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Haemophilus influenzae adhesins and the innate immune response

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LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their roman numerals (I-IV):


III. Brant, M., E. Ronander, A. Forsgren, and K. Riesbeck. 2007. *Haemophilus influenzae* Protein E (PE) is a novel adhesin with the active binding site within the central part of the molecule. Submitted to *Journal of Infectious Diseases*.


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ABSTRACT

*Haemophilus influenzae* is a human specific Gram-negative respiratory tract pathogen. Encapsulated *H. influenzae* strains exist in six different serotypes a-f, of which type b (Hib) is the most virulent. Hib is protected by the polysaccharide capsule when invading the blood circulation and may cause diseases such as meningitis and epiglottitis. Non-encapsulated *H. influenzae* referred to as nontypeable *H. influenzae* (NTHi), are frequently associated with acute otitis media in children and chronic obstructive pulmonary disease (COPD) among the elderly. *H. influenzae* expresses several outer membrane proteins of which the adhesins play an important role in the initial interactions with the airway epithelium.

In this thesis, we have studied two *Haemophilus influenzae* adhesins and their interactions with innate defense mechanisms. Vitronectin is a glycoprotein that regulates the terminal pathway of the complement system by inhibiting the membrane attack complex. We demonstrate that *Haemophilus influenzae* surface fibrils (Hsf) may increase the bacterial survival by interacting with vitronectin in serum.

We also describe the isolation and characterization of a novel *H. influenzae* adhesin designated protein E (PE). PE was isolated by using an IgD myeloma serum which specifically recognized the protein on the bacterial surface. Furthermore, PE was found to be a 16 kDa protein and classified as a lipoprotein. PE displays adhesive activity to three respiratory epithelial cell lines and erythrocytes, both when expressed on the surface of *H. influenzae* and as a recombinant protein in *E. coli*. The active adhesive binding domain has been determined to be located within the central part of the molecule, PE amino acids 84-108. PE shows stimulatory effects when incubated with respiratory epithelial cells by inducing high levels of IL-8 and ICAM-1. These inflammatory mediators play an important role for the innate immune response and the recruitment of neutrophils. Finally, PE promotes bacterial invasion into epithelial cells.

In summary, the work presented in this thesis shows that Hsf may prolong the bacterial survival by interacting with the complement regulator vitronectin. The novel protein PE has been isolated, characterized and shown to be important for interactions of *H. influenzae* with the innate immune system.
ABBREVIATIONS

AOM  acute otitis media
CEACAM-1  carcinoembryonic antigen family of cell adhesion molecules-1
COPD  chronic obstructive pulmonary disease
Hib  *Haemophilus influenzae* type b
Hsf  Haemophilus surface fibrils
Ig  immunoglobulin
ICAM-1  intercellular adhesion molecule-1
IL-6  interleukin-6
IL-8  interleukin-8
kDa  kiloDalton
LOS  lipopolysaccharide
MAC  membrane attack complex
MCP-1  monocyte chemotactic protein-1
MID  Moraxella IgD-binding protein
NTHi  nontypeable *Haemophilus influenzae*
OMPs  outer membrane proteins
ORF  open reading frame
PE  protein E
SDS-PAGE  sodium dodecyl sulphate polyacrylamide gel electrophoresis
TLR  toll like receptor
TNF-α  tumor necrosis factor-α
INTRODUCTION

*Haemophilus influenzae*

**General characteristics**

*Haemophilus influenzae* was first isolated in 1892 by Robert Pfeiffer from the sputum of patients with influenzae during the influenzae pandemics (120). The bacterium was mistakenly considered to be the cause of influenzae, until Smith in 1933 proved that influenzae had a viral etiology (151). The name Haemophilus originates from the Greek haemophilus, meaning “blood-loving” reflecting its growth needs of both hemin and NAD (nicotinamide adenin dinucleotide) that is present in lysed red blood cells (42).

*H. influenzae* is a Gram-negative, small, rod-shaped, facultative anaerobic bacterium that only infects humans. The genus of Haemophilus, which belongs to the family of Pasteurellaceae, comprises more than twenty species including *H. influenzae*, *H. ducreyi*, *H. parainfluenzae*, *H. parahaemolyticus*, and *H. haemolyticus*. *H. influenzae* is the most pathogenic species among these (62).

**Classification**

In 1931 Margret Pittman defined the two major categories of *H. influenzae*, encapsulated and nonencapsulated strains (122). Encapsulated strains were divided into six distinct capsular serotypes, designated a-f, where type b (Hib) is the most virulent (169). The nonencapsulated are also called nontypeable because they do not have the ability to react with antisera against recognized capsulated forms of *H. influenzae*.

**H. influenzae type b (Hib)**

Hib is associated with invasive infections such as meningitis, epiglottitis, and sepsis especially in infants and young children (169). Since the Hib protein-polysaccharide conjugate vaccines were introduced into many industrialized countries in the late 1980s, Hib-related diseases and Hib carriage rates have nearly been eliminated (85, 115). Nevertheless, Hib is still a cause of high mortality among young children and adults in many developing countries due to underdeveloped vaccination programs (26).

*H. influenzae* strains of other capsular serotypes than b have for long time been considered as nonvirulent, and have rarely been reported to cause any disease. Lately, there have been several reports showing an increased incidence of invasive diseases
caused by H. influenzae serotypes a, e and f among the vaccinated population, suggested as being a possible trend towards Hib replacement with another capsule type (2, 22, 171).

Polysaccharide capsule
The polysaccharide capsule of Hib is composed of polyribosyl ribitol phosphate (PRP) and is the principal virulence factor of this organism (34). The capsule facilitates transmission between individuals due to reduced risk of desiccation in the environment. During systemic infection, the capsule inhibits serum bactericidal activity and complement-mediated phagocytosis (117). In vitro, Hib capsulation has been shown to variate according to environmental factors (pH, Mg$^{2+}$, nutrition) (157). Moreover, capsule material is released during growth in broth (7).

The capsule expression is genetically unstable and is dependent on the arrangement of the capsule genes. The capb locus is composed by a duplication of 18 kbp DNA segments joined by a 1.3-kbp bridge region (75, 94). Recombination events between the two copies of the repeat result in deletion of one copy and disruption of the bridge region. Since the bridge region contains a gene encoding a protein that is required for the export of the capsule this event results in loss of capsule expression (75, 93).

Nontypeable Haemophilus influenzae (NTHi)
NTHi is normally found in the human pharyngeal flora, whereas the tissue below the vocal cords, including the lower respiratory tract, is kept sterile or has a low level of colonization in healthy individuals (112).

The asymptomatic carriage rates of NTHi strains in the nasopharynx are high in healthy children; ranging from 25-84 % (20, 168). Later in life, the presence of NTHi decreases and is relatively rare among adults. Immunocompromized individuals due to specific immune defects such as deficiencies in humoral immunity, underlying viral or chronic infections, are more predisposed to NTHi infections as compared to others (14, 129). In addition to its role as a commensal, NTHi is a common cause of respiratory tract infections among children such as acute otitis media (AOM), conjunctivitis, acute sinusitis, children with cystic fibrosis and community-acquired pneumonia, especially in the developing countries (111). In adults, NTHi is implicated in chronic infections of the lower respiratory tract, such as chronic bronchitis and chronic obstructive pulmonary disease (COPD) (112).

Acute Otitis Media (AOM)
AOM is an inflammatory disease that begins in the nasopharynx and continues via the middle ear to the eustachian tube leading to accumulation of fluid that can give
symptoms such as headache, pain and hearing loss. The majority of all bacterial isolates taken from middle ear fluid samples of children affected by AOM are *Streptococcus pneumoniae*, NTHi and *Moraxella catarrhalis* (141). However, the proportion of AOM due to NTHi has increased since the introduction of the *S. pneumoniae* heptavalent conjugate vaccine (17, 35). In many countries the occurrence of otitis media is a burden due to the high prevalence and the high medical costs of treatment (33). These factors are strong indicators that there is a need for a prophylaxis, such as a vaccine.

**Chronic obstructive pulmonary disease (COPD)**

COPD is a condition characterized by airflow limitation and chronic elevated airway inflammatory markers that may lead to severe lung damage and subsequently death (112). In half of all COPD cases bacteria can be isolated from the sputum samples; NTHi, *M. catarrhalis* and *S. pneumoniae* being the most common (149). Known factors that contribute to increased risk of developing COPD are immunosuppression due to tobacco smoke or oxidant stress derived from pollutants (14).

**Outer membrane proteins (OMPs)**

More than 36 different OMPs from *H. influenzae* have been isolated and characterized. The first proteins that were described were in the order of decreasing molecular weight. These are considered to be major OMPs and include P1, P2, and P4-P6 (99). Other proteins such as the transferrin binding protein 1,2 (Tfb1/Tfb2) and protein D belong to the minor OMPs of *H. influenzae* (123). Several of the *H. influenzae* OMPs function as adhesins and are described in more detail on page 17. In Table 1 (p 22) there is an overview of the *H. influenzae* OMPs and their interaction with eukaryotic target molecules.

**Protein 1 (P1)**

P1 is a heat-modifiable protein of 35-50 kDa that accounts for approximately 10% of the OMP content (106). P1 is highly immunogenic and has been shown to induce protective antibodies against NTHi-induced otitis media in chinchillas (108). However, P1 undergoes high antigenic variation in its surface-exposed epitopes which complicates the selection of P1-regions that are important for the pathogenesis (19, 107).

**Protein 2 (P2)**
P2 is a porin that accounts for 50% of the OMP content and is thus the most abundant outer membrane protein of *H. influenzae* (113). According to structural analysis, P2 forms a trimer and allows molecules up to 1400 Da to diffuse across the membrane (32). Moreover, P2 binds to mucin by interacting with sialic acid-containing oligosaccharides (132). The amino acid sequence of P2 is highly variable in NTHi strains (49). During the course of an infection, specific regions of P2 vary at high frequency, in particular a surface exposed loop which undergoes mutations at high rate (38).

It has been demonstrated that P2 plays an important role for the upregulation of proinflammatory cytokines in CNS-cells as tested in the rat brain (54).

**Protein 4 (P4)**

P4 is a lipoprotein that is highly conserved among NTHi and Hib strains, and has been shown to be important for the bacterial growth (59). *H. influenzae* lacks all the enzymes needed for the synthesis of the porphyrin ring, and is therefore unable to synthesize protoporphyrin IX (PPIX), the immediate precursor of heme. Thus, the bacteria has an absolute requirement for PPIX or heme (42). P4 has been found to be one component in the heme-acquisition pathway uptake in *H. influenzae* (105, 133). Moreover, P4 has been proven to be an acid phosphatase and by its enzymatic activity P4 is implicated in the bacterial utilization of NAD, which is another essential growth factor of *H. influenzae* (134, 135).

**Protein 6 (P6)**

P6 is a highly conserved peptidoglycan-associated lipoprotein among *H. influenzae* strains (109). Similar to other lipoproteins that contain an N-terminal cysteine-tripalmitoyl (Cys-Pam₃) motif, P6 shows strong immunoregulatory effects. P6 triggers high release of IL-8 and TNF-α in macrophages (15). In respiratory epithelial cells, P6 upregulates the mucin gene transcription leading to overproduction of mucin, which is a hallmark of diseases such as COPD (23).

*Transferrin binding proteins 1 and 2 (Tfb1 and Tfb2)*

*H. influenzae* have several proteins that are involved in the bacterial acquisition of bound iron from sources such as transferrin in serum (71). The transferrin-binding protein 1 and 2 (Tbp1 and Tbp2) are the most important ones since the lack of either protein severely impairs the bacterial growth (58). Tbp2 serves as surface receptor for transferrin and Tfb2 is responsible of the transferrin transport (123).

**Protein D (PD)**
When PD was isolated it was considered to be an NTHi IgD-binding protein (140). It was later shown that PD bound only to certain IgD-myelomas tested and it was suggested that PD was recognized by the IgD myeloma proteins through its antigen binding fragment (Fab) (144).

PD is a highly conserved lipoprotein that is present in both NTHi and Hib strains (81). PD is not by definition an adhesin, however it indirectly promotes bacterial adhesion and invasion due to glycerolphosphodiester phosphodiesterase activity which is required for the transfer of choline from the host to the LOS of H. influenzae (43, 52, 110). ChoP⁺-LOS variants promote the bacterial invasion by signaling via the PAF-receptor (164, 165). PD has also been shown to promote bacterial adhesion and internalization into human monocytes (4).

PD expression is important for the NTHi virulence in the upper respiratory tract as shown in both in vivo and in vitro experiments. In a rat model of otitis media, a PD-deficient strain displayed a 100-fold decreased virulence as compared to the PD-expressing strain (80). Moreover, a PD-expressing strain was shown to cause significantly higher damage to cilia in a human nasopharyngeal ex vivo tissue culture model as compared to a PD-deficient strain (79). PD has also been shown to induce protective antibodies against NTHi otitis media in rat and chinchilla models (123).

Due to the properties of PD, such as surface localization, high degree of antigenic conservation, wide distribution, pathogenicity, and promising preclinical trials, it was decided to use PD as antigenically active carrier protein in a new 11-valent pneumococcal conjugate vaccine (52). In a randomized double-blind efficacy study, it was shown that by using PD as a carrier protein for pneumococcal polysaccharides, the vaccine induced protection both against pneumococcal otitis and acute otitis media due to NTHi (127).

**Adhesion and invasion**

The adhesion to the human epithelium is a crucial and necessary step for a respiratory tract pathogen in order to survive, multiply, and gain access into deeper tissues. The numerous H. influenzae adhesins that have been characterized reflect the complexity and importance of this virulence property. They are mainly divided into two groups; adhesins that are extending out from the bacterial surface into hairlike appendages are called pili or fimbriae whereas the adhesins that are directly associated with the microbial cell surface are called non-pilus adhesins.

The adherence and the subsequent colonization process is a dynamic event where the bacteria may upregulate the expression of new genes related to various events during the interaction. During NTHi interaction with a lung epithelial cell line, genes that were found to be upregulated were involved in the metabolic processes,
stress responses, gene expression, cell envelope biosynthesis processes, DNA-related processes and cell division, and open reading frames (ORFs) encoding proteins of unknown functions (177).

By sheltering in or between epithelial cells *H. influenzae* can escape antibody-mediated killing and the action of antibiotics from the extracellular space (176). Invasion into host cells is not a rare mechanism among respiratory tract pathogens and has been reported to occur with *S. pneumoniae, M. catarrhalis* and *Staphylococcus aureus* (27, 63, 166).

As early as in 1953 Hers and Mulder examined tissue sections from patients affected by acute and chronic muco-purulent bronchitits and found NTHi organisms between and beneath the epithelial cells (72). Another studie revealed that both capsulated and nonencapsulated *H. influenzae* incubated with adenoidal tissue in organ culture had the ability to cause disruption of epithelial tight junctions and bacteria were later found in clusters between epithelial cells (44).

Hib strains have a reduced ability to adhere to and subsequently invade epithelial cells as compared to nonencapsulated strains (5, 161). One explanation for this is that the polysaccharide capsule constitutes a steric hindrance for the interaction with the host cells (157). Consequently, studies on bacterial invasion mechanisms of respiratory epithelium have predominantly been performed on NTHi strains. A series of electron and confocal microscopic analyses revealed that NTHi incubated with primary bronchial epithelial cells initiated cytoskeletal rearrangements by the extension of microvilli and formation of lamellipodia in a process called macropinocytosis (86). A second invasion pathway was described when NTHi was shown to invade bronchial cells by the signaling through the platelet-activating factor (PAF) receptor via certain glycoforms containing phosphorylcholine on LOS (164, 165). This form of invasion was shown to be strongly dependent on protein D (43, 110). Finally, the last invasion pathway described involved the \( \beta \)-glucan receptors on monocytes since the bacterial entry could be partially blocked by laminarin (Ahren 2000).

It was early defined that the *H. influenzae* invasion is dependent on *de novo* synthesis of proteins and required viable bacteria (160). The ability of adhesins to promote bacterial invasion has been described in other species. *Bordetella bronchispetica*, causing disease in several mammals, has filamentous hemmagglutinin (FHA) and the pertactin of which both act as adhesins as well as invasins into macrophages respectively epithelial cells (145). In NTHi, a number of surface structures have been associated with bacterial invasion e.g. protein D, LOS and Hap (4, 43, 110, 159, 164).

**Adhesins**
Pili
The *H. influenzae* pili are encoded by a gene cluster that contains five genes, designated *hifA-hifE* (121). They have functional and molecular homology to the Pap pili of *E. coli*, and equivalent to the Pap pili, their expression is regulated by phase variation (92, 175). A majority of the Hib strains express pili whereas only a minority of NTHi strains are encoded by the *hif* gene cluster (137). Pili hemagglutinate erythrocytes through the binding of Anton bloodgroup antigen (173). The same epitope mediates attachment to sialyl gangliosides on oropharyngeal epithelial cells (174).

Pili expression is important early in the infection. It has been shown that nonpiliated Hib strains display an impaired ability to colonize the nasopharynx of rats and Rhesus monkeys (8, 182). In addition, piliated Hib strains bind tracheobronchial mucin much better than nonpiliated strains (95). Later in the infection, pili seem to be unfavourable for the encapsulated bacteria since isolates taken from blood and cerebrospinal fluid have been shown to be nonpiliated (102).

Pili promote the bacterial formation of biofilm (114). A novel group of NTHi pili belonging to the subgroup type IV pili (Tfp), was recently characterized and was also found to be strongly associated with biofilm formation as tested in otitis media model in chinchillas (84). Similar to the Tfp expression in other pathogens, NTHi Tfp is important for the twitching motility of the bacteria (12).

Autotransporters
The autotransporter family is a large group of virulence proteins of G-bacteria that have the ability to mediate their own secretion via the outer membrane without the need of energy or accessory proteins (66). After the polyprotein has been exported through the inner membrane the signal-peptide is cleaved off and the C-terminal domain (translocator domain) forms a channel, of β-barrel structure, into the outer membrane and facilitates the translocation of the passenger domain (internal peptide) out to the surface where it remains cell-associated or is cleaved off (66) (Figure 1). Members of autotransporters have various functions, and in *H. influenzae* several adhesins have been found to belong to this group of proteins including Hap, HMW1/HMW2, Hia and Hsf.
Haemophilus adhesion and penetration protein (Hap)
Hap was isolated based on its ability to promote intimate interaction and subsequent invasion into epithelial cells (159). The adhesion of Hap to epithelial cells is mediated via components of the extracellular matrix; fibronectin, laminin and collagen IV (46). Together with the IgA1 proteases of Haemophilus and Neisseria species, Hap belongs to the serine protease autotransporter family (67). Hap and IgA1 proteases were among the first members to be characterized in this group and define the classical autotransporter protein. When the passenger domain has reached the surface it undergoes autoproteolysis mediated by the serine proteases, resulting in extracellular release of the Hap passenger domain (Hap$_s$) (68).

The uncleaved cell-associated Hap mediates adherence and is important for the bacterial aggregation and microcolony formation on the epithelial cell surface (70). An interesting finding is that the secretary leukocyte protease inhibitor (SLPI) inhibits the autoprooteolytic cleavage of Hap$_s$ resulting in increased density of Hap$_s$ on the bacterial surface which consequently leads to an increased Hap-mediated virulence (69). Since SLPI is important for the mucosal homeostasis (by blocking the action of neutrophil elastase) this host defense mechanism is in this particular case used in favour for the pathogen.

High-molecular-weight proteins 1 and 2 (HMW1 and HMW2)
HMW1 and HMW2 are encoded by separate loci, each containing three genes called $hmwA$, $hmwB$, and $hmwC$. The proteins are expressed by 75% of NTHi strains and by a few type a, e, and f strains (129).

HMW1/HMW2 proteins belong to a subgroup of autotransporter proteins that includes Proteus mirabilis HpmB, Bordetella pertussis FHA, and Haemophilus ducreyi HhdB (65). Their secretion is dependent on two additional proteins for their translocation out to the bacterial surface as compared to autotransporters found in other subgroups (155). Despite 80% amino acid similarity between HMW1 and HMW2, they bind to different receptors on epithelial cell lines. HMW1 binds to sialylated glycoproteins on Chang epithelial cells whereas the receptor of HMW2 is still unknown (154).

Haemophilus influenzae adhesin (Hia)
NTHi strains that do not express proteins belonging to the HMW1/HMW2 family of proteins express Hia (137, 162). Hia and the homologous protein Hsf of encapsulated H. influenzae strains demonstrate a similar binding pattern to several human epithelial cell lines (158). The Hia passenger domain has two adhesive binding
domains (BD), BD1 and BD2, both interacting with the same host cell receptor on Chang epithelial cells but with different affinities (101).

Moreover, Hia and the YadA adhesin of Yersinia enterocolitica have characterized a separate subfamily within the autotransporter proteins, known as the trimeric autotransporters defined by their short trimeric C-terminal translocator domain (30). Unlike classical autotransporter proteins such as Hap, trimeric autotransporter proteins remain intact and cell associated on the bacterial surface (156).

Furthermore, crystal structures of Hia BD1 and YadA collagen-binding domain have revealed that both proteins have a three-fold symmetry and three identical faces. This structure is suggested to facilitate a multivalent interaction with the host cell receptor and confers stability towards host defense mechanisms (187).

Haemophilus surface fibrils (Hsf)

Hsf is a large non-pilus protein that can be genetically detected in all Hib strains tested and in strains belonging to serotypes a, c, d, e, and f (158). Hsf is classified as a trimeric autotransporter protein due to significant structural and functional similarities with the Hia protein of NTHi strains (31, 67).

The adhesive activity of Hsf to Chang epithelial cells has been characterized as two separate acidic binding domains, BD1 and BD2 having high homology to the binding domains of Hia. A third domain, BD3, intervened between BD1 and BD2, lacks the acidic pocket which is necessary for adhesion. The importance of the acidic pockets was investigated by introducing point mutations of BD1 and BD2 which resulted in a complete elimination the Hsf adhesion (31).

Protein 5 (P5)
P5 is a heat-modifiable major outer membrane protein that mediates adhesion to a number of ligands on respiratory epithelial cells. P5 is one of several H. influenzae proteins that binds to sialic acid-residues on the respiratory mucin (132). The carcinoembryonic antigen family of cell adhesion molecules 1 (CEACAM1) is another ligand to P5 (73). CEACAM1 plays an important role for the innate immune responses and has been shown to be upregulated in certain epithelial cell lines after a viral infection or treatment with cytokines (83). A third ligand for P5 is ICAM-1, which is upregulated upon infection with H. influenzae. This interaction is suggested to be beneficial for the bacteria by the physical blocking of the ICAM-1, which hinders leukocyte recruitment mediated by ICAM-1 (10).

Based on sequence-analyses on P5 peptides, which originated from NTHi isolates taken from patients affected by chronic bronchitis, it was revealed that four putative surface-exposed regions
showed higher variation due to point mutations and were therefore suggested as being selected under immunological pressure and extraordinary important for the bacterial survival in the airways (37).

Table 1. *Haemophilus influenzae* OMPs interacting with eukaryotic target molecules

<table>
<thead>
<tr>
<th>OMPs</th>
<th>Molecular weight (kDa)</th>
<th>Eukaryotic target molecule</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2</td>
<td>36-42</td>
<td>respiratory mucin</td>
<td>(132)</td>
</tr>
<tr>
<td>P5</td>
<td>27-35</td>
<td>respiratory mucin, CEACAM1, ICAM-1</td>
<td>(132, 73, 10)</td>
</tr>
<tr>
<td>Pili (hifA-E)</td>
<td>22-27</td>
<td>respiratory mucin, anton antigen</td>
<td>(174, 173)</td>
</tr>
<tr>
<td>Hap</td>
<td>110</td>
<td>fibronectin, collagen IV and laminin</td>
<td>(46)</td>
</tr>
<tr>
<td>HMW1</td>
<td>125</td>
<td>sialylated glycoprotein, unknown</td>
<td>(154)</td>
</tr>
<tr>
<td>HMW2</td>
<td>120</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hia</td>
<td>115</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>Hsf</td>
<td>245</td>
<td>Vitronectin</td>
<td>(Paper I)</td>
</tr>
<tr>
<td>Protein D</td>
<td>42</td>
<td>PAF-receptor (indirectly, by acquisition of ChoP from host to LOS)</td>
<td>(43, 52, 110)</td>
</tr>
<tr>
<td>Protein E</td>
<td>16</td>
<td>Unknown</td>
<td>(Paper II-IV)</td>
</tr>
</tbody>
</table>

**Gene diversity**

Variation of the bacterial surface structure is advantageous and sometimes necessary for the bacteria to survive in different host tissues. In an extensive study done by Musser *et al*, a large collection of *H. influenzae* isolates were analysed by multilocus enzyme electrophoresis and it was shown that NTHi strains were more nonclonal and showed greater genetic and phenotypic diversity than the Hib strains (116). In addition, the diversity of NTHi has shown to be higher when isolates have been taken from infections as compared to isolates belonging to the normal flora (172).

*H. influenzae* displays innumerable different phenotypes due to high genetic variability in the OMP content. Proteins such as HMW1/HMW2, LOS, Tfp1, Tfp2 and hifA demonstrate altered gene expression due to phase variation mediated by slipped-strand mispairing (184). Another mechanism is to alter the gene content, which may occur by spontaneous point mutations or horizontal gene transfer, resulting in insertions, deletions or duplications of genes. Spontaneous point mutations occur especially in variable surface-exposed regions and has been detected in P1, P5
and hifA (37, 57, 107, 175). The majority of the NTHi adhesins show high strain-to-strain heterogeneity due to horizontal gene transfer (40, 57).
Hib strains have shown to increase their virulence by amplification of the duplicated genes of the capb locus that encode for the capsule production (94). Three or more copies of the capb locus leads to an increased production of the capsule and thus an increased virulence of the bacterium (29).

**Lipooligosaccharide (LOS)**

The LOS of *H. influenzae* contains a lipid A moiety and an oligosaccharide core, but it possesses considerably shorter oligosaccharide side chains compared to the LPS of enteric gram-negative bacteria (48). In soluble form, LOS is a potent stimulator of proinflammatory cytokines both when incubated with bronchial epithelial cells and with macrophages (15, 25, 88).

An interesting feature of *H. influenzae* LOS is its use of molecular mimicry of the host. The oligo-saccharide component of LOS undergoes phase variation at high frequency resulting in a heterogeneous mixture of LOS molecules on every bacterial cell. The incorporation of phosphorylcholine (ChoP) from the host to certain LOS variant has been shown to contribute to the survival of the bacteria in the nasopharynx (43, 167, 186). However, NTHi ChoP* strains are more serum sensitive than the NTHi ChoP₀ ones since they are targeted by the C-reactive protein that activates the classical pathway of the complement. It is suggested that NTHi switches off the expression of ChoP when the bacteria are invasive to avoid this host counteraction (185). Furthermore, LOS of NTHi may incorporate sialic acid from the host, and thus increase the bacterial serum resistance (76).

**IgA1 protease**

Nearly all *H. influenzae* strains secrete an extracellular endopeptidase, IgA1 protease, that cleaves the mucosal immunoglobulin A1 specifically at the hinge region leading to the release of Fc and Fab fragments (89, 91). The IgA1 protease of *H. influenzae* is encoded by two genes, the iga, present in most *H. influenzae* strains, and the igaB gene, that is present in one-third of *H. influenzae* strains. Strains containing both genes have been correlated with significantly higher levels of IgA1 protease activity as compared to strains containing only the iga gene (45). Moreover, invasive disease-causing strains have shown higher level of IgA1 protease activity as compared to isolates taken from asymptomatic carriers, implying that the IgA1 protease plays a role in the pathogenesis of *H. influenzae* (178).
Innate immune responses in the respiratory tract

First line of defense: respiratory epithelium

The epithelial cells lining the respiratory tract are constantly exposed to airborne particles such as dust, pollutants, bacteria and virus. The mucociliary escalator is the most prominent barrier against invading pathogens. It is mainly composed by pseudostratified columnar ciliated cells and goblet cells that produce mucus (74). Invading pathogens are trapped in the mucus and the ciliary action attempts to remove them towards the pharynx with the help of mechanical forces such as sneezing and coughing.

Apart from providing an interface between the extracellular and intracellular milieu, the mucosal epithelium contributes actively in immune responses by producing antimicrobial peptides including lysozyme, lactoferrin, surfactant proteins, SLPI and \[\beta\]-defensins that have the ability to neutralize bacteria (96). For instance, lactoferrin mediates several antimicrobial mechanisms, it inactivates both \textit{H. influenzae} IgA1 protease and Hap (128). In addition, lactoferrin can bind to iron and makes this essential nutrition for the bacteria unaccessible (96).

Secretory (sec) IgA are part of the adaptive immune system and is an important local immune mechanism of the airways (90). Subclass IgA1 accounts for over 90% of all IgA present in the respiratory tract. \textit{H. influenzae} may resist the agglutination activity of sec IgA by secretion of IgA1 proteases that specifically cleaves the J-chain of IgA1 (89). In Table 2 (p29), there is an overview of the components of the innate immune system.

Inflammation

The primary phagocytic cells in the conducting airways are the macrophages and the neutrophils. Specific receptors on the phagocytic cells bind to ligands on the pathogens surface followed by engulfment and proteolytic processing of the pathogen. Recognition receptors that lead to phagocytosis are either cellulary attached including the mannose receptor, integrins (e.g. CD11b/CD18) and scavenger receptors, or humoral such as the mannose-binding protein (MBL) (3).

The macrophages and the epithelial cells may activate both the innate and the adaptive immune system through proinflammatory cytokines and chemokines. The chemokine IL-8 is a strong activator of PMNs, and the first PMNs that arrive to the area of inflammation are the neutrophils. The neutrophils migrate to a chemotactic gradient out from the vasculare system, through permeabilized endothelial cells via
It has been demonstrated that *H. influenzae* induces a high inflammatory response in respiratory epithelial cells by the upregulation of TNF-α, IL-6, IL-8, monocyte chemotactic protein-1 (MCP-1) and intercellular adhesion molecule-1 (ICAM-1) (25). The expression of ICAM-1 on epithelial cells have proven to be crucial for the neutrophils to attach with their counterreceptors M2-intergrins (CD18b/CD11) and subsequently mediate phagocytic killing on *H. influenzae* (25, 53, 78) (Figure 2).

Studies of *H. influenzae* ability to cause inflammation has a clinical relevance since several reports show that chronic airway infections such as COPD, cystic fibrosis and AOM are characterized by an acute inflammatory response mainly mediated by IL-8, interleukin 1-beta (IL-1β) and TNF-α (18, 103). Moreover, measurements on bronchoalveolar lavage (BAL) components have shown that COPD patients colonized by bacteria in the airways have elevated amounts of IL-8 and neutrophilic airway inflammation as compared to healthy individuals, smokers and individuals not colonized with bacteria (112, 148).
Figure 2.
The inflammatory response in the airways. Bacterial-stimulated epithelial cells release IL-8, which attracts neutrophils. Activated macrophages and neutrophils release high amounts of reactive oxygen intermediates and proteolytic enzymes leading to tissue destruction of the lung parenchyma. Bacterial attachment increases to damaged epithelium (130, 131). Local immunoglobulins are inactivated, the muco-ciliary function and the complement-mediated phagocytosis gets impaired leading to elevated amounts of mucus production (129). Environmental factors such as tobacco smoke, pollutants or underlying viral infections also contribute to an impaired immune response. Figure adapted from (87).
**Toll like receptors (TLRs)**

Recognition of pathogens is mediated by germline encoded receptors, so called pattern recognition receptors (PRRs). PRRs recognize constitutive and conserved patterns on the pathogen, referred to as pathogen-associated molecular patterns (PAMPs) (for reviews see (170)).

PAMPs are found on most pathogens (G+ and G- bacteria, yeast, viruses and fungi) and include structures such as dsRNA, LPS, peptidoglycan, porins, flagellin and lipoproteins whereas TLR4 recognizes LPS from G- bacteria.

Similarly the mannose-binding lectin (MBL) is a secreted PRR, whereas the macrophage mannose receptors and the nucleotide-binding oligomerization domain proteins (NOD-like receptors) belong to the endocytic group of PRRs. The TLRs are the most characterized PRRs and belong to signaling receptors.

TLRs are transmembrane proteins that are homologous to the interleukin-1 receptor (IL-1R). In humans there are in total 10 different TLRs (TLR1-10). The upregulation of various genes, triggered via the TLRs, are predominantly regulated at a transcriptional level, thus the transcription factors play an important role, such as nuclear factor kappa-B (NFkB) and activator protein-1 (AP-1). NFkB regulates the expression of an array of genes that encode proinflammatory cytokines (e.g. IL-1, TNF-α, IL-6) chemokines (e.g. IL-8, MIP-1, MCP-1, eotaxin), adhesion molecules (e.g. ICAM-1, VCAM-1, E-selectin) and inflammatory enzymes (e.g. inducible nitric oxidesynthase, inducible cyclooxygenase-2) (16).

The most studied TLRs are TLR2 and TLR4. TLR2 recognizes components from both G- bacteria and G+ bacteria including lipoteichoic acid, peptidoglycan and lipoproteins whereas TLR4 recognizes LPS from G- bacteria.

Similarly to LPS in E. coli, the biologic activity of LOS in H. influenzae resides in the lipid A moiety. The released form of LPS is more potent than the membranebound and requires at least four molecules in order to be recognized. The acute phase protein LPS binding protein (LBP) accessorizes with LPS before it is loaded by the glycosylphosphatidylinositol (GPI) linked coreceptor CD14 onto the receptor complex consisting of TLR4 and the accessory protein MD-2. The expression of TLR4 has been shown to be crucial for the clearance of H. influenzae from the lung in mice (181). In the absence of LOS, the host response due to signaling via TLR4 is shifted towards a TLR2-driven response implying that the NTHi proteins signaling via the TLR2 replaces the LOS-driven inflammatory response (100).
Most bacterial lipoproteins are triacylated at the N-terminus cysteine including *H. influenzae* lipoproteins P6 and P2 whereas a few known lipoproteins are diacylated such as the macrophage-activating lipopeptide-2 (MALP-2) in *Mycoplasma fermentans* (6, 150). The signaling of lipoproteins containing two acylchains are recognized by heterodimerized TLR2/TLR6 and lipoproteins that contain three acylchains are recognized by TLR2/TLR1.

P6 purified from NTHi signals via TLR2 to upregulate the mucin (MUC5AC) gene transcription in the respiratory epithelia by activating both transcription factor NF-κB and activating protein-1 (23). Similiarly to porins of other G-negative bacteria such as in *Neisseria meningitis*, and *Shigella dysenteriae*, P2 in *H. influenzae* demonstrates immunostimulatory properties by signaling via the TLR2 and upregulating TNF-α and IL-6 both in human and murine cells (6, 55).

<table>
<thead>
<tr>
<th>Component</th>
<th>Main functions</th>
</tr>
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<tbody>
<tr>
<td>Cellular</td>
<td></td>
</tr>
<tr>
<td>epithelial mucosa</td>
<td>Anatomical/physical barrier,</td>
</tr>
<tr>
<td></td>
<td>Production of antimicrobial peptides</td>
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</tbody>
</table>
and cytokines.

<table>
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<tr>
<th>Macrophages, neutrophils</th>
<th>Phagocytosis</th>
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</thead>
<tbody>
<tr>
<td>Scavenger receptors, integrins, mannose receptors, mannose binding proteins, TLRs, NOD-like receptors</td>
<td>Host cell-interactions, pattern recognition of pathogens</td>
</tr>
<tr>
<td><strong>Humoral</strong></td>
<td></td>
</tr>
<tr>
<td>Antimicrobial peptides; lysozyme, lactoferrin, surfactant proteins, SLI</td>
<td>Neutralization and killing of microbes</td>
</tr>
<tr>
<td>LBP, CD14</td>
<td>Enhancement of LPS-induced activation</td>
</tr>
<tr>
<td>Complement</td>
<td>Bactericidal, opsonization, induction of chemotaxis</td>
</tr>
<tr>
<td>Immunoglobulins (IgA, IgG, IgM)</td>
<td>Neutralization, activation of complement, enhancement of phagocytosis</td>
</tr>
</tbody>
</table>

**The complement system**

The complement system has a central role in host defense against microorganisms. It interconnects the innate and adaptive immune system by taking part in several inflammatory responses. Traditionally, the complement is classified as a part of serum. However, proteins belonging to the complement, coagulation and fibrinolysis cascades play also a role in the innate immune defense of the airways. These proteins reach the airway lumen by plasma exudation from permeabilized intercellular epithelial cells junctions (60).

The complement is composed of a large groups of proteins organized into three activation pathways; the classical, the alternative and the lectin pathway. Early in the activation, opsonins (C3b) are generated to coat the surface of the foreign cell, and thus target it for destruction by activating phagocytic cells. The central event of all three pathways is the enzymatic cleavage of C3 by the C3 convertase. The complement also produces anaphylatoxins (C3a, C4a and C5a) in order to recruit inflammatory cells to the site of inflammation. All three activation pathways result in the formation of the membrane attack complex (MAC) and lysis of the bacterial cell (for review see (179, 180)).

**The classical pathway**

Activation is initiated by the recognition of the C1-complex (C1qr₂s₂) that binds to antibodies (IgG or IgM) that are attached to the surface of a pathogen or complexed
with antigens (146). This leads to an enzymatically active C1s. Thereafter, C1s binds and cleaves C4 and C2. The C4b may form covalent bonds to the pathogen’s surface and be stabilized, and subsequently act as a binding site to C2a.

The covalently attached complex C1sC4bC2a is the C3 convertase. The C3 convertase cleaves C3 into C3b and C3a. C3 convertases are usually generated on "protected" surfaces, as a pathogens surface, or immune complexes. This means that they are protected from degradation, which would occur on a host cell. Thus, C3b gets deposited on a protected surface and binds C5, which is cleaved by the C5 convertase, i.e., C4b2a3b.

The assembly of the MAC is a non-enzymatic reaction. Cleavage of C5 results in C5b, which serves as an anchor for the assembly of C6, C7 and C8. The resulting complex C5b678 is inserted into the lipid bilayer of the pathogens membrane. The lysis of the cell is not completed until at least twelve C9 monomers have bound to the C5b678 and generates a pore-forming molecule.

C1q can also bind in an antibody independent manner to C-reactive protein, the lipid-A component of Gram-negative bacteria, and to several other targets.

The alternative pathway
Exposed thioester bond of native C3 makes it susceptible to spontaneous hydrolysis. There is a steady, low level, spontaneous C3 activation in plasma, generating activated C3i(H2O). Factor B binds C3 in the presence of Mg 2+ to form C3iB. This complex is cleaved by Factor D to release Ba. Because the activated C3iBb complex, i.e., C3 convertase, operates in fluid phase, most of the generated C3iBb is hydrolysed and inactivated. If C3iBb comes in contact with "protected" surfaces, where C3b has a higher affinity to factor B than factor H, it will bind covalently (77). An amplification loop will be triggered. More C3b will bind to the C3 convertase and generate the C5 convertase, C3bBb3b.

The lectin pathway
The lectin pathway is initiated by the mannann-binding lectin (MBL) that binds to terminal mannose groups found in many microbial carbohydrates. MBL interacts with the so called MASP1 and MASP2 (mannann-binding protein-associated serine proteinase) which leads to the formation of the enzymatically active MBP-MASP complex that is able to cleave C4 and C2 (24). The subsequent activations are comparable to the classical pathway.

Regulators of complement activation
The complement system must be tightly regulated to prevent any potential damage that an uncontrolled activation of the complement may cause the host. But also, not to deplete the system if an appropriate target would appear. The regulators of the
complement activation are divided into fluid-phase regulators and membrane-bound regulators depending on their site of action. All regulators operate to prevent an excessive reaction by binding and accelerating decay-processes except for properdin, which is the only positive regulator of the complement. In Table 3 (p 32) there is summary of the complement inhibitors.

<table>
<thead>
<tr>
<th>Fluid-phase regulators</th>
<th>Function</th>
</tr>
</thead>
</table>
| C1 inhibitor            | Dissociates C1r:C1s from C1q  
                          | Binds C3b  
                          | Inactivates MASPsv |
| Factor I                | Inactivates C3b  
                          | Inactivates C4b |
| Factor H/              | Inactivates C3b  
Factor H-like protein 1 (FHL-1) | Cofactor to factor I in inactivation of C3b |
| C4-binding protein (C4BP) | Inactivates C4  
                          | Cofactor to factor I in inactivation of C4b |
| Properdin               | Stabilizes the C3 convertase |
| Vitronectin             | Prevents C5b-7 membrane insertion |
| Clusterin               | Prevents C5b-7 membrane insertion |

<table>
<thead>
<tr>
<th>Membrane-bound regulators</th>
</tr>
</thead>
</table>
| CR1 (complement-receptor 1) | Inactivates C3b  
                          | Inactivates C4b  
<pre><code>                      | Cofactor for factor I in the inactivation of C3 convertase |
</code></pre>
<p>| MCP (also CD46)            | Cofactor to factor I in inactivation of C3b and C4b |
| Decay accelerating factor (DAF) | Dissociates C3/C5 convertase by binding C4b and C3b |</p>
<table>
<thead>
<tr>
<th>CD59 (also protectin)</th>
<th>Inhibits MAC formation by binding C8 in the C5b-8 complex</th>
</tr>
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<tbody>
<tr>
<td>Homologous restriction factor (HRF)</td>
<td>Inhibits MAC formation by preventing the binding of C9 to C5b8</td>
</tr>
</tbody>
</table>

**CD59 (also protectin)**
Inhibits MAC formation by binding C8 in the C5b-8 complex

**Homologous restriction factor (HRF)**
Inhibits MAC formation by preventing the binding of C9 to C5b8

**Vitronectin**
Vitronectin, also known as S-protein, is a glycoprotein. It is found in the extracellular matrix (ECM) with its complete molecular form of 75 kDa. In the plasma it is found both as a single chain of 75 kDa and as two chains of 65 and 10 kDa, joined by a disulfide bond (147). Vitronectin is implicated with several physiological processes. In the ECM, vitronectin affects the movement and attachment of epithelial cells by communicating with various ligands of the integrin family (125). In fluid phase, vitronectin interacts both with the complement and the coagulation cascades (147).

**Bacterial interactions with complement**
Microorganisms interact with complement components in order to increase their survival chances in the host. A number of bacteria bind C4BP to increase their serumresistance including NTHi, *M. catarrhalis, Streptococcus pyogenes, N. meningitis* and *E. coli* (61, 82, 124, 136). In *M. catarrhalis* the UspA1/UspA2 proteins have been shown to be extraordinary important for the complement resistance since they interact both with C4BP and C3 meaning that they can inhibit both the classical as well as the alternative pathway (136).

The UspA2 also interacts with vitronectin, which additionally contributes to the bacterial serumresistance (9). Binding to vitronectin as a serum resistance mechanism has been reported in *H. ducreyi* mediated by the OMP DsrA and DltA (1, 28). Some bacteria use vitronectin to facilitate their attachment and invasion into host cells such as *Pseudomonas aeruginosa* and *Neisseria gonorrhoeae* (36, 97).

**The adaptive immune system**
The adaptive immune system provides the second line of defense against invading pathogens. The main effector cells are the B and the T cells. Unlike the cells of the innate immune system, the B and the T cells may differentiate into memory cells which have the ability to respond more rapidly and more efficiently to secondary challenge with a specific antigen. The B cells provide the humoral immunity by producing antibodies (immunoglobulins) whereas the T cells perform cell-mediated immunity. When a B cell has been activated by an antigen, it internalizes and degrades the antigen so it can be presented on the major histocompatibility complex (MHC) class II to the T cell receptor (TCR) of T helper cells. Other antigen
presenting cells (APCs) include dendritic cells, macrophages, monocytes and epithelial cells. Thus, the cells of the innate immune system are also important activators of the adaptive immune system. The T cells that interact with APC are triggered to direct the immune response by producing cytokines and signaling back to the APC.

**Immunoglobulins**

Immunoglobulins (Igs) are glycoproteins that are secreted by activated B cells. There are five classes of Igs: IgG, IgA, IgM, IgE and IgD. IgG and IgA are further subdivided into four (g1-4) respectively two (a1-2) subclasses. Since IgG was the first Ig to be characterized, it represents the prototypic structure of the four-chain Ig monomer which comprises two identical heavy (H) and light (L) chains each. The constant regions of the H chains display low sequence variations and the number of domains in the H chains varies between the different Ig classes. In addition, the different Igs have various amounts of carbohydrates at different locations of the Igs. The L chains are always composed by two domains and may be either of kappa (κ) or lambda (λ) class.

The Ig molecule is divided in two separate regions; two Fab-regions that comprise the variable regions and have the ability to adapt to various antigen epitopes, and the Fc-region that mediates effector functions such as complement activation and antibody-dependent phagocytosis. The Fab and the Fc-region are connected via the CH1 and the CH2 by a structure referred to as the hinge region that allows flexibility of the Ig molecule.

The variable domain of the N-terminal globular structure of the H- and L-chains is composed by three hypervariable regions, so called complementarity-determining residues (CDRs). These regions are responsible for the majority of the antigen interaction.

Upon B cell activation, the CDRs undergo somatic recombinations, random insertions or deletions of nucleotides in the V-regions of B cells in order to produce antigen specific antibodies.

In summary, the Igs of the different classes have two major functions 1) to recognize epitopes on antigens with the antigen-specific Fab domains and 2) to mediate different effector functions with the Fc region leading to an inflammatory response and subsequent elimination of the antigen/pathogen.

*IgD in the respiratory tract*
The IgD molecule was discovered in serum from a myeloma patient in 1965 by Rowe and Fahey (138). It was later found that membrane bound IgD serves as an antigen B cell receptor (BCR) together with IgM on all mature B cells (139). B cells that express IgD on their surface are naïve, meaning that they have not been exposed to an antigen. Together with a pair of heterodimeric proteins (IgD/IgM), IgD and IgM form the BCR complex. The interaction of the BCR and the antigen is strengthened by coreceptors expressed on the B cell surface (CD19, CD21 and CD81). Antigens that bind to mIgDs or mIgMs must crosslink several Ig molecules in order to trigger a signaling cascade within the B cell that will lead to internalization of the antigen. The processed antigen is subsequently presented on MHC II. The antigen presenting indicates that the B cell is ready to be activated by T helper cells and to proliferate into an Ig-secreting plasma cell.

In the respiratory tract, there are plasma cells that produce immunoglobulins by all classes. IgG is the most abundant immunoglobulin in the airways (12 mg/ml) whereas IgD is found in minute amounts (≤30 μg/ml). The exact role of secIgD is still uncertain, however it is known that there is an increased amount of IgD-secreting plasma cells in the upper respiratory tract as compared to spleen lymph nodes and glandular tissue of the gastrointestinal tract (21). Furthermore, patients suffering from tonsillar disease, AOM and COPD have increased amounts of IgD-producing cells as compared to healthy individuals (104, 152, 163).

Before there were any available cell-lines that expressed IgD, studies involving IgD were performed with IgD myeloma serum that originated from myeloma patients. IgD multiple myeloma (MM) is a rare condition that accounts for approximately 2% of all myeloma cases (126). IgD MM is a form of plasma cell neoplasia which means that a malignant monoclonal plasma cell tumor shows a continuous clonal evolution in contrast to the tightly regulated plasma cells. Patients with multiple myeloma display an unusually large amount of the M component, i.e. the monoclonal antibody (153).

Nearly three decades ago it was shown that H. influenzae and M. catarrhalis display a high affinity for soluble IgD and are strong stimulators of B cells proliferation (13, 51). These findings together with the fact that both H. influenzae and M. catarrhalis often cause disease in tissues of elevated IgD amounts indicate that there might be a connection between IgD and the humoral immunity of secretions from the upper respiratory tract.

The M. catarrhalis IgD-protein was discovered through its ability to bind IgD (50). MID is a large protein of 200 kDa and was shown to bind to all IgD as tested including two purified IgD myeloma proteins, four IgD myeloma sera and one IgD standard serum. The smallest IgD binding domain was encirculated to comprise MID amino acids 962-1200 (118). It was later found MID962-1200 mediated specific
binding to the C_{H1} region of the IgD molecule of which amino acids 198-206 were crucial (51, 143). Thus, MID binds to IgD in a nonimmune manner, i.e. not to the antigen-binding site of the Ig molecule. Biologically, MID962-1200 activates B-cells through the IgD B cell receptor leading to a strong antibody response (119).

Interestingly, the binding of *H. influenzae* type b strains to IgD was recently characterized and it was found that Hib strains bound to the C_{H1} region of IgD, i.e. the same binding site as for MID, whereas NTHi strains were shown to be nonbinders of IgD (142) (**Figure 3**). These data correlate well with previously published material within this field. When protein D was isolated from NTHi it was considered to be an IgD binding protein (140). However, it was later found that protein D only bound to one IgD myeloma, and it was suggested that the IgD myeloma used to isolate protein D contained specific antibodies against PD (110).

**Figure 3.**
Schematic drawing of the IgD molecule and the binding domain of MID and Hib strains.
THE PRESENT INVESTIGATION

Aims

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

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

• To identify an *H. influenzae* protein detected by an IgD (□) myeloma and to study its interaction with human host cells.

• To study the binding of *H. influenzae* protein E (PE) to epithelial cells and to encircle the active binding site within the PE molecule.

• To analyse the innate immune response in respiratory epithelial cells exposed to *H. influenzae* PE.


Results and Discussion

H. influenzae binds to vitronectin via Hsf and thus resists complement dependent killing (Paper I)

In order to successfully persist and not be cleared out by the defense system, the invading bacteria use a wide variety of mechanisms that involve interactions with both soluble and membrane-bound host molecules. In the literature, several pathogens have been described to interact with vitronectin; both by using vitronectin as a bridging molecule that helps the bacteria invade subepithelial tissue (e.g., N. gonorrhoeae), and by using vitronectin as a sheltering mechanism leading to increased serum resistance (e.g., M. catarrhalis) (9, 36).

Previous studies by Eberhard and Ullberg showed that H. influenzae strains display a high affinity for the heparin binding domains of immobilized vitronectin and that the bacterial binding is not dependent on pili (39). Hsf is a non-pilus protein and due to its large size it has been suggested that it protrudes from the capsule enabling interaction with the environment (158). Moreover, Hsf seems to be necessary for a persistent bacterial colonization of encapsulated H. influenzae strains to respiratory epithelial cells, especially in non-piliated strains (56). Since Eberhard and Ullberg showed that the H. influenzae binding to vitronectin is not dependent on pili, we decided to investigate whether Hsf was responsible for the ability of H. influenzae type b to bind vitronectin.

The gene encoding for hsf was knocked out in two Hib strains, Eagan respectively RM 804, by introducing a kanamycin cassette in the N-terminal region of the hsf ORF. In addition, Hsf was recombinantly expressed on the surface of E. coli. With these strains, we could show that the Hsf-expressing bacteria bound significantly higher amounts of soluble vitronectin as compared to the mutant counterparts in flow cytometry experiments. The binding to vitronectin due to Hsf was shown to be dose dependent both when Hsf was expressed on the surface of Hib and on E. coli. Furthermore, by using immobilized vitronectin on glass slides that were subsequently incubated with bacteria, we could study the Hsf dependent binding to vitronectin after gram-staining.

By using two different species, tested in two different assay systems, we had strong evidence to conclude that Hsf was the principal vitronectin binding protein of H. influenzae. In contrast to the studies of Eberhard and Ullberg, we showed that H. influenzae also has the ability to interact with soluble vitronectin. The reason for this difference is unclear. There is a possibility that the choice of method was the determining factor; we used native vitronectin, whereas the other group used iodine-labeled vitronectin (39).
We wanted to investigate which part of the vitronectin molecule that was responsible for the Hsf binding. Vitronectin is a well-defined molecule regarding its functional domains and interactions with other proteins. For instance, vitronectin has an Arg-Gly-Asp (RGD) motif of which it interacts with the extracellular matrix via specific integrins (147). Furthermore, vitronectin can regulate the blood coagulation through the binding of heparin, plasminogen, plasminogen activator inhibitor-1, and thrombin-inhibitor complexes (98).

Vitronectin has three heparin-binding domains; one is located at the N-terminal, the second is at the C-terminal and the third is in the central part of the molecule. The binding of Hsf to vitronectin could be inhibited by the addition of heparin at increasing concentrations. Interaction with vitronectin has been reported with other pathogens including S. pyogenes group G and A, S. aureus, and E. coli. All these pathogens interact with the heparin-binding domains except for S. pyogenes group A. Therefore, we decided to use S. pyogenes group A as a negative control in order to underline the specific interaction of H. influenzae with the heparin-binding domains. Moreover, we could exclude one out of the three heparin-binding domains as being involved in the interaction with H. influenzae Hsf since preincubation of full length vitronectin with peptides covering the C-terminal heparin-binding domains did not affect the bacterial interaction with vitronectin. Thus, Hsf most likely interacts with the N-terminal heparin-binding domains.

Vitronectin regulates the terminal pathway of the complement by binding to the MAC complex. Since Hsf binds to vitronectin, we wanted to investigate whether this interaction prolonged the survival of the bacteria in serum. The H. influenzae strains deficient of Hsf expression showed to be more susceptible to complement-mediated killing, as compared to the Hsf-expressing H. influenzae. Thus, by binding to vitronectin the bacteria can prevent lysis mediated by the MAC and increase its survival.

To in detail study what part of the Hsf molecule that was responsible for the vitronectin interaction, a series of recombinant proteins were produced covering the entire molecule. In accordance to work by Cotter et al who showed that Hsf mediates binding to Chang cells via three crucial domains, we could show that Hsf amino acids 608-1351 respectively 1536-2414 displayed the strongest binding to vitronectin which contains the crucial binding domains to Chang cells (31). In addition, the strongest binding domain, Hsf608-1351 interacts specifically with vitronectin since cold Hsf608-1351 competes with iodine-labeled Hsf608-1351, as shown in a competition inhibition assay. This implies that Hsf uses the same binding domains to bind to the eukaryotic receptor as to bind to the vitronectin molecule, indicating that vitronectin is a putative eukaryotic receptor for Hsf. However, more experiments need to be done to confirm this theory.
The isolation and characterization of an H. influenzae surface protein detected by an IgD (\(\text{IgD}^\text{M. catarrhalis}\)) myeloma protein (PaperII)

M. catarrhalis and Hib strains have the ability to bind IgD in a non-immune manner (51, 144). M. catarrhalis binds to the C\(_h\)1-region of the IgD molecule via the MID protein, whereas the IgD binding protein of Hib strains is still unknown (51, 143). In a recent investigation from our laboratory, a panel of Hib strains were screened for their non-immune binding to IgD by flow cytometry. NTHi strains were included as negative controls. Fifty percent of the Hib strains bound the C\(_h\)1-region of the IgD molecule, whereas the NTHi strains were negative (142).

We also found that both Hib and NTHi strains bound a certain IgD (\(\text{IgD}^\text{M. catarrhalis}\)) myeloma serum, originating from an IgD myeloma patient. We decided to run a western blot (WB) on OMP- preparations from the tested bacteria, and probed the membrane with the IgD (\(\text{IgD}^\text{M. catarrhalis}\)) myeloma serum. Intriguingly, a strong signal from one protein band at a molecular weight of 16 kDa appeared in all lanes, indicating that the IgD (\(\text{IgD}^\text{M. catarrhalis}\)) myeloma serum specifically recognized the 16 kDa protein. (Figure 4).

In initial attempts, we tried to isolate the protein recognized by the IgD (\(\text{IgD}^\text{M. catarrhalis}\)) myeloma serum from Coommasie-stained SDS-PAGE and silver-stained 2-D-Gel analyses. Since the protein was difficult to evisualize, we decided to isolate the protein from a genomic library based upon NTHi 772. Three clones were selected by colony immunoassays using the IgD (\(\text{IgD}^\text{M. catarrhalis}\)) myeloma serum. One of the three positive clones was sequenced and shown to encode four proteins; HI0175 to HI0178, according to the physical map of H. influenzae KW20 (47). Each protein was cloned into E. coli and tested for IgD (\(\text{IgD}^\text{M. catarrhalis}\)) myeloma binding. The only transformant that bound to the IgD (\(\text{IgD}^\text{M. catarrhalis}\)) myeloma was HI0178, which we designated as protein E (PE). (Figure 5).

Since our analyses revealed that PE is produced in small amounts, we decided to recombinantly express the protein. PE devoid of the signal peptide (PE22-160) was
cloned into pET26 and could be expressed in large amounts. The IgD (\(\square\)) myeloma serum detected PE22-160 as efficiently as native PE, as shown by WB analysis.

Full length PE (amino acids 1-160) was expressed on the surface of *E. coli* using the vector pET16. *E. coli*-PE could be recognized by the IgD (\(\square\)) myeloma serum in flow cytometry experiments. These results confirmed that that we had isolated the protein in question and that PE is surface attached.

**Figure 5.**
Genetic organization of the insert sequence comprising HI0175-HI0178 that was isolated from the genomic library from strain NTHI 772. ORF HI0178 encodes PE.

The next step was to construct a mutant of PE in strain NTHI 3655. This was done by introducing a kanamycin cassette in the N-terminal part of the gene. By using the NTHi 3655\(\square\)pe we could confirm that PE was the only protein recognized by the IgD (\(\square\)) myeloma serum. We could also show that no other IgD preparation bound to PE
except for the IgD (l) myeloma serum. The NTHi 3655pe was also tested for its growth conditions as compared to NTHi 3655. No difference was found between the two strains. This result gave us an indication that PE does not seem to be essential for the growth, but has perhaps a role for the virulence of the bacteria.

PE is a lipoprotein, according to prediction peptide sequence analysis. Lipoproteins are characterized by a distinct so called lipobox at the C-terminal of the signal peptide with the consensus sequence [LVI][ASTVI][GAS][C] including the invariable cysteine residue (11). After posttranslational modification, lipoproteins are anchored to the membrane through N-acyl-S-diacylglyceryl-cysteine. (Figure 6). Several lipoproteins in H. influenzae have been characterized including P2, P4, P6 and protein D. Lipoproteins may have a wide variety of functions such as structural, enzymatic, immunostimulatory, adhesive and invasive properties.

```plaintext
lipobox
PE signal peptide

[ LVI ] [ ASTVI ] [ GAS ] [ C ]

MEKIILTLSL GLLTAC
```

Figure 6.
A comparison of the signal peptide of PE with the consensus sequence i.e., the lipobox of lipoproteins (11).

We found that when we incubated NTHi 3655 respectively NTHi 3655pe with respiratory epithelial cells the binding pattern differed between the two strains. The wildtype showed a significantly higher binding than the mutant, indicating that PE acts as an adhesin. These results were further strengthened when the E. coli-PE strain was tested for binding and showed that the difference between E. coli-PE and E. coli was even higher as compared to the NTHi strains.

An interesting aspect of the present study is that PE is the second H. influenzae protein that has been isolated with an IgD myeloma serum. Protein D was isolated in our laboratorium by using another IgD (l) myeloma serum (140). Another common theme is their classification as lipoproteins. It is tempting to speculate that there are other unidentified IgD myeloma binding proteins in NTHi.

*The central part of the PE molecule displays the strongest binding to epithelial cells (Paper III)*

The adherence of H. influenzae with the human host involves initial interactions with the respiratory mucus followed by direct binding to the epithelial cells (129). H.
*influenzae* strains express an array of various adhesins that can play a complementary role at different stages of the adhesion process. For example, pili and the HMW1/HMW2 seem to be needed in the early contact with the epithelial cells, whereas adhesins that promote disruption of the epithelial membrane, such as Hap and protein D are needed later in the adhesion process (79, 129, 159).

In paper II, we showed that PE acts as an adhesin on the bacterial surface, both in NTHi and as a recombinantly expressed protein in *E. coli*. In paper III, we have in more detail characterized the binding properties of the PE molecule.

By [³H]-thymidine labeling NTHi strains we could show in three different epithelial cell lines, derived from the conjunctiva (Chang), pharynx (Detroit) respectively type II alveolar cells (A549), that PE plays an essential role for the bacterial binding. The binding was decreased by 50% with the NTHi 3655*pe* strain as compared to the wildtype strain. When we performed the same set-up of experiments with *E. coli*-PE, we could observe that the difference in adhesion between the *E. coli*-PE and the wildtype counterpart was even more pronounced than between the two NTHi strains. These results clearly indicate that despite its small size of 16 kDa, PE is important for the adhesion, and also that PE alone has the ability to enhance the bacterial adherence when expressed in another strain. In other words, PE does not depend on other adhesins or factors in order to mediate adherence.

The construction of an NTHi mutant involves permeabilizing the cell membrane by a defined medium that will lead to an increased bacterial competence. To ensure that the cell structure of NTHi 3655*pe* was not affected in a way that could influence the bacterial capacity to adhere, PE was inserted into NTHi 3655*pe* by complementation using the vector pSPEC. Both expression levels of PE and the adherence capacity of the complemented NTHi 3655*pe* strain showed to be increased.

In order to analyse what part of the PE molecule showed strongest binding, we used recombinantly produced PE22-160 (devoid of the signal peptide) and a series of peptides that covered the full length PE molecule. All proteins were iodine-labeled and incubated with the three epithelial cell lines (Chang, Detroit, A549), and erythrocytes. After measuring the bound radioactivity, the results revealed a distinct binding pattern with all three epithelial cell lines. PE84-108 was found to be the strongest binding domain of all the tested peptides including PE22-160. In the binding to the erythrocytes, PE22-160 showed stronger binding than PE84-108. Thus, by using peptides that were 20-30 amino acids long, we could narrow down the PE adhesive domain to 24 amino acids.

We wanted to analyse the specificity of the PE84-108 peptide and investigate whether it could block the binding of PE22-160 to epithelial cells. By preincubating epithelial cells with increasing amounts of PE84-108 and subsequently adding a fixed
amount of iodine-labeled PE22-160, we could show that the binding of PE22-160 decreased at increasing concentrations of PE84-108. In another set of blocking experiments, the bacterial adherence to the epithelial cells could be blocked by 50% when cells were preincubated with PE84-108. Control experiments with non-binding PE domains (PE21-48) were included in the blocking experiments to ensure that the blocking was specific for PE84-108.

Our collected data on the peptide experiments not only point out what region of the PE molecule that shows the most efficient binding, they also underline the specificity of PE, especially since the whole bacterial binding could be blocked by PE84-108.

Several eukaryotic receptors have been identified for the *H. influenzae* adhesins (41). The pili interact with both respiratory mucin and the Anton antigen found on erythrocytes (173, 174). Furthermore, Hap interacts with extracellular matrix proteins whereas P5 interacts with respiratory CEACAM1 and ICAM-1 (10, 46, 73). Both CEACAM-1 and ICAM-1 are cell adhesion molecules that belong to the immunoglobulin super gene family, and are induced in several types of human cells after a viral or bacterial infection. We have shown that the central part of the PE molecule interacts with untreated epithelial cells and erythrocytes via yet unidentified receptor/s. However, the exact mechanisms behind this interaction remain to be elucidated.

*PE induces a proinflammatory response and promotes the bacterial invasion in respiratory epithelial cells (Paper IV)*

In a paper by Frick *et al*, it was shown that *H. influenzae* has the ability to stimulate high release of IL-8 and induce increased amounts of ICAM-1 from respiratory epithelial cells without affecting the upregulation of IL-1β, TNF-α or MHC class I (53). Furthermore, in an *in vivo* murine model it was shown that the increased ICAM-1 expression coincided with increased chemokine levels and neutrophil recruitment in the airways. This response was later found to be crucial in order for the activated neutrophils to attach and mediate phagocytic killing. In addition, none of the well-characterized *H. influenzae* adhesins including LOS were found to be responsible of the observed stimulatory effect (53).

These studies inspired us to investigate whether PE has a stimulatory effect on respiratory epithelial cells. By measuring the released IL-8 in the supernatant of infected cells (A549 or Chang) we could observe that after 4h to 24h postinfection the PE-expressing NTHi 3655 induced a significantly higher amount of IL-8 as compared to the NTHi 3655pe. Since IL-8 and ICAM-1 are interconnected in the inflammatory response, we decided to analyse the infected cells for ICAM-1
expression. In flow cytometry experiments, we found that at all timepoints analysed (0-24h) the wildtype induced significantly higher ICAM-1 expression than the mutant. We also analysed the upregulation of IL-8 on the mRNA level and found a similar pattern.

The next step was to analyse whether the upregulated ICAM-1 was functional for attachment of activated neutrophils in an in vitro assay system. Human neutrophils showed a greater adherence to NTHi 3655-infected cells as compared to cells infected with NTHi 3655\[pe. When the ICAM-1 receptor was blocked (with \[CD54), the neutrophil adherence was reduced by 47%. These experiments indicated that the increased ICAM-1 expression due to the NTHi 3655 has a biological relevance.

Invasion of host cells is a mechanism that pathogens use to avoid human clearing mechanisms and to prolong their survival in the airways. We wanted to investigate whether the PE-expression has a role for the bacterial invasion into respiratory epithelial cells. The NTHi 3655 strain was shown to invade up to three times more as compared to the mutant in A549 cells. Whether this effect was due to the fact that PE acts as an adhesin is not known.

In order to investigate if the difference in the inflammatory response was dependent on a decreased level of adhesion due to the absence of PE in the mutant strain, we performed the same set-up of experiments with recombinantly expressed PE on the surface of E. coli and E. coli (empty vector) as with the H. influenzae strains. Obtained results displayed a similar pattern of IL-8 production and ICAM-1 expression as with the NTHi strains. We concluded that PE has immunostimulatory properties, and therefore decided to stimulate A549 cells with recombinantly produced PE devoid of the signal peptide (PE22-160). As a negative control, we used a recombinantly produced protein derived from MID (MID1000-1200). To eliminate contamination risks due to LPS, we included polymyxin B in the culture medium. PE was found to display a strong immunostimulatory effect on the IL-8 production and the ICAM-1 expression.

In paper III, we defined the active binding region of PE to comprise the central part of the molecule, PE84-108. We were interested to know whether this region was sufficient for the stimulatory effect observed with PE22-160. Initially, we tested the stimulatory effect of all the peptides covering the entire PE molecule (paper III). No peptide displayed any stimulatory effect. The next step was to investigate whether the PE84-108 peptide could block the stimulatory effect due to PE. Therefore, we incubated A549 cells with the PE84-108 peptide and then added NTHi 3655. By measuring the IL-8 production and the ICAM-1 expression after 4h, it was shown that PE84-108 could block the bacterial stimulatory effect by 43% for the IL-8 production and by 50% for the ICAM-1 expression. These results indicate that
although PE84-108 can partially block the PE stimulatory effect, the full length molecule (PE22-160) including the 84-108 region is needed for an inflammatory response.

Lipoproteins are known for their cytokine-inducing ability. In some lipoproteins the lipid modification is essential for the immunostimulatory effect, as for example, lipoprotein OspA from *Borrelia burgdorferi* (183). Nonlipidated OspA produced in *E. coli* shows no stimulatory activity, whereas lipidated OspA produced in *B. burgdorferi* or *E. coli* does. The active moiety lies in the N-terminal tripalmitoyl (Cys-Pam3), which is common to immunoregulatory lipoproteins (64). We have shown that PE displays immunostimulatory effects independently of the signal peptide, i. e., the lipid moiety. The signal peptide of PE is highly hydrophobic and it has therefore been difficult to purify full-length PE from both *H. influenzae* and *E. coli*. Whether purified PE including the lipid moiety would induce an increased stimulatory effect as compared to PE22-160 is not known. Identification of the receptor that PE signals through would be one step in investigating the interesting pathway of PE signaling.
Concluding remarks

*H. influenzae* interacts with several human components that may involve bacterial attachment at the epithelial surface, sheltering from defense mechanisms and induction of signaling in host cells. Characterization of bacterial surface structures and their interactions with the human host will lead to greater understanding of the pathogenesis of *H. influenzae* and hopefully to the discovery of new vaccine candidates.

*H. influenzae* Hsf is an adhesin that belongs to the autotransporter family. Its virulence was first described to involve increased bacterial attachment to epithelial cells. In paper I, we have described the binding of the *H. influenzae* type b protein Hsf to vitronectin and the significance of this interaction. Vitronectin is multifunctional protein that is part of the extracellular matrix (ECM) and a component of serum where it regulates the complement by inhibiting the formation of the MAC. By binding to vitronectin, Hsf may increase the bacterial survival in the airways or in the circulation.

In papers II-IV, we have described a novel *H. influenzae* protein, designated PE. By using an IgD IgG myeloma serum that specifically recognized PE we could target the protein and isolate it. PE is a small protein of 16 kDa, expressed on the bacterial surface and its N-terminal amino acid composition revealed that it is a lipoprotein. Despite its small size, PE was found to be important for the bacterial attachment to epithelial cells. Through specific interactions with the central part of the molecule, PE displayed binding to several human respiratory epithelial cell lines and erythrocytes. Moreover, we found that PE plays an important role for the bacterial induction of inflammatory mediators. A high inflammatory response characterizes several of the diseases caused by *H. influenzae*, including AOM and COPD. PE was shown to upregulate high levels IL-8 and ICAM-1 which are crucial mediators for the human innate immune response. Lastly, PE was found to promote the bacterial internalization into epithelial cells which is a mechanism used by *H. influenzae* to persist in the airways.

PE displays several properties that certainly qualifies it as a virulence factor of *H. influenzae*. One interesting area is its ability to induce an inflammatory response. Whether PE is involved in additional interactions with the human host remains to be further investigated.
FUTURE PERSPECTIVES

In papers II-IV we describe the isolation and characterization of a novel *H. influenzae* protein, designated PE. This protein has been found to be very important for the bacterial adhesion to epithelial cells. It binds specifically with its central part of the molecule to an unknown receptor of three different epithelial cells and erythrocytes. Continued studies on PE should focus on the determination of the target receptor for adhesion on epithelial cells. This protein could be isolated by preparations of cell extracts from epithelial cells or erythrocytes. Analysis of extracted proteins in far western blot experiments may then be used for detection of specific protein-protein interactions, where PE could be iodine-labeled and incubated with the membrane in order to probe the putative eukaryotic receptor. By elucidating the eukaryotic receptor for PE we could learn more about the role of PE in the pathogenesis of *H. influenzae*.

Furthermore, we have shown that PE has a strong ability to stimulate the epithelial production of the two inflammatory mediators IL-8 and ICAM-1. It is known from the literature that *H. influenzae* induces multiple signaling pathways and some of the *H. influenzae* proteins involved in the signaling cascades have been characterized. *H. influenzae* associated diseases such as COPD and AOM are characterized by an inflammatory response. Defining the signaling pathways induced by PE would definitely lead to an enhanced understanding of the underlying mechanisms behind the upregulation of inflammatory genes in COPD and an indication of how the inflammation can be suppressed. One suggestion of an experimental approach to investigate signaling pathways would be to inhibit transcription factors or certain kinases before stimulating cells with recombinant purified PE or PE-expressing bacteria and thereby demonstrating essential proteins involved in the signaling cascade elicited by PE.

In addition to a role in adhesion and inflammatory responses, our data indicate that PE promotes the bacterial invasion into epithelial cells. *H. influenzae* uses different invasion mechanisms to invade epithelial cells such as macropinocytosis, invasion via the PAF-receptor or invasion via the β-glucan receptor. Since viable bacteria have been detected in tissues of COPD patients, it is believed that this mechanism is used in order for *H. influenzae* to persist in the airways. Further exploration of the mechanisms behind PE-mediated invasion could be important to increase our knowledge of factors contributing to bacterial persistence in the airways. Inhibition of certain cellular structures by preincubating epithelial cells with cytochalasin D or colchicine before adding PE-expressing bacteria could be one way to start investigating this mechanism.

Den icke kapslade H. influenzae ger ofta återkommande infektioner hos barn som lider av öroninfektioner (ottit) och hos äldre personer drabbade av kronisk obstruktiv lungsjukdom (KOL), kronisk bronkit och cystisk fibros.


I artikel I har vi studerat hur ett ytprotein från Hib kallat Haemophilus surface fibrils (Hsf) interagerar med immunförsvarat genom att binda till vitronektin. Vitronektin är ett glykoprotein som förekommer dels i det extracellulära matrixet (ECM) i epitelcellerna och dels i löslig form i serum.


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