum bactericidal assay	IV
SDS-PAGE	I, II, III, IV
Surface plasmon resonance analysis	I, II, III

Abbreviations: SCR, short consensus repeat; ELISA, enzyme-linked immunoadsorbent assay; C, complement; SDS-PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis.

Recombinant FH constructs (Fig. 9) were used to localize the binding sites for CRP and microbes on FH. The FH constructs were produced using the baculovirus or the *Pichia pastoris* yeast expression system.

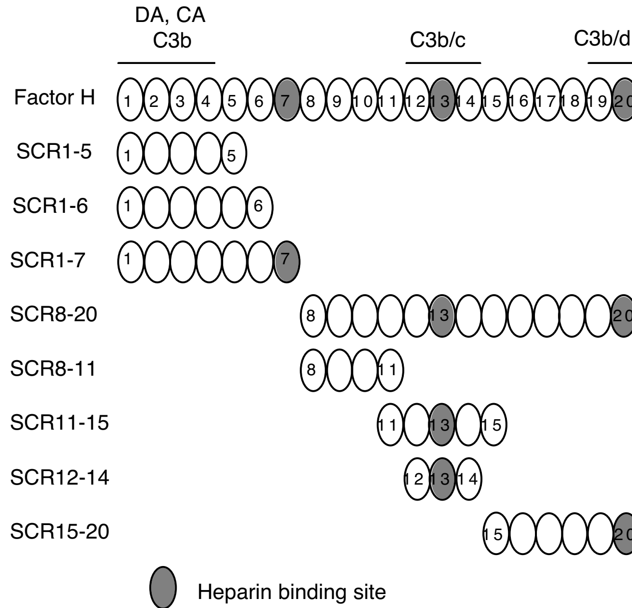

Figure 9. The recombinant constructs of factor H used in studies I-III. The constructs used were produced using the baculovirus expression system (SCR1-5, 1-6, 1-7, 8-20, 8-11 and 15-20) or the Pichia yeast expression system (SCR11-15 and 12-14). All constructs have a poly-Histidine tag. The heparin and C3b binding sites on FH and the constructs are marked. The decay accelerating (DA) and cofactor (CA) activities of FH are located at the N-terminal SCRs 1-4.

The surface plasmon resonance analysis (Biacore®2000 or Biacore®3000 equipment, Biacore AB, Uppsala, Sweden) was one of the main methods used to study direct protein-protein interactions in studies I-III. The appropriate proteins were coupled to the sensor chip flow cells and the putative ligand was injected onto to the flowcell. The binding interaction was monitored real-time and evaluated using the BiaEvaluation 2.0 or 3.0 software. The surface plasmon resonance analysis was used to study CRP – FH, pneumococcal Hic – FH and streptococcal β-protein – FH interactions (I-III). The Biacore® equipment was employed for localization of the binding regions on FH and for studying the effects of e.g. heparin on the binding.

The pneumococcal strains were obtained from Dr. Lars Björck (Lund University, Sweden), the group B streptococcal strains from Prof. Gunnar Lindahl (Lund University) and the meningococcal strains from Dr. Sanjay Ram (Boston University, MA, USA). The strains are shortly described in Table 4 and in more detail in the respective studies (II-IV).

Table 4. Bacterial strains. A more detailed description of the strains used is found in the respective studies.

Strain	Description
Pneumococcal strains (study II)	
HB565	encapsulated, Hic-positive
PR218	nonencapsulated, Hic-positive
FP13	nonencapsulated, Hic-negative
GBS strains (study III)	
A909	type Ia, expressing β
Δ*bac*	β-negative mutant of A909
Δ*bac*/pLZ*bac*	*trans*-complemented strain
BacΔ435-788	deletion mutant of β
BacΔXPZ	deletion mutant of β
BacΔ879-1064	deletion mutant of β
Meningococcal strains (study IV)*	
H44/76	wild type serogroup B, capsule +, LOS sialic acid +
H44/76 *lst*	capsule +, LOS sialic acid -
H44/76 *siaD*	nonencapsulated, LOS sialic acid +
H44/76 *lst/siaD*	nonencapsulated, LOS sialic acid -

*In addition, PorA and PorB negative mutants of the above described four strains were used.
Abbreviations: GBS, group B streptococci; LOS, lipo-oligosaccharide.

A direct binding assay using whole microbes and radiolabeled proteins was used to analyze the binding of C components to microbes (II-IV). Microbes were grown in appropriate broths, washed and the concentrations were adjusted according to the assay used. FH, recombinant constructs of FH, BSA, C4bp or recombinant constructs of C4bp were labeled with ^{125}iodine using the Iodogen method (274). Microbes were incubated with ^{125}I-labeled C components for 30 min at 37°C. After incubation, the microbe-protein suspension was centrifuged through 20% sucrose to separate microbes and microbe-bound proteins (pellet) from unbound protein (supernatant). The sucrose tubes were quickly frozen and cut in two parts and radioactivities in both parts were measured with the γ-counter. Binding was calculated as the

percentage of bound radioactive protein (counts in pellet) vs. total counts (pellet + supernatant). This binding assay was used to study direct binding of FH, C4bp, BSA and recombinant constructs of FH and C4bp to microbes (II-IV). The direct binding assay was also used to study the effects of heparin, CRP, antibodies or unlabeled proteins on the binding of C components.

The C3b or C4b cofactor assays were used to study the effect of CRP on FH function or the functional activity of microbe-bound C regulators FH and C4bp (I-IV). FH acts as a cofactor for factor I-mediated cleavage of C3b, and C4bp is a cofactor for factor I in the cleavage of C4b, and also of C3b (104, 224, 243, 343). The effect of CRP on FH function was studied by incubating FH, factor I and radiolabeled C3b in the presence of varying amounts of CRP (I). After incubation the samples were run in an SDS-PAGE gel under reducing conditions. Cleavage of C3b was detected by autoradiography. To study the functional activity of surface-bound C regulators, microbes were incubated with purified FH or C4bp or heat-inactivated normal human serum (II-IV). After washing the microbes to remove unbound proteins, factor I and C3b or C4b were added and the incubation was continued at 37°C. Cleavage of radiolabeled C3b and C4b was analyzed by SDS-PAGE and autoradiography. If the C regulator (FH or C4bp) was bound to the microbial surface and not removed by washing, C3b or C4b, respectively, should have become cleaved. In controls, the effect of each of the reagents was tested by leaving them out from the incubation mixtures.

Results and Discussion

Regulation of alternative pathway activation by CRP (I)

CRP is an acute phase reactant but its physiological role has remained controversial. Is it required in antimicrobial defense or does it have a homeostatic role during tissue injury and inflammation? CRP binds C1q and activates the CP (325). However, CP activation by CRP does not lead to activation of the terminal pathway (24). Instead, activation leads to the deposition of C3b which is inactivated to iC3b. Previous studies have suggested that CRP inhibits the AP activation, and it has been shown in our laboratory earlier that CRP binds FH (12, 218). However, the functional consequences of this binding had not been analyzed.

By using surface plasmon resonance and microtiter plate analyses it was found that CRP indeed bound FH, and also, vice versa, FH bound to immobilized CRP. With the help of recombinant fragments of FH, two binding sites on FH were recognized: one at SCR7 and a second one within SCRs 8-11. Due to the lability of the SCR11-15 fragment produced in the baculovirus expression system, a third binding site within this region could not be totally excluded. At the time of the experiments we unfortunately did not have access to the Pichia expression system and the more stable middle fragments of FH. The binding reaction between SCR7 of FH and CRP was dose-dependently inhibited by heparin which suggests that the heparin and CRP binding sites on SCR7 at least partially overlap. The binding between SCRs 8-11 and CRP was less affected by heparin. The second heparin binding site on FH is located around SCR13 and its nearness may affect the interaction. CRP did not inhibit the cofactor activity of FH but it had a weak inhibitory effect on the binding of FH to C3b.

These results can explain the previous findings of AP inhibition by CRP. CRP binds to damaged cell membranes and activates complement by binding C1q (325, 327). At the same time, there is a constant deposition of C3b on the target. Factor H affinity for surfaces of injured cells may be diminished by the loss of the integrity of the cell membrane and possible loss of

glycosaminoglycans. Uninhibited C activation causes more inflammation and more tissue damage. This vicious circle can be kept in control if CRP acts as a linking bridge between the injured cell and FH (Fig. 10). Factor H bound to CRP remains functionally active, regulates activation of the amplification loop and C activation is inhibited at the C3b level. This is in accordance with the earlier data that C activation initiated by CRP is stopped at the C3b level (24).

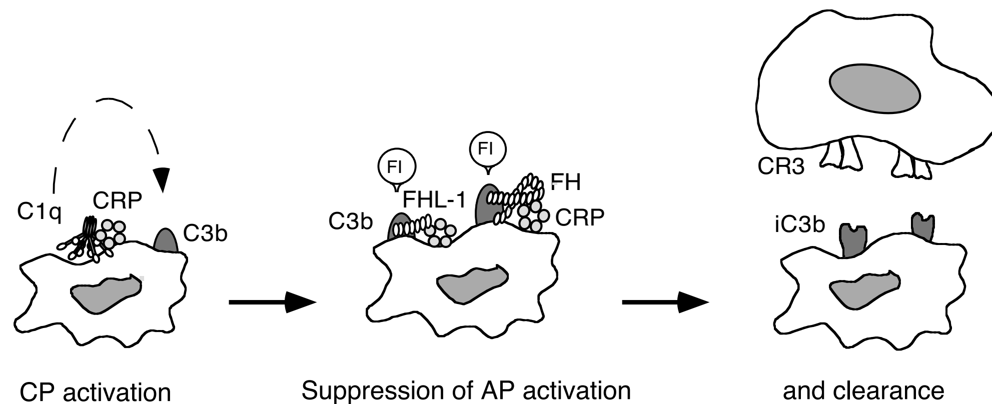

Figure 10. The proposed function of CRP on surface of a damaged cell. Both CRP and C1q are capable of binding directly to damaged cell membranes. CP activation leads to the deposition of C3b on the surface. FH and FHL-1 bind to CRP and the activation of the AP amplification loop is inhibited and C3b is inactivated to iC3b. iC3b is recognized by the CR3 receptor of macrophages and the damaged cell is phagocytosed. Abbreviations: FI, factor I; FH, factor H; FHL-1, factor H-like protein 1; CR3, complement receptor 3; CP, classical pathway; AP, alternative pathway.

CRP and C1q-initiated activation of the CP are presumed to be involved in the removal of apoptotic cells (109, 214). According to our results and previous results by others, the presence of CRP makes it possible for FH to function. CRP binds directly to apoptotic cell membranes and enhances CP activation on the cell surface but the presence of CRP prevents the assembly of MAC (109). This function of CRP required the presence of C1q and FH. When FH is available, C3b becomes inactivated to iC3b which is a ligand for the macrophage CR3 receptor (Fig.10). It has been shown that binding and phagocytosis via macrophage CR3-receptor do not cause leukotriene release or a respiratory burst and can actually downregulate the production of IL-12 and interferon-γ by macrophages (6, 346, 352). Thus, the inflammatory response is kept under control and excessive tissue damage can be prevented.

We thus suggest that the main function of CRP is to focus the clearance activity of CP and to restrict unncecessary tissue damage by directing FH to the site of injury and acting as link between FH and cell surfaces. Thereby, C activation and focused waste clearance would be regulated without excessive inflammation.

Pneumococci, group B streptococci and factor H (II, III)

Pneumococci and GBS are pathogens of world-wide significance. In general, in order for a microbe to be a pathogen, it needs to evade C activation. However, the C evasion mechanisms of both pneumococci and GBS are still incompletely understood. Some pneumococcal proteins have been shown to have an effect on complement activation (PspA and PspC proteins and pneumolysin) (152, 247, 313). The over 90 serotypes and the strain-dependent variation within serotypes make it difficult to generalize findings from one strain to other strains. Furthermore, pneumococci utilize phase variation (336). The expression of several surface antigens depends on the phase, whether colonizing or invasive (264). Many factors have been shown to be important for virulence, and deletion of many of them has rendered the pneumococci avirulent. However, several studies have convincingly shown that the alternative and the classical pathway are of crucial importance in the elimination of pneumococci, and the ability of pneumococci to evade complement attack is probably one of the key reasons behind the pathogenicity of this extremely important group of bacteria (50, 144, 150, 350).

The PspC-protein of serogroup 3 pneumococci (Hic) was shown to bind FH and later Dave et al. showed that FH bound also to serotype 2 PspC but not to PspA (70, 152). In both studies, the PspC-negative pneumococci did not bind FH to their surfaces. Earlier, Neeleman et al. had shown that the inhibition of complement activation and phagocytosis in serotype 3 pneumococci was mediated by binding of FH (229). Binding was inhibited by trypsin pretreatment of the bacteria. Putative FH-binding proteins were identified by immunoblotting experiments. The sizes of these proteins in SDS-PAGE were 88, 150 and 196 kDa. As the sizes of PspC proteins vary between strains, one of these bands could have represented PspC. Neeleman

et al. also studied the FH binding to serotype 3 pneumococci by immunoelectron microscopy (229). They detected two basic modes of FH-binding to the pneumococcal cell wall. FH was seen either directly adjacent to the outer surface of the cell wall or in association with projections extending from the cell wall. The C3b degradation pattern observed by Hostetter et al. on serotype 3 and 4 pneumococci is consistent with the pattern observed when C3b is cleaved by factor I with FH as a cofactor (146). Therefore, this degradation could be explained by FH-binding proteins on the pneumococcal surface.

The interactions between pneumococcal Hic and factor H

The above findings warranted further studies on the interactions of pneumococcal PspC proteins and factor H. We wanted to characterize the Hic-FH binding reaction in more detail and also to study whether this interaction had any significance for the pneumococcal virulence. For the binding assays, we used Hic-positive and -negative pneumococcal strains, recombinant Hic-GST and Hic proteins and recombinant fragments of FH.

Radiolabeled whole FH bound to Hic-positive pneumococci but not to Hic-negative bacteria. However, even a stronger binding was seen using the radiolabeled SCR8-20 fragment, while SCR1-7 did not bind to any of the strains tested. The strong binding seen with SCR8-20 can be explained by the better exposure of the Hic-binding sites on the SCR8-20 fragment than on the whole FH, which has been proposed to have a convoluted structure (13). The binding site on FH was further mapped to SCRs 8-11 and, later, a second binding site was identified on SCRs 12-14. Using a microtiter plate inhibition assay, we had concluded that the SCR8-11 site is the main binding region. However, after obtaining the SCR11-15 and SCR12-14 recombinant fragments of FH we could show with surface plasmon resonance analysis that there is also this second, but a weaker binding site for Hic on FH.

Also CRP binds to the SCR8-11 region but CRP did not affect the binding of SCR8-20 to Hic-positive pneumococci. Interestingly, however, CRP increased the binding of SCR8-20 to Hic-negative pneumococci in the presence of calcium. Thus, it seems that by binding to the pneumococcal C-polysaccharide, CRP can act as a link between PspC-negative pneumococci and FH. In this way, pneumococci could have a double mechanism to evade AP activation – direct binding of FH via the PspC protein and non-direct

binding of FH by using CRP as a linker. It was shown in the early 1980's that CRP increased the interaction of FH and C3b on pneumococcal surface and the AP activation was thus suppressed (218, 219). The fact that phosphorylcholine in pneumococci is usually hidden underneath the capsule means that CRP may bind more avidly to damaged pneumococci than to viable bacteria (72). We also tested the effect of phosphorylcholine on the SCR8-11 – CRP interaction. Phosphorylcholine (10-1000 μg/ml) did not inhibit the binding CRP to SCR8-11 (Jarva et al., unpublished). This suggests that CRP can bind to both pneumococci and factor H at the same time.

Functional activity of pneumococcus-bound factor H

In the C3b cofactor assay, the ability of the Hic-bound FH to act as a cofactor for factor I-mediated cleavage of C3b was studied. Even when the concentration of FH in the reaction mixture was as low as 0.1 μg/ml (compared with the serum level of 500 μg/ml), Hic-positive pneumococci were able to bind enough FH on their surfaces for C3b to become cleaved. The same could be seen using 0.5% serum. The cofactor activity seen after incubation of the bacteria in serum was inhibited by polyclonal anti-FH antibody, suggesting that the cofactor activity seen was explicitly due to FH. Using an ELISA assay, we could confirm that C3b became more efficiently inactivated to iC3b on Hic-positive pneumococci, again indicating the functional activity of FH bound to Hic. The effect of Hic on the ability of pneumococci to evade opsonophagocytosis was studied by FACS and by immunofluorescence microscopy by counting the number of pneumococci phagocytosed by polymorphonuclear leukocytes (Fig. 11). Hic-negative pneumococci were phagocytosed more readily than Hic-positive pneumococci. Thus, we postulate that the Hic-FH interaction is a C evasion and virulence mechanism of pneumococci.

Even though there is considerable strain-dependent variation between PspC-proteins, the initial studies by Janulczyk et al. showed that pneumococcal strains of several serotypes bound FH (152). Also, the results of others showed that at least serotype 2 PspC protein was also capable of binding of FH, and that the binding region on FH is located to SCRs 13-15 (70, 81). We have also screened dozens of clinical strains and found putative FH-binding proteins in most invasive pneumococcal strains (Jarva et al., unpublished).

We can not, of course, exclude that pneumococci express several FH-binding proteins. So far, however, FH-binding has been shown only for PspC.

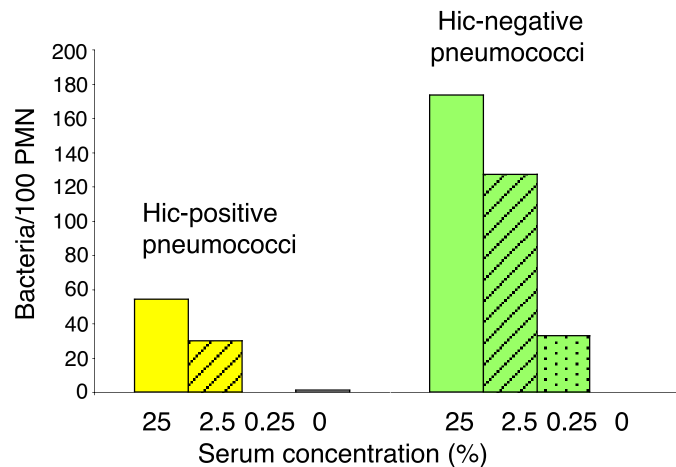

Figure 11. The effect of Hic on the opsonophagocytosis of pneumococci. Equal amounts of Hic-positive (strain PR218) and Hic-negative (FP13) pneumococci were incubated with 0-25% normal human serum and then with freshly isolated polymorphonuclear leukocytes (PMN). The cells were washed and transferred to object slides using a cytospin centrifuge. The slides were stained either with acridine orange or gram-stain and the number of attached/ingested bacteria per the number of PMN was counted.

The use of PspC-family proteins as vaccines

The PspC protein family is so polymorphic that it may not seem like an optimal vaccine candidate. However, the variability between proteins is generated by the eight building domains within the N-terminal part of the molecule (147). Brooks-Walter et al. showed that immunization of rabbits with recombinant PspC elicited cross-protective antibodies also to PspA and conferred immune protection against pneumococcal bacteremia (49). The use of PspC as a vaccine needs further studies. In principle, inhibition of the interactions between PspC proteins and factor H by vaccine-induced antibodies could sensitize the respective pneumococci to complement-mediated opsonophagocytosis.

Characterization of the factor H – β protein interaction

Interestingly, the C-terminal region of the β protein from GBS which was recently shown to bind FH shows some homology to Hic and other PspC proteins (Fig. 2 in III). The β protein was originally identified as an IgA-binding protein expressed by many strains of GBS (191, 271). On β, the

binding site for IgA has been located to a 73-amino acid region in the N-terminal half of the protein (133, 158). In contrast, the factor H-binding region was tentatively located to the C-terminal half of the β protein (11).

Recently, the genome of serotype V GBS was published (301). Gene synteny conservation was more marked between GBS and GAS than between GBS and pneumococci. Despite the relatedness of GAS and GBS genomes, there is no structural homologue for the β protein in GAS.

Knowing that Hic and the β protein are structurally related and both bind FH, we wanted to compare the FH-binding characteristics of these bacterial proteins. We studied the binding of FH and recombinant fragments of FH to GBS strains expressing and not expressing β and also a set of strains where mutations in the β protein spanned the C-terminal region i.e. amino acids 435-1097. Binding studies were also performed with the purified β protein. Using a direct binding assay and surface plasmon resonance analysis, the β binding site on FH was mapped to SCRs 8-11 and to SCRs 12-14, i.e. to the same sites where Hic was found to bind earlier. The binding of the β protein to SCR11-15 was only weakly inhibited by heparin. In order to study whether Hic and β bind to overlapping sites on FH, we tried to look at the possible inhibitory effect of β on the binding of SCR8-11 and SCR11-15 to Hic. However, due to the small amounts of protein we were able to reach only equimolar concentrations of β and of the FH fragments, and no inhibitory effect at this level could be seen. Thus, whether Hic and β bind to overlapping or only nearby sites in the middle region of FH remains uncertain. However, the slight differences in heparin inhibition suggest that the binding sites may differ in structure.

Localization of putative factor H-binding sites on the β protein and Hic

To localize the FH binding site on the β protein the GBS strains expressing mutants of β were used in direct binding assays to test the ability of radiolabeled SCR8-20 to bind to the bacteria. The primary binding region on the β protein was within amino acids 435-788, as the deletion of this region in β almost totally abolished the binding. A peptide spot analysis was used to further map putative binding sites on the β protein and also on Hic. For this analysis, we chose the regions in β and Hic known to be needed for FH binding. We could identify 5 putative binding sites for FH on β and 3 on Hic. Some of these sites are in the homologous regions of Hic and β. As the

3D-structures of these proteins have not been established yet, we can not be certain whether these regions come close together in the final conformation or whether they possibly are separate sites on a filamentous protein. However, based on computer analysis of the predicted secondary structures, both Hic and β are mainly of α-helical structure (133, 152). Also, there are no cysteine residues in Hic or β so no disulphide bridges can be formed. Therefore, it is probable that both proteins have a fibrillar or filamentous structure. If so, the FH-binding sites on β and Hic would be distributed along the filament. Thus several binding interactions between FH and β or Hic could take place and lead to enhanced avidity between FH and the microbial proteins.

Even though both GBS and pneumococci are classified as streptococci, the disease spectrum caused by them differs, and they represent distinct bacterial species. The result that Hic and β, proteins from two different bacterial species bind FH in an analogous manner, is a novel finding. This suggests that the expression of these FH-binding proteins has been conserved through evolution and that binding of FH is an important characteristic for the microbes in order to survive in the human host.

The use of the β protein as a vaccine

Analogously to pneumococcal PspC proteins, the β protein emerges as an attractive vaccine candidate. Antibodies directed against β would promote C activation and opsonophagocytosis and inhibit the acquisition of the C regulator FH onto the GBS surface. The β protein has been tested in a conjugate vaccine composed of GBS type III capsular polysaccharide coupled to β, which is not expressed by type III GBS (196). This vaccine elicited protective antibodies against the type III capsular polysaccharide and the β protein in mice. However, the β protein conjugate vaccines have not yet been tested in clinical trials.

Factor H (I-III)

Our studies on the interactions of CRP with factor H and the C evasion mechanisms of pneumococci and GBS led to the recognition of a novel binding region in the middle of FH. Previously, the SCR8-11 region of FH had no known ligands. Microbes have been shown to bind to SCR7 (M-

proteins of GAS, *C. albicans*) and to the C-terminus (OspE of *B. burgdorferi*, *C. albicans* and gonococcus) of FH (33, 136, 212, 254). All the three FH-ligands characterized in this study (CRP, Hic and β) had two binding sites on FH (Fig. 12). The presence of multiple binding sites on the extended structure of FH also emerges as a new concept from this study. It is likely that multiple binding sites ensure a high binding avidity. Since also the microbial proteins have elongated structures (possibly coiled-coils) one can envisage that the binding partners align longitudinally with each other.

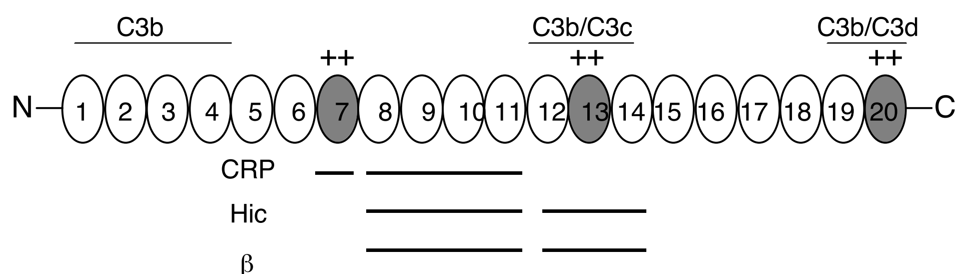

Figure 12. CRP, Hic and β-protein binding sites on factor H recognized in this study. The three binding sites for C3b and for heparin (++) are marked above the circles that represent the 20 SCR domains of factor H.

C4bp and meningococci (IV)

Neisseria meningitidis is an important and feared cause of meningitis and sepsis. In industrialized countries, serogroup B is one of the most common serogroups isolated (267, 319). Mass vaccinations are not a practice and the present vaccines in use, which are all directed against capsular polysaccharides, are not protective against serogroup B meningococci. This is due to the poor immunogenicity of serogroup B capsular polysaccharide. The latter has structural homology with fetal neural tissues in humans (99, 100). Therefore, the goal now is to develop vaccines directed against non-capsular antigens of meningococci. Thus, the non-capsular meningococcal virulence factors are of significant interest.

Complement is essential in the host defense against meningococci. This is underlined by the fact that individuals deficient of AP or terminal pathway components have an increased risk of recurrent meningococcal infections (96). Deficiencies of the CP components do not usually predispose to

neisserial infections. It seems that AP is needed to reject meningococci while meningococci themselves are able to avoid CP. Therefore, we wanted to examine the mechanisms by which meningococci evade CP activation. It had previously been shown that both the AP and CP were activated in meningococcal infection but the relative importance of the pathways is under dispute and probably depends on several factors.

Binding of C4bp to meningococci

Initially we looked at the direct binding of radiolabeled C4bp to 12 randomly chosen clinical isolates of meningococci representing serogroups A (n=2) and B (n=10). The binding of C4bp to the bacteria varied between 12 and 45% under the conditions used. This warranted further studies of the role of C4bp in C evasion by meningococci. Our aim was to locate the binding site for C4bp on the meningococcal surface, and to study whether this binding interaction has any significance in the survival of meningococci in humans. We used 12 serogroup B meningococcal strains with deletions or combinations of deletions of the capsule, LOS and the major outer membrane proteins PorA and PorB. In addition, we had C4bp purified from human plasma, recombinant C4bp with deletions of SCR domains and monoclonal antibodies raised against C4bp. By using the direct binding assay with radiolabeled C4bp we found that deletion of PorA significantly reduced the binding of C4bp to meningococci. Deletion of LOS had no effect on the binding, but the deletion of the polysaccharide capsule enhanced the binding of C4bp to meningococci. The capsule probably presents a steric hindrance, thus its removal increases the binding of C4bp to proteins underneath. The binding of C4bp to strains expressing both PorA and PorB increased from 19% to 38% when the capsule was deleted. However, in the absence of capsule, the binding of C4bp to the PorA-negative strain was also markedly increased to 21% compared to the 4% binding to the encapsulated strain. Thus, our data suggests that PorA is necessary for the binding, but there may be other factors involved which are exposed when the capsule is removed. These factors, however, are of questionable physiological significance since nonencapsulated meningococcal strains are only very rarely isolated from clinical samples. The capsule is thus considered a prerequisite for virulence.

The binding of radiolabeled C4bp to meningococcal strains was dose-dependently inhibited by heparin and unlabeled C4bp but not by BSA. The

results from the direct binding assays using recombinant mutants of C4bp with deletions of single SCR domains and from the inhibition assays with heparin and monoclonal antibodies suggested that the binding site for meningococcus on C4bp is located in SCRs 2 and 3 of the α-chain. The C4bp binding sites for heparin and C4b have been located to the α-chain SCRs 1-3. Thus, a critical question was whether the C4bp bound to meningococci could still bind C4b and exert its C regulatory functions. This was studied by the cofactor assays in which we examined the ability of meningococcus-bound C4bp to act as a cofactor for factor I in the cleavage of C4b and C3b. Indeed, meningococcus-bound C4bp was functionally active and both C4b and C3b became cleaved in the presence of factor I. This can be explained by the fact that C4bp has 7 α-chains, each of which functions individually. Whether a single α-chain of C4bp can bind simultaneously both C4b and meningococcus remains to be studied with monomeric constructs of the α-chains.

We used serum bactericidal assays to further confirm the physiological significance of the interaction between serogroup B meningococci and C4bp. These assays, however, are problematic as meningococci also bind FH and the deletion of PorA does not markedly affect the FH-meningococcus interaction (253, Jarva et al., unpublished). The approach was to compare the serum resistance of a strain expressing both PorA and PorB with a strain where PorA had been deleted. These pairs differed in their ability to bind C4bp but not FH. The PorA-positive strain survived better in 1%, 3% and 10% serum than the PorA-negative strain (Fig. 6 in IV and unpublished). For example, by using 10% serum, 25% of PorA-positive vs 87% of PorA-negative bacteria became killed at 60 minutes. This was most clearly seen with the strains not expressing LOS, which presumably is one of the binding targets for FH. Thus, C4bp-binding seems to play an important role in the C-evasion of meningococci.

Meningococcal PorA – C4bp interaction

On the basis of experiments using deletion mutants of meningococci, the putative target molecule for C4bp binding is PorA. To verify the C4bp-PorA interaction it would be necessary to directly demonstrate a protein-protein interaction. However, because PorA is an integral membrane protein (a porin) with 8 extracellular loops and 16 transmembrane segments, it is difficult to study the interaction directly at the protein level.

Our preparations of purified PorA and PorB were in a detergent solution, and could therefore not be used in the surface plasmon resonance analysis. Also, no binding of radiolabeled C4bp to PorA (or PorB) run into an SDS-PAGE gel and transferred to a nitrocellulose membrane could be detected. These results suggest that the binding of C4bp to PorA is dependent on conformation and proper orientation of the PorA protein on the outer membrane of the meningococcus.

In another approach, we used a peptide spot analysis with peptides representing the surface-exposed loops of meningococcal PorA and PorB and, for comparison, gonococcal Por1A and Por1B. Radiolabeled C4bp bound most strongly to PorA peptides representing loops 1 and 4, i.e. the variable loops (Fig. 13). The most prominent binding regions contained positively charged residues, lysines and arginines. However, the putative region on loop 4 was rich in alanine and valine, small hydrophobic amino acids. On the meningococcal surface, PorA forms trimers in which loops of each molecule come into contact with each other. Therefore, depending on the 3-dimensional conformation of the loops in the porin trimer, the putative binding sites could form 1-6 ligand sites for C4bp. The avidity of the binding interaction is further increased by the redundancy of binding sites on C4bp as each of the seven α-chains could bind to PorA independently.

PorA and meningococcal vaccines

Based on our current data and previous data from elsewhere, PorA emerges as an important virulence and C evasion factor of serogroup B meningococci. PorA has been a candidate in the development of meningococcal vaccines. Antibodies directed against PorA and PorB are produced after meningococcal infection (121, 122). In an animal model, only the bactericidal antibodies against PorA were protective against bacterial challenge (276). However, the expression of PorA is variable, although no antigenic variation during infection has been detected. Occasionally, meningococcal strains not expressing PorA have been isolated from clinical samples, and recently an epidemic (7 cases) with group C meningococci not expressing PorA was described (316). The sequence of PorA varies between strains but the variation is mainly confined to two surface-exposed loops, 1 and 4 (203). In our peptide spot analysis, the main binding region for C4bp appeared to be located on loop 1 and a secondary site was on loop 4. Interestingly, the main binding site on loop 1 was outside the variable area

(Fig. 13). Therefore, antibodies against this conserved region of PorA could inhibit the binding of C4bp to the meningococcal surface and allow CP activation. Thus, our findings have potential significance for protein vaccine development against meningococci.

Loop

1 2 3 4 5 6 7 8

Figure 13. Folding model of PorA of serogroup B meningococcus H44/76. The top part of the model shows the surface-exposed loops, and the central part represents the transmembrane segments. The hypervariable regions in loops 1 and 4 are boxed. Bold letters: a putative C4bp binding site on loop 1. Amino acid residues in the transmembrane segments are shown alternating where they can form a β strand. Adapted from (318). Printed with the permission of the American Society for Microbiology.

C4bp – protein S complex and the pathogenesis of meningococcal disease

Fulminant meningococcal sepsis is characterized by hemorrhages, thrombosis in small vessels and, ultimately, disseminated intravascular coagulation. Procoagulant pathways are activated but the anticoagulant pathways are impaired (245). The plasma levels of the anticoagulant proteins antithrombin, protein C and protein S, and also of C4bp are reduced in meningococcal sepsis (89, 129). Endothelial damage and loss of endothelial

glycosaminoglycans, thrombomodulin and protein C receptor contribute to the dysfunction of the anticoagulant pathways (89, 171, 239).

As much as 70% of the anticoagulant protein S is in complex with C4bp and functionally inactive (66-69). Based on the results of this study, meningococci bind C4bp both with and without protein S. We don't yet know how much this affects the plasma levels of C4bp or whether the reduction of C4bp plasma levels is simply due to consumption of C4bp because of CP activation. Another question is whether the meningococcus-C4bp interaction has an impact on the plasma levels of free and active protein S. C4bp levels increase during the acute phase reaction (105, 288). During infection, more C4bp is released to circulation and complexed with protein S, whose production may not keep up with the rate of C4bp production. Therefore, the amount of free and active protein S would be decreased, and thus contribute to the dysfunction of the anticoagulant pathway. The possible effects of the meningococcus-C4bp-protein S interactions on the development of the severe disseminated intravascular coagulation syndrome typical for fulminant meningococcal sepsis warrant further studies.

Complement evasion *in vivo*

Animal models have been used to study the importance of CRP in the protection against pneumococcal infections and the virulence and C evasion mechanisms of pneumococci, group B streptococci and meningococci. However, CRP, which binds both pneumococci and factor H, is not an acute phase reactant in mice (175, 248). Models utilizing mice transgenic for human CRP are not necessarily relevant until it is shown that human CRP binds mouse C1q and factor H similarly as respective human proteins. Thus, the data concerning the role CRP in protection against pneumococcal infections obtained from the mouse models can not be extended to humans as such.

The results of current (II-IV) and earlier studies show clearly that C regulators are involved in the C evasion by pneumococci, GBS and meningococci. Mouse and rabbit models have been used in studies on pneumococci, and the infant rat model is widely used in meningococcal

immunization studies. However, a major disadvantage of these models is that the mouse, rabbit or rat C regulators may not behave similarly as the human C regulators and the binding of microbial proteins to these regulators could be species specific. For example, we could not detect binding of FH from mouse serum to pneumococci nor the binding of C4bp from rat serum to the wild type serogroup B meningococcal strain used in our studies (Jarva et al., unpublished). Thus, conclusions drawn from immunization studies and the role of complement evasion in animal models need to be treated with caution.

Complement evasion by pathogenic microbes (II-IV)

As has become apparent from the above, many pathogenic microbes utilize binding of C regulators for their own benefit. In general, microbial surfaces represent activator surfaces for the AP of C, and activation of C on them continues without restriction. By offering binding sites for factor H or C4bp the microbe can transform its role as a target C activator to a nonactivator (Fig. 14).

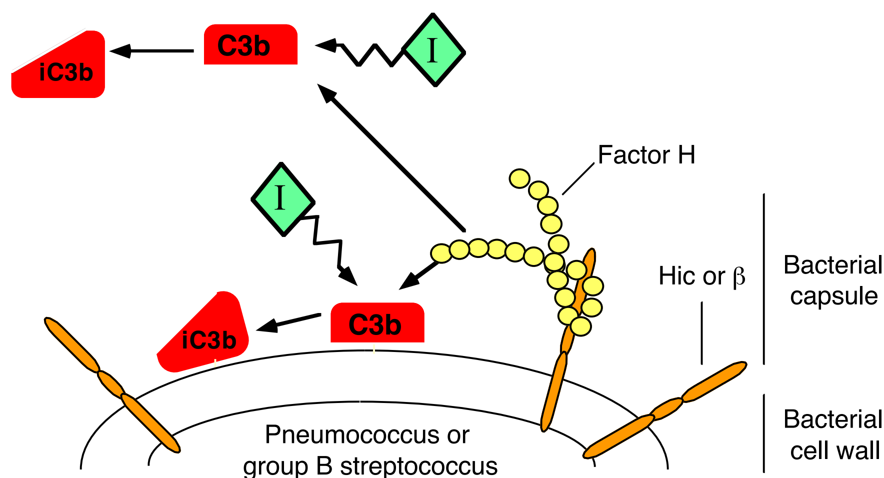

Figure 14. Bacterial evasion of complement by binding factor H. The pneumococcal Hic and B streptococcal β protein are predicted to have extended structures that project out from the cell wall. Both proteins bind FH. Factor H acts as a cofactor for factor I (I) and C3b becomes inactivated to iC3b both on the bacterial surface and in the nearby fluid-phase. Without Hic or β, FH would not bind to the bacterial surface and C activation would continue.

Conclusions and Summary

Complement has a role in the immune defense against invading microbes as well as in the clean-up of the body. CRP is a complement-related molecule involved in both roles. Many of CRP's functions are exerted through activation of the CP. However, while CRP activates the CP, it also regulates C activation at the C3b level via recruitment of FH. The structure of CRP (a pentraxin) has remained conserved through evolution. It is ubiquitous among mammalian species and no deficiencies are known. Microbial structures constantly evolve in response to host immune defense. If the main role of CRP was in the defense against microbes, it would require a capacity to adapt to changing invaders. The conservation of CRP suggests that its primary function is not in host defense but in host clean-up. In this function, CRP collaborates with complement. CRP binds to apoptotic cell membranes, activates the CP and binds FH to prevent C activation beyond the C3b stage. Upon the recruitment of FH, C3b becomes inactivated to iC3b, which is recognized by the CR3 receptor (CD11b/18) on phagocytes. Activation of CR3 leads to an anti-inflammatory type cytokine response, whereby an excessive inflammatory reaction and tissue damage is avoided. Results of this study thus support a new view of a purposeful role of CRP in the response to injury. CRP also reacts with pneumococci, but this is considered to be a secondary function. In fact, pneumococci could themselves utilize FH for their own benefit to avoid C attack.

The exploration of the virulence mechanisms of microbes helps us to understand how microbes evade the host's immune defense and learn more about immune mechanisms of the host. Ultimately, this will also help us to prevent infections. Microbes have survived on earth for over 3 billion years. Since the emergence of animal hosts the microbes have had a long time to develop ways to evade the immune defense mechanisms of their potential hosts. The C evasion mechanisms of pneumococci, GBS and meningococci examined in this study are analogous, yet different from one another. Serotype 3 pneumococcal Hic and group B streptococcal β protein bind the middle part of FH. Via the actions of surface-bound FH the bacteria are subsequently able to avoid opsonophagocytosis. PorA surface protein of serogroup B meningococci seems to be required for binding of functionally active C4bp. Thus, we conclude that the binding of C regulators, either the

AP inhibitor FH or the CP inhibitor C4bp, constitutes an important virulence mechanism of these three bacterial species.

In the course of the above described studies we identified three new ligands for SCRs 8-11 on FH. This region of FH has previously been considered "silent", with no known ligands or C regulatory functions. The results show that SCRs 8-11 have binding sites for CRP, pneumococcal Hic and B streptococcal β, and none of these ligands interferes with the C regulatory functions of FH. This middle region thus emerges as a novel functional region of FH.

One particular aspect in studying microbial evasion of complement and immune attack is the development of vaccines. The goal is to develop protein or conjugate vaccines, which would be immunogenic in infants, adults and in the elderly. The vaccines should elicit cross-protective antibodies. The complement evasion molecules of pneumococci, GBS and meningococci could be ideal protein components in conjugate vaccines. This is because the antibodies directed against the complement-binding proteins would not only recognize an outer surface component but they could also neutralize a virulence property.

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Hanna Jarva

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