Intercellular passage of epithelial cell layers: a pathogenic mechanism for Haemophilus Influenzae infections
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GENERAL INTRODUCTION
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Introduction

The Gram-negative bacterium *Haemophilus influenzae* is a natural inhabitant of the human upper respiratory tract and an important pathogen responsible for respiratory tract infections as well as systemic disease. Isolates of *H. influenzae* occur either encapsulated or nonencapsulated, based on the expression of a polysaccharide capsule (Pittman, 1999). Encapsulated strains express one of six structurally and antigenically different capsular types designated serotypes a to f. Nonencapsulated *H. influenzae* isolates fail to agglutinate with antisera against these capsular serotypes and are nontypeable (Pittman, 1999).

Analysis by multilocus enzyme electrophoresis indicates that encapsulated strains are clonal and can be divided into genetically related clusters which are grouped into two major phylogenetic divisions (Musser et al., 1988). Among 2209 encapsulated isolates, 280 electrophoretic types were distinguished. Nontypeable *H. influenzae* are much more heterogeneous. Of 65 nontypeable isolates analyzed by multilocus enzyme electrophoresis each isolated had its own and unique electrophoretic type (Musser et al., 1986). Comparison of the electrophoretic types revealed no overlap between those of the 65 nontypeable *H. influenzae* isolates and those of 177 *H. influenzae* type b isolates, indicating that nontypeable strains and serotype b strains are genetically quite distinct (Musser et al., 1986). However, a small subgroup of nontypeable *H. influenzae* strains hybridized with a probe containing the type b capsule locus, indicating that these strains might have evolved more recently from a type b encapsulated ancestor (St.Geme et al., 1994b; St.Geme et al., 1998). Using the infant rat model for intranasal colonization it has been found that serotype b *H. influenzae* strains change very readily into nonencapsulated strains (Hoiseth et al., 1985). Capsule deficient *H. influenzae* type b strains have also been isolated from the nasopharynx of children (Hoiseth and Gilsdorf, 1988; St.Geme et al., 1994b).

*H. influenzae* disease

Approximately 95% of cases with invasive *H. influenzae* disease such as meningitis, epiglottitis, cellulitis, arthritis, sepsis, and pneumonia is caused by type b strains (Moxon and Wilson, 1991; Turk, 1984). Systemic infection due to *H. influenzae* type b is assumed to occur after the bacterium has colonized the nasopharyngeal epithelium and from there has invaded into the blood stream (Robbins et al., 1973; Saito et al., 1999). The capsule expression is an important virulence determinant of *H. influenzae* causing invasive disease as it allows intravascular survival (Moxon and Vaughn, 1981).

Infections by nontypeable *H. influenzae* are mostly limited to the respiratory mucosal surface. In children, nontypeable *H. influenzae* is a frequent cause of acute and recurrent otitis media. *H. influenzae* strains causing acute otitis media likely migrate by direct extension from sites in the nasopharynx through the eustachian tube to the middle ear,
possibly facilitated by eustachian tube dysfunction (Murphy et al., 1987; Loos et al., 1989). In adults, nontypeable *H. influenzae* is implicated in lower respiratory tract infections, particularly in the elderly and in patients with chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) (van Alphen. 1992; Murphy and Sethi. 1992; Gilligan. 1991; Murphy and Apicella. 1987; Moller et al. 1995; Groeneveld et al., 1990a). These infections are caused by *H. influenzae* derived from the colonized nasopharyngeal mucosa.

**Carriage of *H. influenzae***

Both *H. influenzae* type b and nontypeable *H. influenzae* colonize the upper respiratory tract. Most children carry *H. influenzae* type b strains in the nasopharynx for a limited period of time during the first 5 years of their life, corresponding with a prevalence carrier rate of 2-4%. The carrier rate for nontypeable *H. influenzae* is up to 80% (Turk. 1984; Murphy and Sethi, 1992). Following colonization of the nasopharynx with *H. influenzae*, the mucosal surface contains sIgA molecules directed against the infecting strain. The predominant subclass of IgA in the respiratory tract is IgA1, which is susceptible to IgA-specific proteases produced by *H. influenzae* (Brandtzaeg, 1995). The impaired capacity of encapsulated variants to colonize for a prolonged period of time is probably due to functional anti-capsular antibodies at the mucosal surface. After initial colonization, encapsulated *H. influenzae* is readily eradicated from the nasopharynx when the host immune response is activated, whereas nonencapsulated variants of these strains persist under these conditions (Kauppi et al., 1993; Hoiseth et al., 1985). Vaccination of large groups of children with a *H. influenzae* type b conjugate vaccine has not only eradicated *H. influenzae* type b systemic disease, but has also reduced oropharyngeal carriage of *H. influenzae* type b (Garpenholt et al., 1996; Mohle-Boetani et al., 1993; Takala et al., 1993). The effect of antcapsular antibodies on colonization of *H. influenzae* type b is most likely due to the inhibitory effect of these antibodies on bacterial growth, or on bacterial adherence to the nasopharyngeal mucosa (Kauppi-Korkeila et al., 1996; van Alphen et al., 1996).

Antibodies against nontypeable *H. influenzae* strains present in human serum and on mucosal surfaces are targeted to outer membrane proteins (OMPs), lipopolysaccharide and IgA proteases all having substantial antigenic heterogeneity (Campagnari et al., 1987; Troelstra et al., 1994; Lomholt et al., 1993). The mucosal immune response against these antigens results in reduction or elimination of *H. influenzae* strains from the mucosal surface of the respiratory tract (Faden et al., 1995; Harabuchi et al., 1994; Sakamoto et al., 1998). The high incidence of carriage of nontypeable *H. influenzae* despite the presence of antibodies on the respiratory mucosa, reflects a high turnover of strains colonizing the nasopharynx (Faden et al., 1995), as well as (Murphy et al., 1987; Lomholt et al., 1993) antigenic variation of the various bacterial surface antigens to which antibodies are elicited, contributing to persistent colonization of one isogenic strain.
Colonization of the lower respiratory tract

The epithelium of the lower respiratory tract has specialized defense mechanisms against bacteria present after aspiration or inhalation. Epithelial cells are covered with cilia that beat coordinately. Overlaying these cells is a cover of mucus containing antimicrobial components including lysozyme, lactoferrin, defensins, and sIgA. Cilia serve to move the overlaying mucus layer upward to the throat in a process called mucociliary clearance.

In healthy persons binding to mucus is of importance for clearance of bacteria from the lower respiratory tract, since mucus is rapidly removed by mucociliary clearance. In COPD and CF patients conditions in the lower respiratory tract are altered due to chronic inflammation which is characterized by infiltration of neutrophils and the up-regulation of the production of cytokines and other inflammatory mediators. This process leads to airway obstruction and impaired mucociliary clearance (Jansen et al., 1995; Gilligan, 1991). Under these conditions, association of *H. influenzae* with mucus may allow bacterial multiplication resulting in persistent colonization (Murphy and Sethi, 1992; Moxon and Wilson, 1991; Gilligan, 1991).

*H. influenzae* has been shown to associate with mucus strands produced by cultured human epithelial cells (Moxon and Vaughn, 1981; Read et al., 1991; Stephens and Farley, 1991; Wilson et al., 1992). *H. influenzae* adheres to mucus sol phase or to purified preparations of mucins (Barsum et al., 1995; Kubiet and Ramphal, 1995; Reddy et al., 1996; Davies et al., 1995). Fimbriae of *H. influenzae* may be involved in adherence to mucus, but the molecules in the mucus which are recognized by the bacteria were not defined (Barsum et al., 1995). Mucins are high molecular weight glycoproteins and major constituents of mucus. Using intact bacteria it was shown that only a subset of nontypeable *H. influenzae* strains adhered to mucins in a specific manner (Davies et al., 1995). There was no evidence that the fimbriae interacted with mucins on the cells. Reddy et al., (Reddy et al., 1996) found that sialic acid containing oligosaccharides in mucins bound to the OMP's P2 and P5 of outer membrane preparations from all *H. influenzae* strains tested. In addition, of two nontypeable *H. influenzae* strains other unidentified proteins bound mucin with a greater affinity than OMP's P2 and P5. Since in this study outer membrane preparations were used, it remains to be shown whether these OMP's are available for adherence to mucus in vivo.

Adherence to epithelial cells

Adherence of *H. influenzae* to human epithelial cells is important for establishment and outgrowth of the bacteria on the epithelial cells. Several adhesins with affinity for various receptors on the eukaryotic cell surface may determine tissue tropism (Stephens and Farley, 1991; Wilson et al., 1992; St.Geme and Cutter, 1996).

Adherence of *H. influenzae* epithelial cells occurs fimbriae- mediated as well as fimbriae- independent. Fimbriae are long filamentous organelles that extend from the
bacterial surface. Fimbriae of the long thick hemagglutination positive (LKP) family are involved in the adherence to oropharyngeal epithelial cells and the agglutination of human erythrocytes (Forney et al., 1992; van Alphen et al., 1988). The agglutination is mediated via the AnWj blood group antigen (van Alphen et al., 1986) and the receptor for the H. influenzae fimbriae on human epithelial cells is a sialic acid-containing lactosylceramide localized in the cell membrane. Fimbriae-mediated adherence to epithelial cells can be demonstrated in vitro in binding assays with nasopharyngeal epithelial cells and can be inhibited with purified ganglioside GM2 (van Alphen et al., 1991). Short thin fibrils are formed by another adhesin mediating adherence of H. influenzae type b to cultured human epithelial cells and having a different specificity than fimbriae (St.Geme and Cutter, 1995). Fimbriae-mediated adherence seems to be especially relevant for adherence of encapsulated H. influenzae strains to different cells during infection (Farley et al., 1990; Loeb et al., 1988; Sterk et al., 1991; St.Geme and Cutter, 1995), since the capsule interferes with the adherence through fibrils and other nonfimbrial adhesins, in contrast to fimbriae mediated adherence (St.Geme, 1996; St.Geme and Cutter, 1996). In addition, H. influenzae type b unable to express fimbriae, showed decreased colonization capacity in primates compared to the fimbriated variant of this strain (Weber et al., 1991).

Only a minority of nontypeable H. influenzae strains from COPD contains a fimbriae gene cluster (Geluk et al., 1998) suggesting a minor role for the fimbriae in the colonization of nontypeable H. influenzae in the lower respiratory tract. Several non-fimbrial proteins have been described to mediate attachment of nontypeable H. influenzae to different cell lines (Barenkamp and St.Geme, 1996a; St.Geme et al., 1994a; St.Geme et al., 1993). The most common adhesins are two immunogenic high molecular weight proteins designated HMW1 and HMW2 (St Gême et al., 1993; Barenkamp and Leininger, 1992), that are detected in 75-80% of unrelated nontypeable H. influenzae strains (Barenkamp and St.Geme, 1996b; St.Geme, 1996). These proteins show homology to filamentous hemagglutinin (FHA) of Bordetella pertussis. This adhesin plays a critical role in B. pertussis colonization of the upper respiratory tract. The two HMW proteins mediate binding to distinct human epithelial cells indicating different receptor specificity (St.Geme et al., 1998; St.Geme, 1996; Hultgren et al., 1993). HMW1 recognizes a sialylated glycoprotein and HMW1-mediated adherence can be inhibited by heparin or dextran sulfate (St.Geme, 1994; Noel et al., 1994b). Some nontypeable H. influenzae isolates lacking the HMW proteins but adhering efficiently in vitro to Chang epithelial cells, express the adhesin Hia (Barenkamp and St.Geme, 1996a). Based on DNA sequence analysis it was concluded that Hia is the nontypeable variant of the fibrils expressed by encapsulated strains. Finally, the Hap protein of H. influenzae was identified (St.Geme et al., 1994a), which mediates low level but intimate adherence to Chang epithelial cells, thereby leading to invasion of H. influenzae into epithelial cells.

In organ culture studies, adherence of nontypeable H. influenzae is enhanced on injured epithelial cells (Read et al., 1991; Wilson et al., 1992). The mechanism behind
this phenomenon is unknown. Recently, adherence of nontypeable *H. influenzae* to human airway epithelial cell appeared enhanced in the presence of neutrophil defensins (Gorter et al., 1998). Neutrophil defensins are released upon neutrophil degranulation and are present in high concentrations in purulent airway secretions from patients with COPD and CF (Gorter et al., 1998). All nontypeable *H. influenzae* strains tested showed this phenomenon irrespective of the presence of adhesins. Therefore, defensin-stimulated adherence may be an important factor in recurrent infections in the airways of these patients.

**Passage of the airway epithelium**

Penetration of bacteria through the respiratory epithelium is normally hindered by tight junctions between the cells. During incubation of *H. influenzae* with organ cultures of respiratory epithelium bacterial products, including the ciliotoxic lipopolysaccharide of *H. influenzae*, disorganize the ciliary beating and damage the epithelium (Johnson and Inzana, 1986; Wilson et al., 1985; Wilson et al., 1992), thereby inhibiting mucociliary clearance. In addition, mucus production is stimulated in the presence of *H. influenzae*, presumably through the bacterial production of histamine (Sheinman et al., 1986).

Several *in vivo* studies indicate that nontypeable *H. influenzae* penetrates the respiratory epithelium during carriage and invasive disease. *H. influenzae* penetration of the upper respiratory tract was detected *in vivo* by in situ hybridization and bacterial viability assays of adenoid tissue from young children who had clinically no infection (Forsgren et al., 1994). Subepithelial localization of *H. influenzae* was also demonstrated in lungs of COPD patients analyzed post-mortem (Hers and Mulder, 1953) and in lung explants from COPD and CF patients obtained during lung transplantation surgery (Moller et al., 1998). Nontypeable *H. influenzae* bacteria resided diffusely between the epithelial cells and in the submucosa. Intracellular bacteria were detected in subepithelial macrophages in adenoid tissue of young children (Forsgren et al., 1994) and in close contact with tissue macrophages in the lungs of COPD and CF patients (Moller et al., 1998). Adherence of *H. influenzae* to macrophages is mediated by HMW protein. Bacteria bound via HMW proteins *in vitro* remained largely extracellular and viable, indicating that efficient uptake of bacteria by macrophages requires additional serum opsonization (Noel et al., 1994a).

In organ cultures incubated with *H. influenzae*, progressive damage of the respiratory tissue allowed *H. influenzae* to interact with the underlying basal cells and the epithelial basement membrane (Farley et al., 1990; Loeb et al., 1988; Read et al., 1991). *In vitro, H. influenzae* adheres to the extracellular matrix via specific interactions with laminin, fibronectin, and various collagens (Virkola et al., 1996). In addition, binding of plasmin to *H. influenzae* led to penetration of bacteria through a basement membrane preparation (Virkola et al., 1996). These mechanisms are likely relevant for spread of *H. influenzae* into subepithelial tissue.
**In vitro** experiments with cultured epithelial cells showed that presence of *H. influenzae* induced the production of pro-inflammatory mediators such as IL-8, IL-6, and TNFα by the epithelial cells, leading to increased neutrophil chemotaxis (Khair et al., 1994; Khair et al., 1996). *H. influenzae* strains persisting in the lower respiratory tract of COPD patients elicited lower responses than strains isolated on only one occasion (Bresser et al., 1997). These results suggest that the presence of *H. influenzae* may affect epithelial cell function and influence the inflammatory reaction of the airway mucosa via induction of pro-inflammatory mediators.

**Persistence of *H. influenzae* in the lower airways of COPD and CF patients**

Non-typeable *H. influenzae* isolates from the lower respiratory tract of COPD and CF patients were characterized in longitudinal bacteriological follow-up studies using genotyping of *H. influenzae* isolates by DNA restriction fragment length polymorphism (RFLP) or random amplified polymorphic DNA (RAPD) patterns, and phenotyping by OMP analysis. Despite the presence of specific antibodies in the sputum and sera of these patients or antibiotic treatment of the patients, persistence of particular *H. influenzae* strains in the lower respiratory tract of COPD and CF patients was shown for periods up to 2 years (Groeneveld et al., 1988; Groeneveld et al., 1990a; Groeneveld et al., 1990b; Moller et al., 1995; Moller et al., 1992).

An effective mechanism for microorganisms to evade from immune defenses of the host is antigenic variation of surface proteins. Antigenic drift of OMPs P2 and P5 of strains persisting in COPD and CF patients has been shown, since the electrophoretic mobility of these OMPs showed variation and preexisting antibodies were unable to kill the new variants in a bactericidal assay (Groeneveld et al., 1988; Moller et al., 1995; Groeneveld et al., 1989). The changes were associated with amino acid substitutions due to non-synonymous point mutations in surface exposed loops (Duim et al., 1997; Duim et al., 1994).

Antigenic variation was also observed for lipopolysaccharide (LPS) of *H. influenzae*, another major target for bactericidal antibodies against *H. influenzae* (Groeneveld et al., 1990a). The molecular basis for this variation is the switching on and off of genes encoding glycosyltransferases and thereby antigenic determinants (Roche and Moxon, 1995; Weiser, 1993). Diversity in LPS is also caused by decoration of the molecule with sialic acid or phosphorylcholine, which causes that LPS mimics host-structures (Weiser et al., 1997; Weiser et al., 1998).

Antigenic drift is indicative for strong immunologic pressure (Duim et al., 1997; Duim et al., 1994; Moller et al., 1995). Exacerbations in COPD patients were associated with re-infection by either exogenous strains with a different genotype, or antigenic variants of endogenous strains (Groeneveld et al., 1990b). In CF patients, OMP variant strains appeared irrespective of the occurrence of exacerbations (Moller et al., 1995). The
appearance of new OMP variants in COPD patients soon after exacerbations suggests that immunologic selective pressure is effective during exacerbations. In support of this hypothesis, Yi et al. (1997) showed that during an exacerbation, COPD patients made new antibodies to a limited number of antigens, including strain specific epitopes of OMP P2. Antigenic variation was studied in a rabbit model using subcutaneous tissue cages were H. influenzae persisted up to 3 years. In this model, immunologic pressure induced by vaccination with the infecting strain resulted in earlier appearance of OMP variants (Vogel et al., 1996). Therefore, antigenic drift is suggested to be a mechanism to escape the antibody dependent immune response and as a consequence to promote persistence of H. influenzae. Despite the immune response, however, the parental strain coexisted with its variants in the subcutaneous tissue cages. This reflects the findings in CF patients where also the parental strain coexisted with its variants (Moller et al., 1995). Apparently, in these cases, the clearance mechanisms are ineffective for the complete removal of bacteria, and immune escape by antigenic drift is not a prerequisite for persistence of H. influenzae.

A general inefficiency in the immune defense mechanisms operating against H. influenzae may also contribute to persistence in COPD and CF patients. Opsonizing antibodies against H. influenzae are most likely not capable of stimulating effective eradication of the bacteria, since viable nontypeable H. influenzae were reported to be poorly opsonophagocytosed in the presence of specific antibodies and complement (Vogel et al., 1994). Since the presence of sialic acid on the surface of bacteria is known to inhibit humoral defense mechanisms, this may be relevant for H. influenzae. Furthermore, the excessive production of mucus in these patients, even stimulated during infection, may prevent trapped and adhered bacteria from being killed, since mucus is a diffusion barrier for antibodies and complement and is impermeable for inflammatory cells. This diffusion barrier may also contribute to inefficient killing of H. influenzae by antibiotic treatment, although selection of isolates with increased antimicrobial resistance during persistence in the respiratory tract of CF patients occurs at low frequency (Moller et al., 1998). However, persistence of nontypeable H. influenzae in the lower respiratory tract of CF and COPD patients occurs despite long periods in which H. influenzae cannot be detected in sputum and throat swabs by culturing and immunological staining, indicating that mucus is most likely not the reservoir for H. influenzae during persistence (Davies et al., 1995; Groeneveld et al., 1990b; Moller et al., 1995; van Alphen et al., 1995). Since penetration of H. influenzae into the subepithelial tissues is characteristic for COPD and CF patients, bacteria may escape eradication by hiding in subepithelial tissues.

Summarizing, the lower respiratory tract of COPD and CF patients is predisposed for H. influenzae infections due to a low level of inflammation. Once present, H. influenzae is not easily eradicated, despite the host response elicited. Poor opsonophagocytosis, antigenic drift, and “hiding” for bactericidal antibodies and antibiotics are likely important factors contributing to H. influenzae persistence (Figure 1).
Predisposed airway epithelia
- low level of inflammation
- PMN's and defensins present

Latency
- bacteria hiding in submucosa

Entrapment of *H. influenzae*
in mucus and adherence
- production of IL-6 and IL-8, and TNF-α by the epithelial cells
- mucociliary damage
- bacterial growth

Full inflammatory response
- influx PMN's
- mucus overproduction
- antibodies against infecting strain
- antigenic variation

*Figure 1:* Model of the steps required for chronic infection of the lower respiratory tract of COPD and CF patients by nontypeable *Haemophilus influenzae*
Aims and outline of the study.

In this thesis we focus on the mechanisms contributing to passage of *H. influenzae* through epithelial cell layers. Passage of *H. influenzae* between epithelial cells has been studied in nasopharyngeal organ culture studies, where the bacteria were found in clusters between the cells (Farley et al., 1990; Read et al., 1991; Wilson et al., 1992; Stephens and Farley, 1991). However, incubation of the organ cultures with *H. influenzae* invariably damaged the respiratory epithelium. The *in vitro* model using cultured human lung epithelial cells on filter supports developed in this thesis allowed studies of passage through intact epithelial cell layers (paracytosis) during long term association of *H. influenzae* bacteria with the cells. In Chapter 2 the passage of *H. influenzae* from the apical to the basolateral side of the epithelial side was studied, as well as the effect of bacterial adherence, and the influence of bacterial metabolism in this model. In Chapter 3 the effect of localization of *H. influenzae* between epithelial cell layers on the susceptibility of *H. influenzae* for antibiotics and antibody-mediated killing during persistence of the bacteria in the system is described. In addition, the effect of immunologic pressure on the appearance of antigenic variants of OMP P2 in the cell system in the presence of specific antibodies against OMP P2 is described.

Since passage of *H. influenzae* through the cell layers was more efficient for adherent strains, adherence of *H. influenzae* isolated from COPD and otitis media patients compared to isolates from healthy controls to two epithelial cell lines was evaluated in the study described in Chapter 4. Bacterial genes that are up-regulated during interaction with the epithelial cells are identified in Chapter 5. In Chapter 6 we describe the cloning and characterization of *H. influenzae* genes involved in penetration into the epithelial cell layer. The significance of the paracytosis for *H. influenzae* infections is discussed in Chapter 7.
References


GENERAL INTRODUCTION


GENERAL INTRODUCTION


