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Survival strategies of the human respiratory tract pathogen Haemophilus influenzae

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Abstract

Haemophilus influenzae is an important respiratory tract pathogen responsible for a variety of infections in humans. Encapsulated H. influenzae belongs to one of six serotypes (a-f), of which type b is the most virulent one causing serious and sometimes life-threatening diseases (e.g., epiglottitis, septicaemia and meningitis). In contrast, non-typeable H. influenzae (NTHi) accounts for the majority of local and upper and lower respiratory tract infections. The pathogenesis of many microorganisms relies on the capacity of pathogens to avoid, resist or neutralise the host defence including the complement system.

We demonstrate that H. influenzae interferes with both the classical/lectin and alternative pathways of the complement system. NTHi binds C4BP, the inhibitor of the classical pathway, and the majority of the H. influenzae tested bound factor H, the inhibitor of the alternative pathway. Importantly, the capacity to bind C4BP and factor H appears to render the bacteria more resistant to serum mediated killing. Furthermore, both C4BP and factor H bound to the surface of H. influenzae retains its cofactor activity as determined by analysis of C4b and/or C3b degradation.

In addition to interacting with the classical/lectin and alternative pathways, we demonstrate that Haemophilus surface fibrils (Hsf), which is expressed by encapsulated H. influenzae, binds vitronectin, a regulator of the terminal pathway of the complement system.

Mapping of the membrane bound Hsf with gold-labelled specific antibodies in transmission electron microscopy (TEM) revealed a double-folded 100 nm long fibrillar structure. Using a series of mutants, we showed that when the C-terminal translocator domain was inactivated, Hsf was not translocated to the bacterial surface. Interestingly, we also show that outer membrane vesicles (OMV) secreted by the bacteria carry Hsf, and that Hsf is secreted into the extracellular milieu.

IgD-binding is another important feature of encapsulated H. influenzae type b. By using a series of different IgD chimeric proteins, the site on the IgD molecule responsible for the interaction with H. influenzae was characterised. The binding site was localised to the CH1 region of IgD.

In summary, H. influenzae binds C4BP, factor H and vitronectin, which are regulators of the complement system. The interaction between H. influenzae and these regulators protects the bacteria and makes them more resistant to the bactericidal activity of human serum. Finally, H. influenzae type b binds human IgD.

Key words: Haemophilus influenzae, C4-binding protein, factor H, vitronectin, Haemophilus influenzae surface fibrils, IgD

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CONTENTS

LIST OF PAPERS

This thesis is based on the following papers, which are referred in the text by their respective Roman numerals:

I: **Hallström, T.,** H. Jarva, K. Riesbeck, and A. Blom. 2007. Interaction with C4b-binding protein contributes to nontypeable *Haemophilus influenzae* serum resistance. *The Journal of Immunology,* in press.

II: **Hallström, T.,** P. Zipfel, A. Blom**,** A. Forsgren, and K. Riesbeck. 2007. *Haemophilus influenzae* interacts with human complement Factor H. *Manuscript*

III: **Hallström, T.,** E. Trajkovska, A. Forsgren, and K. Riesbeck. 2006. *Haemophilus* influenzae surface fibrils contribute to serum resistance by interacting with vitronectin. *The Journal of Immunology* 177(1): 430-436.

IV: **Hallström, T.,** M. Mörgelin, A. Forsgren, and K. Riesbeck. 2007. *Haemophilus influenzae* surface fibrils (Hsf) is a trimeric autotransporter that is both secreted and tethered to the bacterial cell surface. *Submitted to Journal of Bacteriology*

V: Samuelsson, M., **T. Hallström**, A. Forsgren, and K. Riesbeck. 2007. Characterisation of the IgD-binding site of encapsulated *Haemophilus influenzae* serotype b. *The Journal of Immunology,* in press.

ABSTRACT

Haemophilus influenzae is an important respiratory tract pathogen responsible for a variety of infections in humans. Encapsulated *H. influenzae* belongs to one of six serotypes (a-f), of which type b is the most virulent one causing serious and sometimes life-threatening diseases (e.g., epiglottitis, septicaemia and meningitis). In contrast, non-typeable *H. influenzae* (NTHi) accounts for the majority of local and upper and lower respiratory tract infections.
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In summary, *H. influenzae* binds C4BP, factor H and vitronectin, which are regulators of the complement system. The interaction between *H. influenzae* and these regulators protects the bacteria and makes them more resistant to the bactericidal activity of human serum. Finally, *H. influenzae* type b binds human IgD.

ABBREVATIONS

AOM	acute otitis media
C4BP	C4b-binding protein
CCP	complement control protein
COPD	chronic obstructive pulmonary disease
ECM	extracellular matrix
FHL-1	factor H like protein 1
Hib	*Haemophilus influenzae* type b
Hsf	*Haemophilus influenzae* surface fibrils
Ig	immunoglobulin
kDa	kiloDalton
LOS	lipooligosaccharide
LPS	lipopolysaccharide
MAC	membrane attack complex
MBL	membrane bound lectin
MID	Moraxella IgD-binding protein
mIg	membrane-bound Ig
NHS	normal human serum
NTHi	non-typeable *Haemophilus influenzae*
OM	outer membrane
OMP	outer membrane protein
OMV	outer membrane vesicle
RCA	regulators of complement activation
SCR	short consensus repeat
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
TEM	transmission electron microscopy

INTRODUCTION

HAEMOPHILUS INFLUENZAE

General properties

Haemophilus is a member of the Pasteurellaceae family, which includes *H. influenzae, H. haemolyticus, H. parainfluenzae, H. parahaemolyticus, H. ducreyi, H. aphrophilus, H. paraphrophilus, and H. segnis* (168). *Haemophilus influenzae* was first discovered by Robert Pfeiffer in 1892 (164). The bacteria were first considered to be the cause of influenza, therefore the characteristic name. However, in 1933 Smith showed that influenza was caused by a viral agent (206).

H. influenzae is a Gram-negative, rod-shaped, facultative anaerobic, human specific pathogen, which requires media supplemented with the two growth-stimulating factors contained in blood: haemin (X factor) and NAD (V factor). Thus, *H. influenzae* can be cultured on chocolate agar where both factor V and X are available. *H. influenzae* grows in a humid atmosphere with 5-10 % CO_2 and at an optimal temperature of 35-37 °C (105). The bacteria are oxidase positive, catalase positive, ferment glucose and can reduce nitrate.

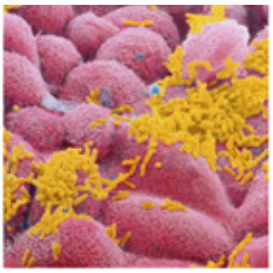

Figure 1. *H. influenzae* attached to respiratory epithelium.
(This picture is taken from; http://sitemaker.umich.edu/medchem6/files/influenzae.jpg)

Prevalence

Approximately 10 % of the normal flora, in the respiratory tract, consists of different *Haemophilus* species (105). *H. influenzae* is part of the normal flora and is commonly present

throughout the year in the nasopharynx of healthy children (85). However, the presence of *H. influenzae* is higher in the wintertime compared to the summer season. The colonisation rates of healthy individuals are approximately 40-80 % among children and adults and carriage of *H. influenzae* in the respiratory tract decreases with age (119). Colonisation of one particular strain may persist for weeks or months and during that time most individuals remain asymptomatic (224). Respiratory viral infections, underlying lung disease (i.e., COPD, bronchiectasis and cystic fibrosis), and exposure to cigarette smoke are factors that predispose individuals to be infected by unencapsulated *H. influenzae* (209). Transmission of the bacteria occurs by inhalation of aerosolised respiratory droplets or by direct contact with respiratory secretions (30).

Infections caused by *H. influenzae*

H. influenzae can be divided into encapsulated strains and unencapsulated strains according to the presence of a polysaccharide capsule (165). The polysaccharide capsule is the major virulence factor of invasive *H. influenzae* strains. Encapsulated strains belong to one of six serotypes (a to f), where type b (Hib) is the most virulent one (165, 226). The most serious and sometimes life-threatening conditions caused by encapsulated *H. influenzae* are septicaemia, meningitis and epiglottitis (28). Historically, Hib strains were the major cause of bacterial meningitis in children. Invasive diseases caused by Hib have, however, decreased dramatically since the introduction of the Hib vaccine. The present Hib vaccines consist of type b capsular polysaccharide conjugated to one of several non-*H. influenzae* immunogenic carrier proteins (104). The vaccines only protect against Hib and are not able to protect against the other capsular types or unencapsulated *H. influenzae* strains. Thus, invasive infections caused by non-type b encapsulated strains have recent years increased in frequency (1, 161, 228). Moreover, the Hib vaccine is not commonly used in developing countries, making the population susceptible to all types of *H. influenzae* infections.

In contrast, unencapsulated and hence non-typeable *Haemophilus influenzae* (NTHi) commonly causes local disease in the upper and lower respiratory tract (e.g., bronchitis, sinusitis and acute otitis media (AOM)). NTHi is after pneumococci the second most common pathogen isolated from children with AOM (226). In addition, NTHi is also the main cause of acute exacerbations in patients with chronic obstructive pulmonary disease (COPD) and bronchiectasis (63, 232, 243). In contrast to encapsulated *H. influenzae*, NTHi strains are only

sometimes invasive and 25 % of all invasive NTHi occur in adults (51). The majority of these patients have underlying medical problems (e.g., COPD, malignancy, and human deficiency virus type 1 (HIV-1) infection) that predispose them to infection (30).

Pathogenesis and virulence factors

A crucial factor in the pathogenesis of both encapsulated and unencapsulated *H. influenzae* involves the initial adherence to the mucosa in the respiratory tract (140). If the bacteria manage to overcome the host clearance mechanisms including the mucociliary escalator and local immunity, they may colonise and cause damage to epithelial cells and breakdown of tight junctions (182, 244). Adherence to the epithelium is a mechanism to circumvent the mucuciliary clearance, and penetration of the bacteria between host cells may facilitate evasion of the immune system. Moreover, the bacteria can reach the basement membrane and the extracellular matrix (ECM) and may penetrate into deeper tissue layers and consequently into the circulation. *H. influenzae* expresses a number of surface structures that influence the process of adherence and colonisation. If the microbe overcomes the local immune response and colonises, this can result in a contiguous spread to other sites of the body. In some cases bacteria can enter host cells where they survive intracellularly. Some of the relevant factors involved in the virulence of *H. influenzae* are outlined in Table 1, shown in Figs. 2-3 and described below.

Polysaccharide capsule

A capsule composed of repeating units of polyribose-ribitol phosphate (PRP) (39) surrounds the encapsulated strains. This capsule is a well characterized virulence factor and it makes the strains more resistant to phagocyte mediated killing (152). Antibodies raised against the specific polysaccharides of the capsule are poorly immunogenic. To overcome this event, the vaccine developed against Hib is based on covalently linkage of an immunogenic protein to the capsular polysaccharide antigens (104). This elicits a strong immune response to the capsular polysaccharides and induction of memory. *H. influenzae* expressing the type b capsule have been shown to more likely cause meningitis and sepsis in humans compared to the other capsular serotypes and NTHi (227). Studies with non-immune rats have demonstrated that Hib strains expressing a capsule are more resistant to clearance from the blood stream than their corresponding unencapsulated mutants (240).

Table 1. Relevant outer membrane proteins of *H. influenzae*

OMP	Serotype	Function(s)	Size (kDa)	Reference(s)
Pili	All serotypes	Adhesion	Variable	(229)
Hsf	Encapsulated	Adhesion/Serum resistance	245	(211, Paper III)
Hia	NTHi	Adhesion	115	(14)
HMW1	NTHi	Adhesion	125	(213)
HMW2	NTHi	Adhesion	120	(213)
Hap	All serotypes	Adhesion	155	(212)
IgA1 protease	All serotypes	Cleaves secretory IgA	169	(106, 128)
P2	All serotypes	Porin activity and adhesion	40	(80)
P5	All serotypes	Adhesion	27-35	(80)
Protein D	All serotypes	Adhesion and cilia toxin	42	(96, 97)
Tbp1	All serotypes	Iron acquisition	95	(87)
Tbp2	All serotypes	Iron acquisition	68-85	(87)

Pili

Pili are helical structures up to 450 nm long (215), which promote adherence to respiratory mucus and bronchial epithelial cells (71, 117). They have mainly been characterised and observed on the surface of encapsulated *H. influenzae,* but a subset of NTHi strains also expresses pili that mediate binding to oropharyngeal cells (229). In addition, studies have shown that pili expressed by Hib interact with ECM proteins such as fibronectin (233). Pili have been sequenced and cloned from both Hib and NTHi and are encoded by a gene cluster containing five genes designated *hif A-hifE* (68, 231). After colonisation in the nasopharynx, *H. influenzae* loose the expression of pili suggesting a role for pili in the initial stage of infection (11). It has been demonstrated that Hib isolates found in blood and cerebrospinal fluid are non-piliated (131).

Fimbriae

Fimbriae are thin and non-hemagglutinating filaments expressed by NTHi strains (205). The gene encoding for fimbriae shows homology with the gene encoding for OmpA of other Gram-negative bacteria but not to the gene encoding for the subunit in pili. A fimbriae deficient mutant was demonstrated to exhibit reduced adherence to oropharyngeal cells and reduced virulence in a chinchilla model. Moreover, fimbriae enhanced the attachment of NTHi to erythrocytes and respiratory tract mucus (15).

LOS

Lipopolysaccharide (LPS) is present in the outer membrane of Gram-negative bacteria and belongs to a family of toxic glycolipids (139). LPS is a strong inducer of inflammatory responses and a well known virulence factor, composed of an O-specific polysaccharide chain, a core oligosaccharide and lipid A. In contrast to some other Gram-negative bacteria, *H. influenzae* produces lipooligosaccharides (LOS), which lacks the O-chain (repetitive side chain) of LPS (187). The oligosaccharides are highly variable from one molecule to another (176). Bacteria releases LOS in the surrounding medium during growth and therefore LOS has been suggested to play an important role in the pathogenicity of AOM caused by NTHi (78). In addition, released LOS has been shown to be a more potent inducer of inflammation compared to surface bound LOS.

Phosphorylcholine (ChoP) is a phase variable molecule linked to different hexoses on the LOS molecule depending on the particular strain (239). Phase variation is a phenomenon, which allows bacteria to turn on and off selected genes in order to regulate the expression of different molecules. ChoP expression is associated with more efficient colonisation of the nasopharynx in an experimental infant rat model. On the other hand, $ChoP^+$ organisms are more susceptible to the bactericidal activity of human serum compared to $ChoP^-$ strains. Nevertheless, the ability of *H. influenzae* to vary the expression of ChoP correlates with its ability to colonise and persist on mucosal surfaces ($ChoP^+$) and to cause invasive infections by evading the innate immunity ($ChoP^-$).

IgA1 protease

IgA is the predominant immunoglobulin produced by mucosal tissues and is involved in the human host defence, including inhibition of bacterial adherence and invasion, and inactivation of bacterial toxins (107). IgA1 is the subclass that represents over 90 % of all IgA present in the respiratory tract (26). To overcome local immune mechanisms including the presence of secretory IgA1, *H. influenzae* expresses an extracellular endopeptidase called IgA1 protease responsible for degrading the molecule (106, 128). IgA1 protease cleaves the IgA in the hinge region, which separates the antigen binding Fab domains from the Fc portion resulting in elimination of the agglutinating activity of both free and antigen bound IgA1 (166). Almost all *H. influenzae* strains express IgA1 protease, but higher levels of protease activity have been found in disease associated isolates compared with isolates from asymptomatic carriers (234).

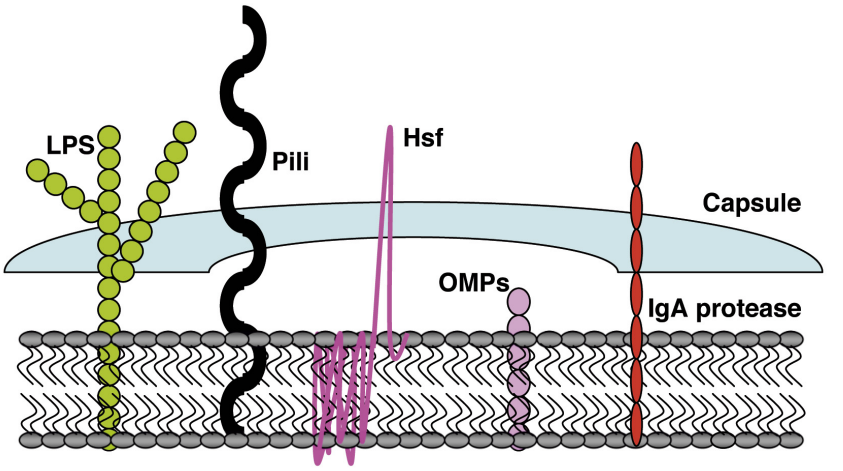

Figure 2. Schematic picture of some important virulence factors of encapsulated *H. influenzae*.

Haemophilus surface fibrils (Hsf)

The major non-pilus adhesin in *H. influenzae* type b is a 245 kDa protein designated *Haemophilus* surface fibrils (Hsf) (211). The *hsf* gene is highly conserved among encapsulated *H. influenzae* strains and encodes an approximately 2,414 amino acids long protein consisting of three repetitive domains with high sequence similarity (Fig. 3). Hsf is found as short, thin surface fibrils at the bacterial surface and is associated with adherence to epithelial cells. Hsf contains three binding regions with striking homology to the binding domains of Hia responsible for interaction with epithelial cells (38), which is situated in the N-terminal (BD2), the middle region (BD3) and the C-terminal (BD1) part of the molecule. Two of these binding domains (BD2; Hsf $^{537\text{-}652}$ and BD1; Hsf $^{1904\text{-}2022}$) were shown to bind Chang epithelial cells (38).

Figure 3. Schematic outline of Hsf. The hatched boxes indicate regions with sequence similarities.

Haemophilus influenzae adhesin (Hia)

Haemophilus influenzae adhesin (Hia), a homologue to the Hsf protein, can be found in approximately 25 % of all unencapsulated strains (14, 133). The *hia* gene, which is shorter than the *hsf* gene, encodes for a protein with a size of 1,098 amino acids and harbours only one domain that corresponds to the three repetitive domains in Hsf (211). However, Southern blot analysis has revealed that *hsf* and *hia* are alleles of the same locus with 81 % similarity and 72 % identity (211). The two proteins are most similar in the N-terminal and C-terminal ends. Hia was not able to be visualised by transmission electron microscopy (TEM), whereas Hsf was seen as thin surface fibers on the surface of the bacteria, maybe according to their differences in size (14). Furthermore, it has been suggested that Hia and Hsf are ligands for the same receptor on epithelial cells (38). It has been reported that Hia possesses two distinct binding domains (BD1 and BD2), which stands for the binding of the same host cell receptor on the epithelial cells but with different affinities (120). Furthermore, Hia belongs to the autotransporter family and is characterised as a trimeric autortansporter (37). In contrast to most known autotransporters, which undergo processing and is released into the extracellular milieu, Hia remains uncleaved at the C-terminus and is fully cell associated (45, 84, 210).

High molecular weight proteins (HMW) 1 and 2

NTHi strains lacking the expression of Hia (75 %) express filamentous high-molecular weight proteins called HMW1 and HMW2, which have been shown to bind respiratory epithelial cells and macrophages (14, 151, 213). Studies have demonstrated that in some cell lines, HMW1 mediates high level of adherence compared to HMW2 and vice versa (92). HMW1 is a 125 kDa protein responsible for adherence to Chang epithelial cells and HMW2 (120 kDa) mediates binding to oropharyngeal epithelial cells (92, 213). The HMW proteins are also members of the autotransporter family (82).

Haemophilus adhesion and penetration protein (Hap)

Mutants lacking HMW1/2 or Hia remain capable of adherence to epithelial cells, suggesting the presence of additional adhesins. *Haemophilus* adherence and penetration protein (Hap) is an 155 kDa autotransporter protein with homology to the IgA1 protease, involved in adhesion to epithelial cells (212). The protein was originally identified in NTHi, but is also present in Hib (86, 212). In addition, a study by Rodriguez and coworkers showed that the *hap* gene is found in all encapsulated *H. influenzae* types, suggesting that Hap plays a major role in colonisation of *H. influenzae* in the nasopharynx (188). Furthermore, Hap mediates bacterial

aggregation and microcolony formation, and interacts with ECM proteins, including fibronectin, laminin and collagen IV (58, 86).

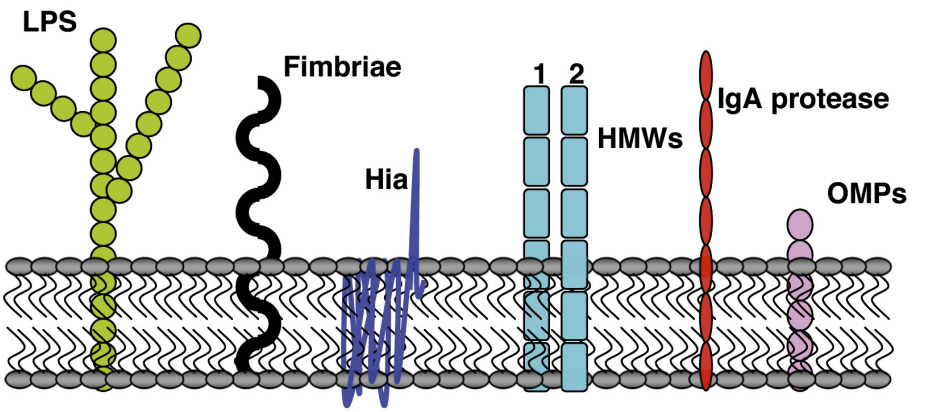

Figure 4. Examples of some important virulence factors found in unencapsulated *H. influenzae* (NTHi).

Other OMPs

The major outer membrane proteins of *H. influenzae* are P1-P6, of which P2 is the most abundant one (170). P2 is highly variable and immunogenic and the variability allows the bacteria to evade protective antibodies (59, 79). P2 is a porin capable of interacting with mucin and this binding may facilitate the establishment of infection (183). P5 is another major outer membrane protein, also showing antigenic variability and capability of binding mucin (183). It has been suggested as an adhesin while strains lacking P5 expression showed reduced adherence.

Protein D is a conserved 42 kDa lipoprotein found in Hib and NTHi strains and was originally characterised as an IgD binding protein, but was later shown to be detected by the IgD myeloma itself (96, 97, 198). Protein D has been suggested to be a promising vaccine candidate since the protein is antigenic conserved in all *H. influenzae* strains (6). Studies have shown that a mutant lacking Protein D expression was 100-fold less infectious compared to the wild type NTHi strain in a rat AOM model, suggesting Protein D as a crucial virulence factor (98).

Opacity-associated protein A (OapA) is a surface associated protein reported to contribute to the adherence of *H. influenzae* to epithelial cells (173). The adherence to Chang epithelial cells was shown to decrease three- to nine-fold with a mutant deficient in OapA expression compared to the wild type counterpart. In addition, OapA is also responsible for the transparent-colony phenotype and is required for efficient colonization of the nasopharynx in an infant rat model (238).

H. influenzae requires iron for bacterial growth. Transferrin binding proteins 1 and 2 (Tbp1/2) are responsible for binding of human transferrin (87). The growth of *H. influenzae* is severely impaired if either of the Tbp proteins is lacking (74).

Outer membrane vesicles (OMV)

H. influenzae releases outer membrane vesicles in vitro during growth in broth. Other Gram-negative bacteria (e.g., *Proteus mirabilis, Neisseria gonorrhoeae, Pseudomonas aeruginosa,* and *Moraxella catarrhalis*) release OMV from the cell surface during growth in various environments, including liquid culture, solid culture, and biofilms (21, 118). Blebs are thought to carry some periplasm together with OMP, porins, receptors and LPS from the outer membrane layer. Many functions have been attributed to blebs in general, for example, interaction with host cells deep in tissues that are not accessible by infecting bacteria, activation of immune cells and induction of leukocyte migration, and finally escape of immune detection during colonisation (5, 67, 163).

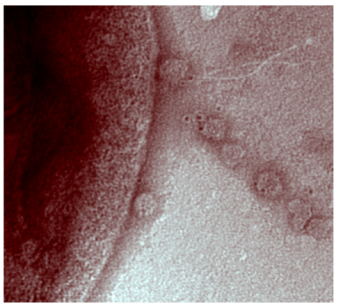

Figure 5. Outer membrane vesicles secreted from *H. influenzae* as seen in TEM. OMVs are discharged from the surface of the bacterium (Hallström et al, unpublished). Dark spots represent gold-labelled antibodies directed against Hsf.

GRAM-NEGATIVE SECRETION MECHANISMS

In order to survive and multiply within a host, bacterial pathogens have to execute an array of complex functions. Virulence determinants are often proteins, which are either secreted to the bacterial cell surface or released into the extracellular milieu. Gram-negative bacteria have developed five major secretion systems, by which secreted virulence proteins pass through their outer membranes (81-83).

The *type I secretion system* requires three accessory proteins. These proteins comprise a channel, which spans the inner and outer membranes (52, 69). The secretion of *Escherichia coli* hemolysin (HlyA) is one typical example of this system.

The *type II secretion system* involves 14 accessory proteins, which are encoded on a single continuous operon (155). The macromolecular and multi-component structure probably also spans the inner and the outer membrane. One example using the type II pathway is pullulanase (PulA) of *Kleibsella oxytoca* and secretion of this protein is *sec* dependent (177).

The *type III secretion system* involves proteins, which are assembled into an oligomeric structure spanning the inner and outer membranes (32). The *sec* machinery is required for translocation of some of the components of the secretion apparatus of the inner membrane. The first identified proteins using this pathway are the Yop proteins of *Yersinia* spp.

The *type IV secretion system* requires at least nine proteins (82). These proteins are associated with both the inner and outer membranes and are localised in the periplasm and cytoplasm. The secretion of *Bordetella pertussis* toxin (PT) is one of the best characterised type IV systems (29).

The type *V secretion system* is probably the least complicated of the pathways, including proteins secreted via the autotransporter system (Table 2) (82, 83). Proteins secreted by this system, mediate their own translocation across the membrane without any accessory proteins, and are called autotransporters. Since Hsf (Papers III and IV) is suggested to be an autotransporter belonging to the type V secretion system (37, 38), this group is further described in detail below.

Table 2. Examples of autotransporters (type V secretion pathway) and their functions.

Species	Protein	Function(s)	Reference(s)
Escherichia coli	Tsh	Hemagglutinin/ haemoglobin binding	(156)
	AIDA-1	Adhesin	(17)
Haemophilus influenzae	IgA1 protease	Cleavage of IgA1	(171)
	Hsf	Adhesin/serum resistance	(211, Paper III)
	Hia	Adhesin	(14)
	Hap	Adhesin	(212)
Helicobacter pylori	VacA	Toxin	(185)
	BabA	Adhesin	(94)
Moraxella catarrhalis	UspA1	Adhesin/ ECM binding	(121, 220, 222)
	UspA2	Serum resistance/ ECM binding	(121, 141, 220,
	MID	Adhesin/hemagglutination/	222)
		IgD binding	(60)
Neisseria spp.	IgA1 protease	Cleavage of IgA1	(169)
Yersinia enterocolitica	YadA	Adhesin	(191)

General structure of autotransporters

The autotransporters consist of three domains (Fig. 6). These include, (i) an N-terminal signal sequence, targeting the protein through the inner membrane into the periplasm, (ii) a C-terminal translocation unit responsible for membrane anchoring and transport of the protein to the bacterial surface, and finally (iii) an internal passenger domain, which is surface exposed and harbours different effector functions of the protein (84). The proteins are exported from the cytoplasm to the periplasm, the signal peptide is cleaved off and the C-terminal inserts into the outer membrane. It has been suggested that the passenger domain is translocated through the C-terminal β domain. Once the passenger domain is translocated to the surface of the bacteria, it can be cleaved off and released into the extracellular milieu or remain in contact with the bacterial surface (45). An autotransporter is not necessarily cleaved off but can also remain intact. Hia is an example of this phenomenon; here the passenger domain is only attached to the cell surface and not cleaved off (210).

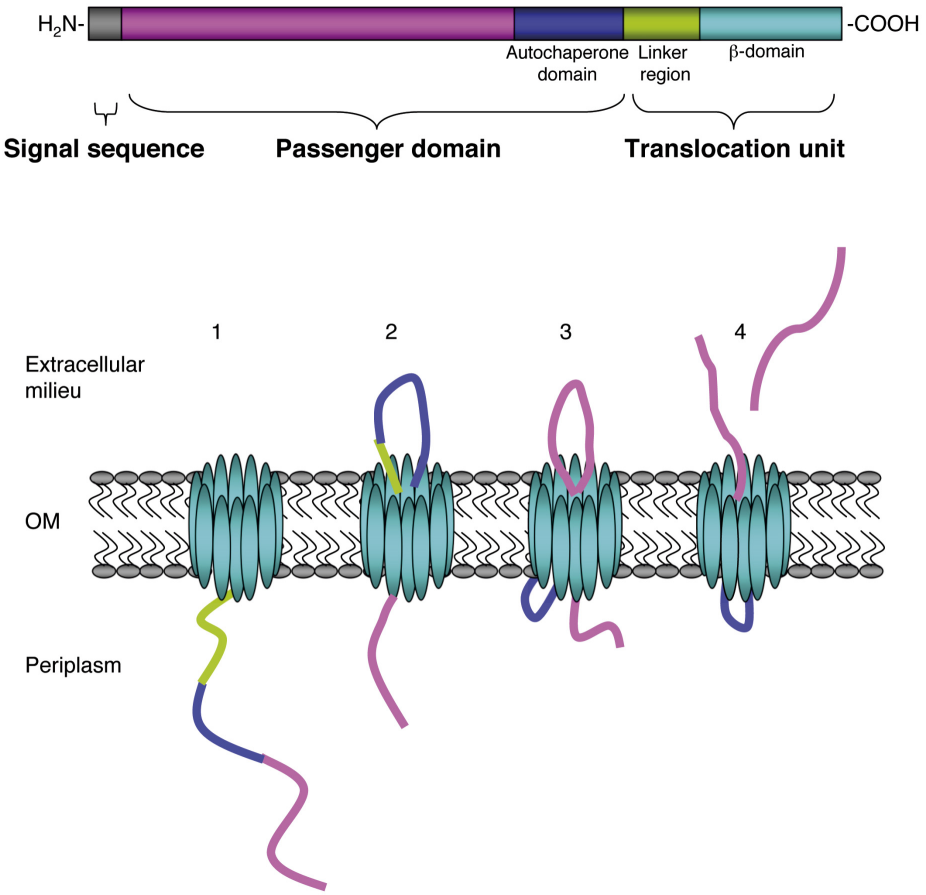

Figure 6. Schematic outline of an autotransporter and the secretion pathway. Step 1: The β-domain is inserted into the outer membrane forming a β-barrel pore. Step 2: The secretion is lead through the pore by the linker region. Step 3: The passenger domain starts to fold and emerges from the β-barrel by a triggering signal from the autochaperone domain. Step 4: The passenger domain is exposed on the cell surface and is either tethered to the surface or released into the extracellular milieu.

THE IMMUNE SYSTEM

The immune system protects our body against invading microbes or infectious agents. It is a complex network consisting of tissues, cells and molecules working together to eliminate intruders. When a microbe enters the host it must overcome both mechanical and chemical barriers, which block initial attempts to cause disease. However, some microbes are able to damage the epithelium and invade the host, whereas others cause infections without having to penetrate the host. The immune system consists of the innate and the adaptive (acquired) parts, which are tightly linked together. The innate immune system includes the complement system, lysozymes, and phagocytes. The adaptive immunity is distinguished from the innate counterpart by having specificity for particular foreign antigens. In addition, the adaptive immune system also involves the ability to distinguish between self and non-self. A previously encountered antigen results in a more rapid and vigorous response due to the immunological memory. The mediators of the adaptive immune system are lymphocytes, which can be divided into B and T cells. The T cells are involved in cell-mediated immunity whereas the B cells are involved in the humoral immunity producing antigen specific antibodies (immunoglobulins).

THE COMPLEMENT SYSTEM

The complement system is the first line of defence and is an essential part of the innate immune system. This system has three main functions; (i) defend the host against invaders by opsonisation and lysis of microbes, (ii) bridge innate and adaptive immunity and, (iii) dispose immune complexes and the products of inflammatory injury (237). It consists of more than 30 soluble and membrane bound proteins. Activation of complement leads to a cascade of protein activation and deposition on the surface of the pathogen, resulting in formation of the MAC and opsonisation of the pathogen followed by phagocytosis. Invading pathogens activate complement either spontaneously due to differences in envelope/membrane composition compared to host (alternative and lectin pathways) or through antibody binding (classical pathway). All three pathways lead to the formation of C3 convertase and thereafter they follow the same terminal pathway, which is a key step in producing an inflammatory response. A schematic overview of the activation of the complement system is presented in Fig. 7.

Activation of the complement system

The classical pathway

The classical pathway is activated when IgG or IgM interacts with an antigenic surface (36, 103). C1q is the first component of the classical pathway and is composed of six subunits, each of which is comprised of a collagen-like tail at the N-terminus and a globular head at the C-terminus (184). It circulates in complex with the catalytic subunit C1s-C1r-C1r-C1s ($C1qr_2s_2$), and this complex is calcium-dependent (36). The classical pathway is initiated when the Fc region of the antigen-bound IgG or IgM binds and activates the macromolecule C1. C-reactive protein, nucleic acids and damaged cell membranes are other activators of the classical pathway (35, 101, 145). The binding of C1q to the Igs results in a conformational change of C1q, leading to proteolytic activation of C1r (46, 73). The proteolytic active C1r then cleaves C1s, producing an active enzyme capable of interacting with and cleaving C4 and C2. C1s in the activated complex binds and cleaves C4 producing C4a, a small anaphylatoxin, which is released and C4b, which binds covalently to the antigenic surface. Attachment of the C4b molecule to antigenic surfaces is inefficient and most of the C4b is quickly hydrolysed and consequently, inactivated in fluid phase. C4b bound to the surface of the antigen acts as a receptor for C2 and the molecules are cleaved by C1s. The cleavage products are C2b, which is released from the complex and C2a, which remains bound to C4b forming the C3 convertase (C4b2a) of the classical pathway. The C3 convertase has the ability to cleave C3 into C3a and C3b. C3a is released from the C3b molecule and has various inflammatory promoting properties. C3b can bind covalently to the complement activating surface and C4b2a forming the classical pathway C5 convertase, C4b2aC3b, which in turn binds C5.

The lectin pathway

Binding of mannose binding lectin (MBL) is the initiation of the lectin pathway. In contrast to the classical pathway, this pathway does not require antibodies to get activated. MBL binds mannose-containing carbohydrates present on microbial surfaces. MBL is a large molecule that is structurally related to C1q, which is associated with three proteases called MBL associated protease 1, 2 and 3 (MASP-1/2/3) (34). MBL undergoes conformational change when it binds mannose residues on the microbe, leading to activation of the MASPs.

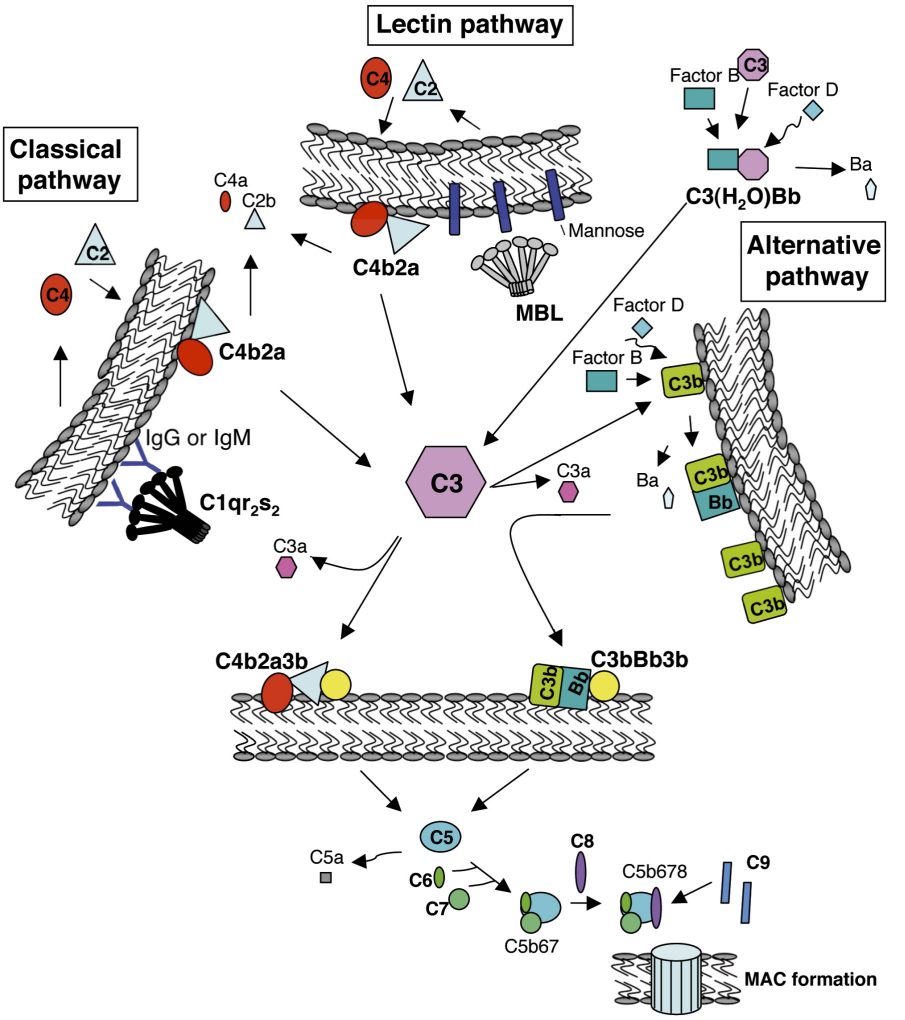

Figure 7. An overview of the complement system.

Furthermore, this MBL-MASP complex cleaves C4 and C2, resulting in membrane bound C4b2a (C3 convertase) and the activation of this pathway follows the same route as the classical pathway.

The alternative pathway

C3 is the central molecule of the alternative pathway. It is constantly hydrolysed in the circulation at a slow rate forming C3(H_2O), which is a form of C3 with C3b-like properties

(159). The plasma protein factor B binds C3(H_2O) and the resulting complex is then cleaved by the enzymatic action of factor D (124). Factor D is a serine protease, which cleaves factor B to Ba that is released, and Bb which forms the fluid phase C3 convertase (C3(H_2O)Bb) of this pathway (123). This fluid phase C3 convertase cleaves C3 to C3a and C3b, which can be deposited on nearby surfaces (148). C3b is quickly hydrolysed, unless it finds a surface to attach to. Once attached to a surface, C3b binds factor B in the presence of Mg^{2+}. Subsequently, this complex is cleaved by factor D resulting in a surface-bound C3 convertase, C3bBb. This C3 convertase will cleave more C3 in an amplification loop. The activation of the alternative pathway augments activation initiated by the classical pathway. This occurs after classical pathway activation has lead to the deposition of C3b on surfaces.

C3b binds surfaces in a non-discriminative manner, while it can bind both non-activating (self) surface, and activating (non-self) surfaces. However, amplification of the activation usually occurs only on foreign surfaces while activating surfaces favour binding of factor B and non-activating surfaces favours binding of the inhibitor factor H to surface bound C3b (90).

The surface bound C3 convertase (C3bBb) is relatively unstable and easily decays, but an increased stability and extended half-life is provided when properdin, a positive regulator of complement system, binds the C3bBb (54, 55, 103). When additional C3b is attached to C3bBb, the C5 convertase is formed and the substrate has changed to C5.

The terminal pathway

The C5 convertases, C4b2a3b of the classical/lectin pathways and C3bBbC3b of the alternative pathway, binds and cleaves bound C5. This cleavage leads to a release of the anaphylatoxin C5a (235) and the larger fragment C5b, which binds C6. This C5b6 complex is capable of non-covalently interacting with biological membranes and binding of C7, the next component of the complement cascade. The C5b67 insertion into the membrane is now initiated and binding C8 further incorporates it into the membrane and causes some leakage (219). This is followed by addition of up to 14 C9 monomers forming a pore in the membrane. These channels, called membrane attack complex (MAC), are the endpoint of the complement cascade. They reduce osmotic pressure and causes lysis of the target cell.

Regulation of the complement system

The complement system is tightly regulated, otherwise it could cause extensive tissue damage in the host. Both soluble and membrane bound regulators keep the system in control and protect our cells from unwanted damage. Regulators of complement activation (RCA), is a group of proteins, including C4BP, factor H, FHL-1, CR1, CR2, MCP and DAF (110). The regulators and their functions are summarised in Table 3 and described below.

Table 3. Soluble and membrane-bound regulators of complement

Regulator	MW (kDa)	Recognition	Function
Soluble			
C1-inhibitor	76	C1r, C1s	Dissociation of C1s and C1r from C1 complex, binds to activated MASPs
C4BP	570	C4b	Inhibits the formation and accelerates the decay of C3 convertase of the classical pathway, cofactor for factor I
Factor H	150	C3b	Inhibits the formation and accelerates the decay of C3 convertase of the alternative pathway, cofactor for factor I
FHL-1	47	C3b	Inhibits the formation and accelerates the decay of C3 convertase of the alternative pathway, cofactor for factor I
Factor I	88	C3b, C4b	Degradation of C3b and C4b
Properdin	53		Stabilises the C3 convertase of the alternative pathway
Vitronectin	65-75	C5b-7, C5b-9	Prevention of C5b-7 membrane insertion
Clusterin	80	C5b-7, C5b-9	Prevention of C5b-7 membrane insertion
Membrane bound			
CR1 (CD35)	200	C3b, iC3b, C4b	Inhibits the formation and accelerates the decay of C3/C5 convertases, cofactor for factor I
MCP	45-65	C3b, C4b	Act as a cofactor for factor I
DAF	70	C3b, C4b	Decay accelerating activities for C3/C5 convertases
CD59 (protectin)	18-23	C8, C9	Inhibits the incorporation of C9 into MAC
HRF	65	C9	Prevents C9 to bind to C5b-8

Soluble regulators

C1 inhibitor

The C1 inhibitor is a 105 kDa regulator of the classical pathway. It is regulating the initial step by acting as a substrate to the enzymatic C1r and C1s. When C1r and C1s cleaves the C1

inhibitor, it remains bound and inactivates the complex (248). In addition, the C1 inhibitor is also able to block the lectin pathway by binding activated MASPs (132).

C4b-binding protein (C4BP)

C4b-binding protein (C4BP) is a 570 kDa fluid phase inhibitor of the classical and the lectin pathways of complement and inhibits the formation and accelerates the decay of C3 convertase (C4bC2a) (41). It also serves as a cofactor to factor I in the proteolytic degradation of C4b (65, 66, 70). C4BP is a large plasma glycoprotein that exists in several forms varying in subunit composition. The major form consists of seven identical α chains (70-kDa subunits) and one β-chain (45 kDa) (Fig. 8) (42). The α- and β-chains are composed of repeating domains of ~60 aa known as complement control protein (CCP) domains (40, 154). Each α-chain is composed of eight CCPs and the β-chain three CCPs. The C4b binding site is located between CCP1 and CCP2 of the N-terminal part of the α-chain (25). C4BP is also linked to the coagulation system since the β-chain is bound to the vitamin K-dependent anticoagulant protein S. Most of C4BP in blood circulates in a 1:1 high affinity non-covalent complex with protein S (42).

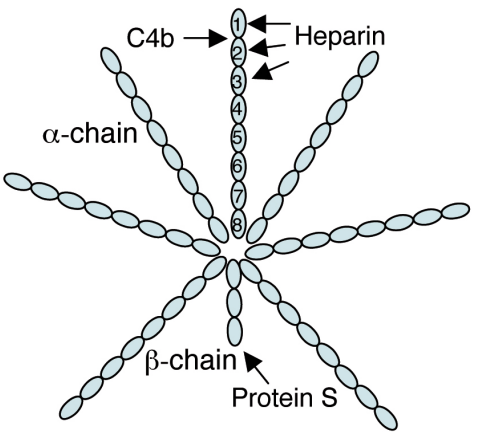

Figure 8. Schematic picture of C4BP and sites where interacting molecules attach.

Factor I

Factor I is a 90 kDa fluid phase protein that inhibits both the classical and alternative pathway convertases. Factor I cleaves C3b and/or C4b in the presence of either C4BP, factor H, FHL-1, membrane cofactor protein (MCP), or complement receptor 1 (CR1).

The factor H family

Factor H is a 150 kDa fluid phase protein that regulates the alternative pathway of the complement system (189). It is a glycoprotein found in human plasma composed of 20 repetitive units of 60 amino acids, which is designated CCPs or short consensus repeats (SCRs) (Fig. 9) (186). The alternative pathway is regulated by factor H via a binding of C3b, accelerating the decay of the alternative pathway C3-convertase (C3bBb) and acting as a cofactor for the factor I-mediated cleavage of C3b. Factor H regulates the complement system both in fluid phase and on cell surfaces, C3b is inactivated by factor H in the fluid phase, whereas inactivation of surface bound C3b by factor H is dependent on the chemical composition of the surface to which C3b is bound. Factor H has three binding sites for C3b, i.e., SCRs 1-4, SCRs 12-14, and SCRs 19-20 (202). Furthermore, factor H has an important role in the discrimination between self (non-activating) and non-self (activating) surfaces. Factor H has high affinity for C3b when the molecule is deposited on human cells (non-activators), which are coated with sialic acid and glycosaminoglycans (135). This keep the alternative pathway activation under control on self surfaces. However, the complement activation proceeds if the C3b coated surface lacks the polyanions.

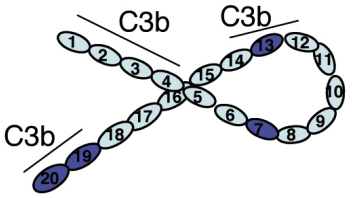

Figure 9. The factor H molecule. The C3b and heparin (dark blue) binding sites are indicated.

In addition to factor H, the factor H family consists of six multidomain and multifunctional serum proteins (250). Factor H-like protein 1 (FHL-1) and five factor H-related proteins (FHR1-5) are the additional members of this group (Fig. 10).

FHL-1 also acts as a regulator of the alternative pathway and is a product of alternative splicing of the factor H gene on chromosome 1 (50). FHL-1 is a 42 kDa protein composed of seven SCRs identical to the N-terminal SCRs of factor H, and four unique amino acids at the C-terminal of the protein (249). The protein also displays cofactor and decay-accelerating activity. The plasma concentration of FHL-1 is approximately 10-50 fold less than the concentration of factor H.

Genes located in the RCA gene cluster encode the FHRs, and the proteins are all composed of SCRs. There are five FHRs and their SCRs show structural similarities to factor H and to each other (Fig. 10) (250). Despite the fact that FHRs are known to bind C3b and heparin, the functions of the different FHRs are still unknown and are under extensive investigation.

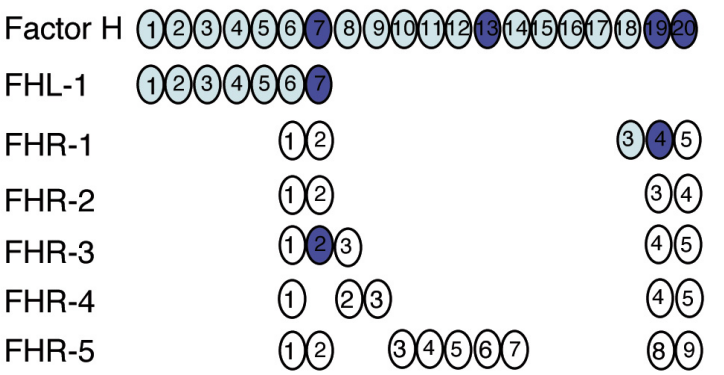

Figure 10. The factor H protein family. Each member is composed of SCRs and the SCR are aligned vertically by their similarities. The dark blue SCRs indicate heparin-binding domains. Modified from ref. 250.

Vitronectin

Vitronectin is a regulator of the terminal pathway by inhibiting MAC (200), It is found as a single chain (75 kDa) and a truncated form of 65 and 10 kDa in human plasma. Vitronectin binds the C5b-7 complex at its membrane-binding site and thereby inhibits the insertion of the complex into the cell membrane and prevents cell lysis of the microbe (174). This complex is still able to bind C8 and C9 to form SC5b-8 and SC5b-9 complexes but the latter is non-lytic. Furthermore, vitronectin blocks the tubular formation by binding C5b-9 (167, 175).

In addition to the complement regulatory activities, vitronectin is involved in cell adhesion, spreading and migration, coagulation and fibrinolysis (174, 200). Furthermore, it is present in the extra-cellular matrix (ECM) as a 75 kDa protein.

Clusterin

In addition to vitronectin, clusterin is also an inhibitor of MAC of the terminal pathway (100). It is a 70-80 kDa glycoprotein, which binds C5b67 and C5b678 complexes and thereby prevents insertion into cell membranes (225).

Membrane-bound regulators

Complement receptor (CR1) is a receptor for C3b and C4b and is primarily found on peripheral blood cells (53). CR1 acts as a cofactor for factor I-mediated cleavage of C3b and C4b and accelerate the decay of C3/C5 convertases (93).

Membrane cofactor protein (MCP) is a glycoprotein that is expressed on all cell types except erythrocytes (126). In addition to CR1, MCP acts as a cofactor for factor I-mediated cleavage of C3b and C4b (201).

Decay accelerating factor (DAF) is a membrane bound glycoprotein, which is found on most human cells (109). DAF binds to and dissociates both the classical (C4b2a) and the alternative (C3bBb) C3 convertases (147).

CD 59 (protectin) and homologous restriction factor (HRF) are membrane bound regulators of the terminal pathway. Protectin is present on practically all cell surfaces and it inhibits MAC formation by binding the C5b-8 complex and thereby blocking the incorporation of C9 (134). In addition, HRF prevents C9 to bind the C5b-8 complex (247).

Functions of the complement system

The best known function of the complement system is the ability to trigger a powerful, coordinated repertoire of anti-microbial reactions, including inflammation, opsonisation and cell lysis. Activation of the components in the complement cascade releases small cleavage products, C3a, C4a and C5a. These are called anaphylatoxins and are powerful chemotactic agents (64). They have different functions, including induction of smooth muscle contraction, enhancing vascular permeability and causes release of histamine and other vasoactive substances from basophils. In addition, C5a has been shown to be strongly chemotactic for phagocytic cells.

C4b, C3b and cleavage products of C3b are components of the complement system called opsonins, which are deposited on the surface of the target cell following activation of the cascade. Recognition of these opsonins by phagocytes enhances

phagocytosis. The phagocytes express receptors for C3b and C4b (CR1) and iC3b (CR3 and CR4) (56). Binding of the opsonised microbe results in phagocytosis and degradation of the microbe.

The end product of the complement system is the formation of MAC. The incorporation of MAC into the membrane of the pathogen causes lysis of the cell and, consequently, death. This process is very important in the defence against Gram-negative bacteria. Gram-positive bacteria have a thick peptidoglycan layer, which effectively resists the formation of MAC.

Another function of complement is its involvement in the clearance of apoptotic cells. C1q binds directly to surface blebs on the apoptotic cell, resulting in activation of the classical pathway (145). This activation leads to C3b deposition and inactivation of C3b (iC3b). CR3 on macrophages binds the iC3b and this leads to phagocytosis of the apoptotic cell.

Microbes and the complement system

In order to be a successful pathogen, the microbes have to overcome the host defence. One essential part of the innate immune system is the complement system. Bacteria have evolved an array of strategies to survive the complement system and be able to colonise the host and cause disease. The pathogenesis of many microorganisms therefore relies on the capacity of pathogens to avoid, resist or neutralise the complement system (114, 193, 246). Some pathogens are able to inactivate complement components, whereas other produce proteins or other components that mimic complement inhibitors.

Group A Streptococci (GAS) express a 31 kDa protein called Streptococcal inhibitor of complement (SIC), which mimics the action of the terminal inhibitors; vitronectin and clusterin, by inhibiting the insertion of C5b-7 into the membrane (4). Borrelial CD59-like protein is expressed by *Borrelia burgdorferi* and shares functional and antigenic similarities with CD59 (protectin) (160). Both proteins inhibit formation of MAC and thereby prevent cell lysis.

Another frequent strategy used by some pathogens is binding of complement inhibitors such as C4BP, factor H and vitronectin, which protects them from complement attacks. These regulators are captured on the bacterial surface in such way that they are still complement regulatory active. *M. catarrhalis, Neisseria gonorrhoeae and E. coli* K1 are bacteria, which express surface molecules (UspA1/A2, Por1A/1B and OmpA, respectively)

capable of binding C4BP and thereby protecting the bacteria against the classical and lectin pathways (153, 179, 245). Another pathogen able to bind C4BP is *Streptococcus pyogenes* (31). The interaction is mediated by the M protein and it has been correlated to phagocytosis resistance. Many pathogens also bind the inhibitors of the alternative pathway; factor H and/or FHL-1, in order to protect them from complement attacks by accelerating the decay of alternative pathway C3 convertase and inactivation of C3b. In fact, a number of microorganisms, including *Streptococcus pneumoniae*, *S. pyogenes, B. burgdorferi*, *Neisseria meningitidis* and *Candida albicans* have been reported to bind factor H (91, 99, 137, 199, 236). Several microbes, e.g., group A streptococci, gonococci, *B. pertussis* and *C. albicans* have also been shown to bind both C4BP and factor H (19, 20, 137, 180). When these regulators are bound to the surface of the microbes, they maintain their complement regulatory activity and protect the microbes against direct lysis. In general, the binding of complement inhibitors may contribute to serum resistance and prevention of opsonophagocytosis (181). Many *M. catarrhalis* strains are resistant to the bactericidal activity of human serum. In addition to C4BP-binding (153), *M. catarrhalis* ubiquitous surface protein A2 (UspA2) has been found to bind vitronectin, the inhibitor of the terminal pathway, and this interaction significantly contributes to serum resistance (12). Furthermore, *Staphylococcus aureus*, *E. coli* and β-hemolytic streptococci are efficient binders of vitronectin (33). In addition to rendering the bacteria more resistant to complement induced lysis, the bacteria/vitronectin interaction also contributes to the attachment and invasion into host cells. The vitronectin binding of *Pseudomonas aeruginosa* is involved in internalization of the bacteria into human epithelial cells (122). Blocking this interaction with anti-vitronectin antibodies inhibited the bacterial invasion. In addition, *N. gonorrhoeae* probably uses vitronectin as a bridge for attachment and invasion of human cells (43).

Gram-positive bacteria are in general resistant to MAC induced cell lysis. They are shielded by a thick cell wall comprised of peptidoglycan (102). In contrast, the majority of Gram-negative bacteria are susceptible to the action of the terminal pathway (MAC formation and cell lysis). Thus, the capsule of some of the species makes them more resistant to complement mediated attacks and opsonophagocytosis than the unencapsulated counterparts.

The capacity to adhere to mucosal epithelium is crucial for the virulence of many pathogens. Different regulators of the complement system have been shown to mediate adherence and ingestion to epithelial and endothelial cells. The binding of C4BP by *C. albicans* has been shown to mediate adherence to endothelial cells and the binding of *S.*

pyogenes to FHL-1 mediates and enhances the ingestion of the pathogen into epithelial cells (136, 158).

Some pathogens expresses enzymes capable of cleaving complement components and thereby avoid complement activation or restrict the inflammatory reaction. *P. aeruginosa* and *Helicobacter pylori* are two species expressing enzymes capable of cleaving C1q and/or C3 (89, 192).

Table 4. Interaction between pathogens and complement regulators.

Pathogen	Protein	Regulator	SCRs required for binding	Reference
B. burgdorferi	BbCRASP-1	Factor H, FHL-1	SCRs 5-7, SCRs 19-20	(112, 113)
	BbCRASP-2	Factor H, FHL-1	SCRs 6-7, SCRs 19-20	(112, 113)
	BbCRASP-3/4/5	Factor H	SCRs 19-20	(112, 113)
E. coli K1	OmpA	C4BP	SCR 3	(172)
M. catarrhalis	UspA1/UspA2	C4BP	SCRs 2, 5 or 7	(153)
N. gonorrhoeae	Por1A	C4BP	SCR 1	(179)
	Por1B	C4BP	SCR 1	(179)
S. pneumoniae	Hic	Factor H	SCRs 8-11, 12-14	(99)
	PspC	Factor H	SCRs 13-15	(47)
S. pyogenes	Fba	Factor H, FHL-1	SCR 7	(157, 158)
	M protein	Factor H. FHL-1	SCR 7	(111)
	M protein	C4BP	SCRs 1-2	(24)

Complement in the respiratory tract

For many microorganisms, including *H. influenzae*, the primary interaction with the human host is through colonisation of the mucosal surface of the respiratory tract. Therefore, it is of highest interest for the host to exploit an efficient defence at these surfaces. The complement system is classified as a part of serum, but there are several studies demonstrating the presence of complement in various sites of the body. Reports of the presence of complement components in the respiratory tract of healthy individuals are scarce. Functionally active C3, C4 and factor B are complement components that have been detected in human saliva (10). However, there are several studies indicating the importance of complement in the respiratory tract during infections. During inflammation, the permeability of the mucosa increases and plasma, including complement proteins, immunoglobulins and components of the coagulation

and fibrinolysis systems, enters the airway lumen (75, 76, 162). This process designated plasma exudation, has been suggested to be the first line of the mucosal defence system.

Complement components, i.e., C3a and C5a, have been found in the mucosa of the nose and lower airways in various respiratory tract challenges, including allergy and influenza virus infection (9, 23). In patients with chronic otitis media with effusion (OME), local complement activation in the middle ear mucosa was observed, including an intense deposition of C3 (143). In addition, factor H, FHL-1 and FHR-1/2/3/4/5 are complement components found in middle ear effusions of patients with OME (142). Marc and coworkers found increased concentrations of C5a in patients with COPD, suggesting the involvement of complement in the pathogenesis of the disease (130). Furthermore, highly elevated levels of C3a has been observed in middle ear effusions, indicating ongoing complement activation (144).

H. influenzae ***and the complement system***

An important feature for a pathogen to cause invasive diseases is the ability to avoid and resist the bactericidal activity of the complement system in human serum. A pathogen having the capability to survive in human blood, also has the potential to spread to other sites of the body. Hib is capable of activating both the classical/lectin and alternative pathways of the complement system. By using capsular polysaccharide or encapsulated bacteria, complement-dependent bactericidal antibodies were efficiently absorbed from serum indicating presence of antibodies initiating the classical pathway (8). Both NTHi and Hib are capable of activating the alternative pathway, suggesting the cell wall rather than the capsule as a target for the alternative pathway (178). The concentration of polysaccharide seems to be important for the bacterial survival, since an increased production of polysaccharide is associated with increased resistance to lysis (217). Hib has been shown to be more resistant to the bactericidal effect of complement. To analyze whether there are differences in resistance to the actions of complement depending on the capsular serotype, Swift and coworkers used capsule deficient mutants that were identical with respect to outer membrane proteins, LPS and antibiotic susceptibility (218, 251). The capsule types a, b and e transformants were shown to be equally resistant to the complement activity as compared to the Hib wild type.

In addition to encapsulated *H. influenzae*, NTHi has been found to be invasive and resistant to the actions of human serum (242). In that study the serum resistant strains delayed the C3 deposition on the cell surface, resulting in prevention of MAC accumulation.

Furthermore, a recent study demonstrated that serum resistance is facilitated in a NTHi strain by a delay of C4b deposition (88). When the LOS biosynthetic gene *lgtC* was inactivated the C4b deposition increased and the survival in serum and blood was reduced, suggesting an involvement of LOS in the interaction with the complement system. As mentioned before, the ChoP associated with LOS is involved in serum resistance of *H. influenzae* (239). $ChoP^+$ strains were more susceptible to the bactericidal actions of human serum than the $ChoP^-$ counterpart. It was shown that C-reactive protein mediated this susceptibility to serum by binding directly to the $ChoP^+$ strain and thereby activating the classical pathway. Furthermore, the complement system was suggested to have a central role in the innate immune defence against NTHi in experimental AOM (57). When depleting complement in chinchillas, two otherwise avirulent *siaB* mutants (defective in their ability to sialyate LPS) caused EOM with severity similar to their wild type counterparts. In addition, a higher deposition of C3 was detected on the serum sensitive mutants compared to the more serum resistant wild types.

Moreover, to conquer the innate immune system, some pathogens collaborate in a sophisticated way. In a recent study performed by our group, Tan *et al*. showed that OMV produced by *M. catarrhalis* contributed to an increased survival of *H. influenzae* in human serum (221). This observation may explain why these two pathogens are often found together in the human respiratory tract. Finally, patients with complement components (including C2 and C3) deficiencies have been shown to be associated with increased susceptibility to Hib (7, 223).

IMMUNOGLOBULINS

Antibodies belong to a group of glycoproteins also known as immunoglobulins (Igs). Igs are present in tissue fluids, human serum and on the surface of B cells and they are important effector molecules of the adaptive immune system. Thus, their function is to recognise and bind foreign antigens. Furthermore, they are also involved in the innate immunity by their ability to initiate the classical pathway of the complement system. Five different Igs belong to the family; IgG, IgA, IgM, IgD, and IgE. IgM and IgG are the Igs responsible for the primary and secondary response to antigens, whereas IgA is the predominant Ig protecting mucosal surfaces. IgE is involved in allergy and in the protection against parasitic worms. IgD is further described in the next section.

The antibodies all differ in size, carbohydrate and amino acid composition, and function. Nevertheless, all Igs consist of two Fab regions and one Fc part, responsible for antigen recognition and mediation of effector function, respectively. The Igs are composed of two heavy (H) chains and two light (L) chains hold together by disulphide bonds (Fig. 11). Two disulphide bonds link the mid-region of the two H chains, and are designated the hinge region, displaying a high flexibility. The outer parts of the H and L chains comprise the variable regions containing the antigen-binding site. This variable region varies between the different antibody isotypes and recognises and attaches specifically to a particular antigen. The remainder of the H and L chain is called the constant (C) region and this region is almost the same in all antibodies of the same Ig class. Moreover, the L chains exist in two forms named lambda (λ) and kappa (κ).

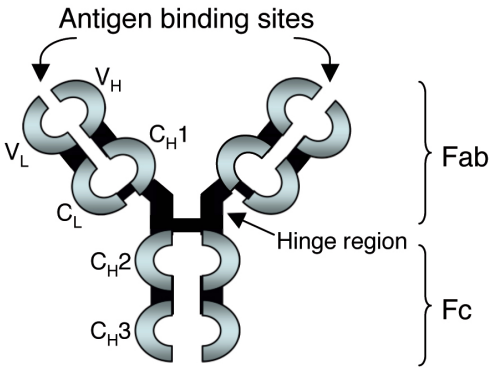

Figure 11. Schematic picture of an Ig molecule.

Immunoglobulin D (IgD)

In 1965 Rowe and Fahey first discovered IgD by studying serum from a myeloma patient (194). After a couple of years it was shown that IgD is expressed on mature B cells together with IgM (230). In contrast, immature B cells and memory cells lack the expression of membrane bound IgD (mIgD) (146). IgD and the other Igs have structural similarities. However, IgD has an unusually long hinge region, which probably makes it susceptible to proteolytic degradation and hence the short half-life in serum (77). Thus, IgD is found in serum at such a low concentrations as 30 μg/ml compared to 12 mg/ml of IgG. The role of soluble IgD still has to be established and IgD secreting plasma cells are relatively

uncommon. However, the number IgD secreting plasma cells have been shown to be increased in nasal and salivary glands (27). Patients suffering from frequent tonsillitis has higher levels of IgD secreting plasma cells and a spontaneous production of IgD by tonsils cultured in vitro has been observed (125, 216). Furthermore, children with recurrent AOM have high concentrations of IgD in the middle ear effusions and nasopharyngeal secretions (207, 208). It has been shown that higher concentrations of serum IgD can be found in patients suffering from COPD as compared to healthy individuals (138). IgD is also, as mentioned earlier, together with IgM expressed on mature B cells and recognises and binds foreign antigens. In the presence of T cells, this event leads to activation of the B cell. The signal arisen from crosslinked mIgD is both stronger and more prolonged compared to that of mIgM (108). To further investigate the role of IgD as a BCR, IgD knockout mice have been generated. These mice showed independently of mIgD expression normal development of the immune system and a normal antibody response (150, 190). However, these knockouts had a certain delay in the affinity maturation in the early primary response. Thus, this maturation is important in the defence against pathogens (190). In IgM-knockout mice, mIgD replaced mIgM function and showed normal B cell development and maturation indicating similar functions of IgD and IgM (127). Taken together, these findings strongly indicate that both secreted and mIgD are important for the immune response against pathogens in the upper respiratory tract.

Bacterial non-immune immunoglobulin binding

Immune binding is a process where an antibody binds its antigen through the variable region. Several pathogens are able to bind immunoglobulins in a non-immune manner, i.e., the binding does not involve the normal antigen binding sites of the antibody. Several surface proteins responsible for the interactions have been identified and the first protein binding an immunoglobulin in a non-immune fashion was protein A (SpA) from *S. aureus* (62). Non-immune binding of Igs is a feature shared by several pathogens but is more prevalent among the Gram-positive than the Gram-negative bacteria.

Non-immune immunoglobulin binding to Gram-positive bacteria

The first characterised bacterial Ig binding protein was isolated from *S. aureus* and is called Protein A (SpA) (62). SpA binds the Fc region of IgG1, 2 and 4, and the binding region is located between C_H2 and C_H3 (44, 116). In addition, a weak binding between SpA and the Fab

region in all Ig molecules has also been demonstrated (95). The capacity of SpA to bind IgG is widely used as a purification method for IgG.

Group C and G streptococci express Protein G, a protein with affinity for all IgG subclasses (22). Protein G has been shown to bind the Fc region of IgG with high affinity and the Fab region (C_H1 domain) with low affinity (49). It is able to bind both regions simultaneously.

Protein L is a protein expressed by *Peptostreptococcus magnus* with affinity for Igκ-light chain of all Ig classes (2). Protein L was shown to bind the variable region of the κ-light chain. This interaction has been shown not to interfere with the capacity of the antibodies to bind antigens (149).

S. pyogenes is another Gram-positive pathogen capable of binding Igs, including IgG and IgA. Protein H is the protein expressed by *S. pyogenes* responsible for the interaction with IgG, whereas Protein Sir binds both IgA and IgG (3, 214).

Non-immune immunoglobulin binding to Gram-negative bacteria

In contrast to Gram-positive bacteria, non-immune Ig binding to Gram-negative bacteria is much less frequent. However, there are a few examples of Gram-negative bacteria that bind Igs in a non-immune manner. *Haemophilus somnus* and *E. coli* have been shown to bind human IgG and IgA, respectively (197, 241). In addition, *E. coli* has also been demonstrated to bind human IgG. Interestingly, both *M. catarrhalis* and *H. influenzae* bind IgD (61). *Moraxella* IgD-binding protein (MID) was isolated from *M. catarrhalis* in our laboratory (60). The MID-IgD interaction is located to the C_H1 region of IgD (196). Protein D isolated from *H. influenzae,* identified by Ruan and colleagues, was shown to bind an IgD myeloma serum (195). However, later on it was shown that Protein D was merely detected by the IgD myeloma used, suggesting another IgD binding protein in encapsulated *H. influenzae* (198).

Importance of bacterial non-immune binding

The reason why several bacterial pathogens have acquired the ability to bind Igs in a non-immune fashion is not fully understood. There are some indications of involvement in the pathogenesis. By binding IgG in a non-immune manner, Protein H inhibits complement activation (18). Similarly, SpA interferes with the complement system by inhibiting the classical pathway (129).

A B cell superantigen is an antigen capable of stimulating B cells independently of their antigenic specificity (203). Some of the Ig binding proteins belongs to this family of proteins. SpA and Protein L are two superantigens known to activate B cells (13, 115). MID of *M. catarrhalis* stimulates B cell proliferation by binding mIgD, and is thus considered to be a superantigen (72). The biological effects of these B cell superantigens are unclear. However, the superantigens are capable of interacting with the specific humoral immune response by activating B cells independently of their antigen specificity. A polyclonal response is induced by the superantigens, resulting in production of unspecific antibodies that most likely are not directed against the pathogen in question. The interaction of the superantigens with the B-cell receptor may lead to anergy or apoptosis of the B cell. In fact, SpA has been shown to induce apoptotic B cell death in a mouse model (204). However, the findings reported by Silverman and collaborators have not been possible to reproduce with human tonsillar B cells in vitro (16).

THE PRESENT INVESTIGATION

AIMS

The aims of the studies upon which this thesis is based were as follows:

- To investigate whether *H. influenzae* interacts with the classical pathway of the complement system by binding C4BP and to analyse the biological importance.

- To examine whether *H. influenzae* interacts with the alternative pathway by binding factor H and to analyse the functional importance.

- To analyse whether *H. influenzae* interacts with the terminal pathway by binding vitronectin and to identify the key molecule important for the interaction.

- To characterise the structural features of Hsf and provide evidence that it belongs to the autotransporter family.

- To identify the region of IgD responsible for the interaction with *H. influenzae* type b.

RESULTS AND DISCUSSION

H. influenzae *interferes with the classical pathway by binding C4BP (Paper I)*

In paper I, we describe an interaction between different NTHi isolates and the complement inhibitor C4BP. Complement resistance is crucial for bacterial virulence and binding of complement inhibitors such as C4BP and factor H is an efficient strategy used by several serum resistant pathogens (114, 193). The complement resistance mechanisms of *H. influenzae* have not yet been completely resolved. It has previously been shown that serum-resistant NTHi strains prevent MAC accumulation by delaying the synthesis of C3b through the classical pathway (242). By using both flow cytometry and a direct binding assay, it was shown in Paper I that most of the NTHi strains tested bound C4BP, whereas the majority of the typeable strains did not. The binding occurred also when serum was used as a source of C4BP. There was a significant difference in C4BP binding between two NTHi strains (506 and 69) and this difference may explain why one C4BP-binding strain 506 was serum resistant while the non C4BP-binding strain 69 was sensitive to killing by human serum. When C4BP was depleted from serum, a statistically significant decrease in survival was seen with NTHi strain 506, suggesting that binding to C4BP indeed protected the bacteria against the complement system. Our findings are in contrast to what has been shown in a recent study, in which no binding of *H. influenzae* to C4BP was found (88).

Several different experiments, including bactericidal assay, antibody and C3 deposition in flow cytometry were performed to ascertain the relevance of the classical pathway. When inhibiting the classical pathway with Mg-EGTA, a significant increase in the survival of the NTHi strain investigated was observed. By using flow cytometry, a significant decrease in C3b deposition was also seen when the classical pathway was inhibited, which further corroborated this hypothesis. Moreover, it is a well known fact that the classical pathway is initiated when antibodies bind to bacteria. In our experiments, both IgG and IgM were deposited on the surface of the bacteria, suggesting an initiation of the classical pathway.

The ability of *H. influenzae* to bind C4BP suggests that the species uses the capacity of C4BP to inhibit complement-mediated attacks. A critical question was whether C4BP was still active and able to exert its complement regulatory functions when bound to the bacterial surface. To analyse this, cofactor assays were performed. C4BP bound to the surface of *H. influenzae* maintained its activity to degrade C4b, and C3b in the presence of factor I, which affects all three pathways of complement (Fig. 12). Consequently, such degradation prevented C4b and C3b from participating in the opsonisation of the pathogen. In

addition, the surface bound C4BP also binds C4b and thereby inhibits C3 convertase formation and accelerating its decay.

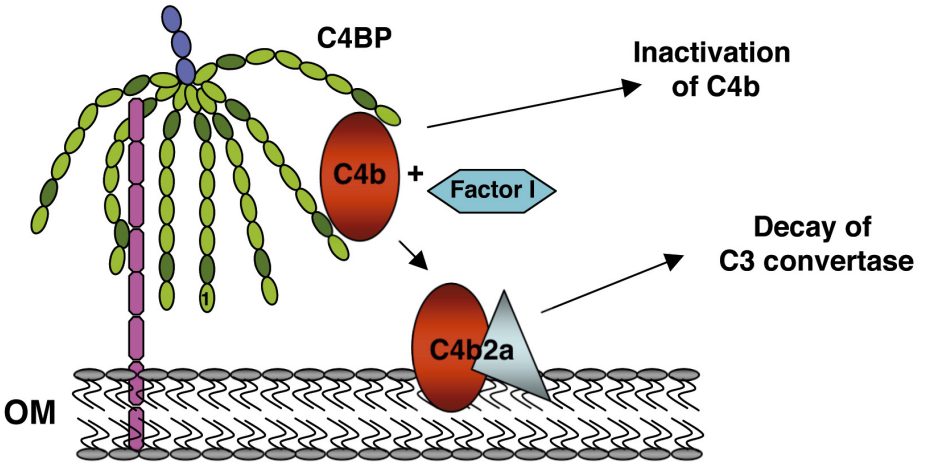

Figure 12. Bacterial evasion of complement by binding C4BP. C4BP bound to the surface of NTHi retains its functions, including inhibition of C3 convertase formation and acceleration of its decay. The binding domains CCP2 and CCP7 are indicated by dark green.

To further analyse the interaction between NTHi and C4BP, we wanted to localise the binding site on the C4BP molecule. Binding experiments in whole cell ELISAs with rC4BP mutants, each deficient in one CCP domain, suggested that CCP2 and CCP7 were involved in the binding. Previously, many of microbial binding sites on C4BP have been localised to CCPs 1-3. As this is the same region required for the binding of C4b, one could speculate that binding to microbial ligands inhibited the regulatory function of C4BP. However, C4BP is a polymer of 7 identical α-chains, which allows simultaneous binding of several different ligands even if they utilize overlapping binding site.

H. influenzae *interferes with the alternative pathway by binding factor H (Paper II)*

In this paper we investigated whether *H. influenzae* interacts with the alternative pathway by binding the regulator factor H. A collection of different *H. influenzae* strains was incubated with [^{125}I]-labelled factor H followed by separation of unbound ligand in a direct binding assay. Interestingly, the majority of the *H. influenzae* strains bound factor H. Our findings are in contrast to what has been shown in a recent study, in which no binding of *H. influenzae* to

factor H was found (57). Amongst all the strains tested, *H. influenzae* types b (Hib), c and f showed the highest factor H binding, whereas the other *H. influenzae* strains bound factor H to a varying extent. The Hib strains used in this study showed strong factor H binding and were chosen for further studies. When serum was used as a source of factor H, all the Hib strains tested bound factor H and some of them bound FHL-1.

To evaluate the physiological significance of the interaction between factor H and *H. influenzae*, a serum bactericidal assay was performed. The approach was to compare a low factor H-binding and a high factor H-binding strain in terms of survival. The results showed that the low-binding strain *H. influenzae* type d (Hid 555) was more sensitive to killing by human serum than the high factor H-binding strain Hib 541, suggesting that the bacteria use the capacity of factor H to inhibit complement-mediated attacks. After 7.5 min, more than 50 % of Hib 541 survived as compared to Hid 555 that only showed an 11 % survival. Thus, the *H. influenzae*-dependent interaction with factor H is important for bacterial serum resistance.

Factor H regulates the alternative pathway activity by serving as a cofactor for factor I in the degradation of C3b, which results in the appearance of the cleaving fragments α´68-kDa, 46 kDa, and 43 kDa. It is very important for the pathogen that the regulator maintains its regulatory functions when bound to the surface of the bacteria. Functional analysis showed that factor H maintained cofactor activity when bound to the surface of Hib and promoted factor I-mediated C3b inactivation (Fig. 13). The cleavage of C3b was determined by the detection of the three cleavage products (α´68-kDa, 46 kDa, and 43 kDa) after separation in SDS-PAGE.

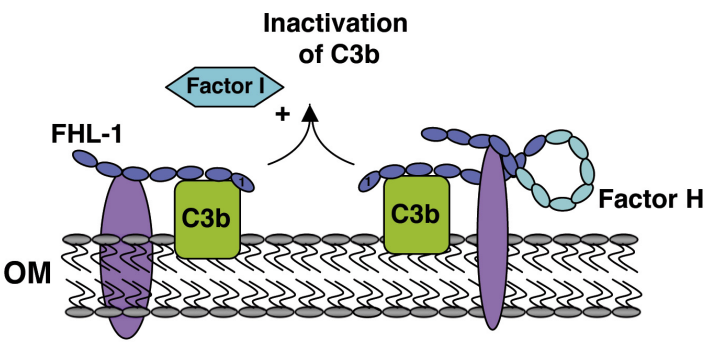

Figure 13. Factor H bound to the surface of *H. influenzae* retains its functions, including inactivation and acceleration of the decay of the C3 convertase of the alternative pathway. The SCRs important for the interaction with Hib is coloured in blue.

To investigate which part of the factor H molecule involved in the interaction between Hib and factor H, we used whole cell ELISAs to test the ability of different fragments spanning the entire factor H molecule to bind Hib. Interestingly, two binding domains were identified within the molecule; one binding site common to both factor H and FHL-1 was located in the N-terminal, whereas one was located in the C-terminal SCRs 15-20 that only can be found in factor H. Heparin inhibited the interaction between Hib 541 and factor H in the direct binding assay using [^{125}I]-factor H, suggesting involvement of the heparin binding domains. The heparin binding domains of factor H is located in SCRs 7, 13 and 19-20 and in FHL-1 in SCR 7 (189).

Hsf binds vitronectin and thus interferes with the terminal pathway (Paper III)

Some pathogens have been shown to interact with vitronectin, the inhibitor of the MAC of the terminal pathway (12, 33). To analyse whether Hib bound soluble vitronectin, flow cytometry was performed. The results show a strong binding to vitronectin. We wanted to investigate whether the outer membrane protein *Haemophilus influenzae* surface fibrils (Hsf) was responsible for the interaction with vitronectin. To analyse this, *H. influenzae* type b strains (RM804 and Eagan) were mutated by introduction of a kanamycin resistance gene cassette in the gene encoding for Hsf. Resulting mutants were confirmed by PCR, flow cytometry and Western blot. The two latter methods showed a lack of Hsf expression. Flow cytometry analysis of the Hsf-deficient mutant revealed that Hsf is the major vitronectin binding protein in encapsulated *H. influenzae*. These findings were in contrast to a previous study, where no binding to soluble vitronectin could be detected (48). In addition, we showed *H. influenzae*-dependent binding to immobilised vitronectin. To further confirm that Hsf bound vitronectin, *E. coli* transformants expressing Hsf was constructed, and experiments with these transformants demonstrated a Hsf-dependent binding to both soluble and immobilised vitronectin.

To analyse the biological significance, the Hsf-deficient mutants and their corresponding wild types were analysed in a serum bactericidal assay. The *H. influenzae* devoid of Hsf had a markedly reduced survival as compared to the wild type when exposed to human serum. The ability to bind vitronectin suggests that Hsf uses the capacity of vitronectin to inhibit complement-mediated attacks.

The vitronectin molecule contains three heparin-binding domains: residues 82-137, 175-219, and 348-376. To analyse which part of the vitronectin molecule involved in the

Hsf interaction, inhibition experiments with heparin were performed, using flow cytometry. Heparin inhibited the binding between *H. influenzae* and vitronectin, and blocking experiments using peptides encompassing vitronectin $^{341-370}$ suggested that the other two regions (residues 82-137 and 175-219) were involved in the interaction.

Vitronectin is also a component of the ECM, and binding to exposed ECM components may facilitate adherence, which is an essential step in bacterial pathogenesis. Moreover, to investigate which part of Hsf that was involved in the vitronectin binding, several recombinant fragments spanning the entire molecule were constructed. Hsf $^{608-1351}$ and Hsf $^{1536-2414}$ displayed the highest binding to vitronectin in ELISA. These binding sites also contain amino acids involved in the attachment to epithelial cells (38). As a consequence, the Hsf molecule may be able to bind two vitronectin molecules and stabilising adherence despite the physical forces in the respiratory tract, including the mucociliary escalator, sneezing, and coughing.

Hsf is a double-folded autotransporter protein (Paper IV)

Outer membrane proteins of Gram-negative pathogenic bacteria comprise an important group of virulence determinants highly specialised in a variety of functions most of which are targeting the host. Hsf is an outer membrane protein of approximately 245 kDa belonging to the autotransporter protein family. The aim of the present study was to in detail study how Hsf is expressed at the bacterial surface and to further prove that it belongs to the autotransporter family. Initially we analysed the Hsf expression in a collection of encapsulated *H. influenzae* strains by using flow cytometry. The results showed a large variation in Hsf expression. The majority of the *H. influenzae* type b strains expressed Hsf on the surface of the bacteria, whereas the Hsf expression of types c and type d varied. Types a and f were negative for Hsf when analysed by flow cytometry. The differences in Hsf expression suggested that phase variation occurred, since all isolates contained the *hsf* gene. The presence of *hsf* was analysed by Southern blot. The conservation of the gene indicates that the product plays an important role in the life cycle of the bacteria (211). It is very important to analyse virulence factors by the different serotypes. Thus, since the introduction of the vaccine against serotype b, the other serotypes have been more frequent causes of invasive diseases (1, 161, 228).

Hsf has been suggested to be a trimeric autotransporter, but this suggestion was mainly based on sequence alignment with the related outer membrane protein Hia (38). We wanted to further evaluate Hsf as an autotransporter. Three mutants were constructed by inserting antibiotic resistance cassettes in the N-terminal and/or the C-terminal ends of the

gene (Fig. 14). When the translocating C-terminal end of the *hsf* gene was inactivated, Hsf could be detected in the outer membrane extractions as analysed by Western blots. However, flow cytometry and transmission electron microscopy (TEM) showed a lack of expression on the bacterial surface, suggesting an expression of the protein but inhibition of the membrane insertion.

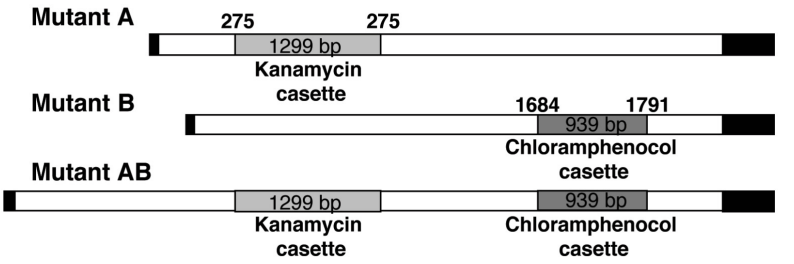

Figure 14. A Schematic drawing of the three mutated *hsf* genes with antibiotic resistance gene cassettes indicated.

Three immuno-purified and gold-labelled antibodies were used in TEM to in detail examine the ultrastructural features of Hsf. These antibodies were directed against the N-terminal, C-terminal and the middle region of the Hsf molecule. The results showed a 100 nm, double-folded structure (Fig. 15). Both the antibody directed against the N-terminal and the C-terminal bound close to the membrane of the bacteria. In addition, an antibody used against the middle part of the molecule bound at the very tip of the protein.

Both outer membrane vesicles (OMVs) and free Hsf was observed in the surroundings of the bacteria when analysed by TEM and specific anti-Hsf antibodies. OMVs (or blebs) are produced in vitro during growth in broth and are thought to carry some periplasm together with OMP, porins, receptors and lipopolysaccharide (LPS) from the outer membrane layer (21, 118). Blebs are suggested to have many functions, for example interacting with host cells deep in tissues that are not accessible by infecting bacteria, activate immune cells and induce leukocyte migration, and escape immune detection during colonisation (5, 67, 163). Both Western blot and TEM showed a presence of Hsf on the blebs, suggesting a role outside the bacteria. Furthermore, Hsf was both tethered to the surface of the bacteria and was released into the extra-cellular milieu, also proven by both Western blot and TEM. Most known autotransporters undergo processing and is released into the extracellular

milieu (45, 84) and so does Hsf, further supporting the hypothesis as an autotransporter protein.

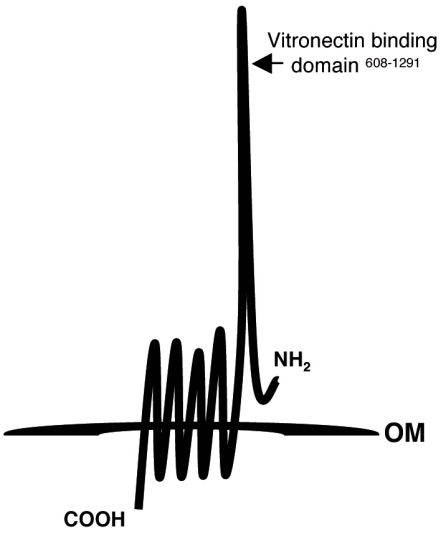

Figure 15. Schematic illustration of the Hsf protein on the bacterial surface. TEM showed that Hsf has a 100 nm long, double-folded structure with one of the vitronectin binding domains at the tip of the protein.

Haemophilus influenzae ***type b binds human IgD (Paper V)***

Previous studies have shown that *H. influenzae* bind human IgD in a non-immune fashion (61, 198). The ligand responsible for this interaction has not yet been identified. A collection of different NTHi and Hib isolates were analysed for IgD binding by using a whole-cell ELISA and flow cytometry. The results revealed that 50 % of the Hib strains displayed IgD-binding capacity, whereas the NTHi lacked this ability. These results confirmed previously published data by Sasaki and coworkers (198). Two strains of the Hib collection were chosen for further studies; one isolate demonstrating high IgD binding and one moderate IgD binding. ELISAs were performed to characterise the binding region on the IgD molecule responsible for the interaction with *H. influenzae*, using a series of recombinant IgD chimeras. A part of the IgD molecule was replaced by part of an IgG molecule in these chimeras. Replacing amino acids 157-224, 167-206 or 185-224 of the IgD molecule by the corresponding IgG fragments, resulted in an abolished binding by Hib. In contrast, the replacement of IgD amino acids 157-

197 with IgG did not affect the interaction. Thus, these results show that the C_H1 region of the IgD molecule, namely amino acids 198-224, is responsible for the interaction between Hib and IgD. Flow cytometry analysis confirmed these results. *M. catarrhalis* is another respiratory pathogen shown to bind IgD (60). Interestingly, MID is responsible for the interaction with IgD and the binding site on the IgD molecule is located to the same region as *H. influenzae*, i.e., the C_H1 region (196). We have shown an interaction between *H. influenzae* and IgD, and together with the previously reported *M. catarrhalis*-IgD interaction, these observations suggests that these interactions may be involved in the pathogenesis of respiratory pathogens.

CONCLUDING REMARKS

Microbes often interact with human complement regulatory proteins in order to conquer the innate immune system. Complement resistance is crucial for bacterial virulence and binding of complement inhibitors such as C4BP, factor H and vitronectin is an efficient strategy used by several serum resistant pathogens. In Papers I-III we studied the interactions of *H. influenzae* with the classical/lectin, the alternative and the terminal pathways of the complement system. We demonstrated interactions with C4BP, factor H and vitronectin, all regulators of the complement system. All these interactions contributed to increased survival of *H. influenzae* in human serum and, consequently, most likely contributed to their pathogenicity. Hsf was shown to be the protein responsible for the vitronectin-binding of Hib (Paper III). Vitronectin is also a component of the ECM. Thus, the interaction between Hsf and vitronectin, not only prevent complement induced cell lysis, but may also contribute to bacterial colonization and spread of *H. influenzae*. We further characterised the structure of Hsf and its properties as an autotransporter (Paper IV). Hsf has a double-folded structure exposing one of the vitronectin-binding domain at the tip of the protein. Hsf was also present at the surface of OMVs and secreted into the extracellular milieu. To in detail studying the virulence mechanisms of microbes helps us to understand how the pathogens evade the host immune defence and how our immune system works. This may give us tools to be able to prevent infections. Binding complement regulators are important virulence mechanisms of *H. influenzae*. The best way to block a cascade for a pathogen is to inhibit every subsequent step in the cascade and the best evasion strategy is to interact with many of the attacking steps.

Finally, we have also been able to prove an interaction between *H. influenzae* and human IgD using recombinant material and to further characterise the binding domain of

IgD involved (Paper V). Further studies need to be done to evaluate the functional importance of this interaction.

FUTURE PERSPECTIVES

In the first two papers we show that *H. influenzae* interacts with both the classical and the alternative pathway of the complement system. These interactions affect the survival of the bacteria. In the first paper, we identified that NTHi binds the regulator C4BP and it would be of high interest to find the ligand in NTHi responsible for the interaction. In addition, we demonstrated an interaction of *H. influenzae* and the inhibitor of the alternative pathway, factor H (Paper II). The protein responsible for the binding of factor H is also unknown and would be very interesting to identify.

Hsf has been identified as the major vitronectin binding protein in *H. influenzae* type b (Paper III) and this interaction contributes to serum resistance. Vitronectin is an inhibitor of MAC in the terminal pathway of the complement cascade and it is also a component in the ECM. Hsf is an adhesin and has been shown to interact with epithelial cells, but the receptor for the interaction is not known. Vitronectin has been found on the surface of some epithelial cells. Thus, investigations to evaluate the role of vitronectin in the Hsf-dependent adhesion of *H. influenzae* would be interesting experiments.

Hsf has been suggested to be a trimeric autotransporter based on sequence analysis (38). In paper IV we have mapped the ultrastructure of Hsf by using gold-labelled antibodies and investigated whether Hsf is an autotransporter. By using scanning transmission electron microscopy (STEM) combined with the molecular weight and the precise length of the protein, the oligomeric structure of Hsf could be established. In addition, another proof of Hsf as an autotransporter would be to identify the translocator domain of Hsf. *H. influenzae* secretes OMVs expressing Hsf and additional experiments evaluating the biological importance of this mechanism are needed.

The respiratory pathogens *M. catarrhalis* and *H. influenzae* bind IgD (61). An interaction between recombinant IgD with a defined antigen binding site and *H. influenzae* type b was identified (Paper V), and it would be of highest interest to find the protein responsible for the interaction. The function of IgD is not fully understood and it would be tempting to further evaluate the role of the *H. influenzae*-IgD interaction.

SVENSK POPULÄRVETENSKAPLIG SAMMANFATTNING

Haemophilus influenzae är en bakterie som orsakar flera olika sjukdomar hos människa. Den kan delas in i typbara och icke-typbara beroende på förekomsten av en polysackaridkapsel. De typbara kan delas in i 6 olika typer (a-f), där typ b är den som är mest virulent och ger svårast sjukdomar. *H. influenzae* typ b (Hib) kan bla orsaka struplocksinflammation (epiglottit) och hjärnhinneinflammation (meningit). Dock har antalet sjukdomsfall orsakade av typ b sjunkit drastiskt sedan ett vaccin infördes på 1990-talet. De icke typbara saknar kapseln och orsakar mindre svåra sjukdomar som tex bihåleinflammation (sinuit), ögoninflammation (konjunktivit) och öroninflammation (media otit). Patienter med kronisk obstruktiv lungsjukdom (KOL) och cystisk fibros blir ofta infekterade med icke typbara *H. influenzae*. Denna icke typbara är efter Pneumokocker en vanlig orsak till öroninflammation hos barn. Det finns ännu inget vaccin mot dessa bakterier. Det finns ett stort forskningsintresse för att utreda hur och varför dessa bakterier överlever vårt immunförsvar och kan orsaka sjukdom.

När en inkräktare kommer in i kroppen så möter den vårt immunförsvar. En av de första delarna av detta försvar är en kaskad av proteiner som kallas komplementsystemet. Aktivering av denna kaskad leder till att olika proteiner samverkar med varandra med målet att oskadliggöra inkräktaren. Komplemensystemet kan delas in i tre vägar beroende på hur de aktiveras. Den klassiska vägen aktiveras av antikropparna IgG och IgM, den alternativa av bindning till mikrobens yta och lektinvägen av bindande till sockermolekyler på bakteriens yta. Alla dessa vägar går ihop till den terminala vägen, som slutar i att en stor por bildas. Denna por tar hål på bakteriens membran (lyserar) och dödar den. Detta system är strikt kontrollerat av regulatorer så att det inte ger sig på och skadar våra egna celler. En av dessa regulatorer heter C4BP (C4b-binding protein) och vi visar i artikel I att icke typbara *H. influenzae* binder till denna regulator resulterade i längre överlevnad i humant serum. C4BP hämmar den klassiska vägen och lektinvägen av komplementsystemet och C4BP bundet till de icke typbara *H. influenzaes* yta är fortfarande funktionellt aktivt, dvs regulatorn kan fortfarande hämma komplementsystemet och på så sätt skydda bakterien mot lysering.

Detta är bara två av vägarna som leder till döden för bakterien så därför var det mycket viktigt att undersöka om den alternativa vägen också kunde blockeras. Vi såg att *H. influenzae*, både typbara och icke typbara, band faktor H (en hämmare av den alternativa vägen) (artikel II). Vi såg även här att faktor H fortfarande är aktiv (inhiberar alternativa

vägen) när den är bunden till bakteriens yta och att denna bindning gör så att den överlever längre i serum om man jämför med en icke-bindande stam.

De tre vägarna i komplementsystemet går alla ihop till en gemensam väg som kallas den terminala vägen. Denna vägen regleras också, bla av ett protein som heter vitronektin. Vitronektin hämmar uppbyggnaden av poren som lyserar och dödar mikroben. I artikel nummer III så visade vi att Hib binder till vitronektin och att proteinet som står för bindningen heter *Haemophilus influenzae* surface fibrils (Hsf). Bindningen mellan Hsf och vitronektin bidrog till att bakterien överlevde bättre i humant serum om man jämför med en mutant som ej uttrycker Hsf. Mutanten dog mycket fortare än vildtypen. Vitronektin är även en viktig komponent i extracellulär matrix och bindningen till vitronektin kan förmodligen hjälpa *H. influenzae* när den ska adherera till epitelcellerna i början av en infektion.

Bakterier uttrycker olika proteiner på sin yta och dessa proteiner interagerar med värden på olika sätt för att överleva. Det är viktigt att karaktärisera dessa proteiner för att få mer kunskap om hur bakterien klarar av att starta en infektion och överleva vårt försvar. Om man har kunskap om dess struktur och med vad den interagerar med hos värden har man kommit en bra bit på vägen för att kunna tillverka bättre läkemedel som kan angripa bakterien. Med hjälp av elektronmikroskopi och antikroppar riktade mot olika delar av proteinet så karaktäriserade vi Hsf. Resultatet visar att Hsf är 100 nm långt, dubbelvikt och sitter ankrat med den C-terminala delen i membranet. Vidare så klyvs proteinet (Hsf) också av och utsöndras i den omgivande miljön.

Vi har visat att *H. influenzae* typ b binder humant immunoglobulin D (IgD) som är en av fem olika typer av antikroppar vi har i kroppen. Dessa antikroppar är medlemmar av vårt immunförsvar och de känner igen främmande ämnen (antigen) som kommer in i kroppen och hjälper till att eliminera dem. IgD finns både membranbundet på humana B-celler och lösligt i plasma men man vet inte riktigt funktionen hos lösligt IgD. *H. influenzae* binder C_H1 regionen av IgD och det är samma region som en annan viktig luftvägspatogen, *Moraxella catarrhalis*, binder till. Att två olika luftvägspatogener binder i samma område visar att det är en viktig interaktion för bakterien. Vidare forskning krävs för att undersöka betydelsen av denna interaktion.

Sammanfattningsvis så presenteras i denna avhandling hur *H. influenzae* interagerar med komplementsystemet genom att binda tre av dess inhibitorer och därmed förbättra sin överlevnad i humant serum. Vi karaktäriserar även proteinet Hsf och visar att *H. influenzae* binder humant IgD.

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