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

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GENERAL DISCUSSION
Characteristics of H. influenzae passage through lung epithelial cell layers

Passage of H. influenzae through the respiratory tract epithelium occurs in the onset of systemic disease as well as during persistent lower respiratory tract infections in CF and COPD patients (Hers and Mulder, 1953; Moller et al., 1998; Moxon et al., 1974). After infection of the lower respiratory tract, H. influenzae persists despite antibiotic treatment of the patients and its susceptibility for the majority of the antibiotics used to treat lower respiratory tract infections. In addition, immunological pressure due to high concentration of specific antibodies against H. influenzae exerting complement dependent bactericidal activity in vitro, is inefficient to clear the bacteria (Moller et al., 1995; Groeneveld et al., 1990a). During periods that H. influenzae strains are not detected in sputum specimens or throats swabs from COPD and CF patients (Groeneveld et al., 1990b) the bacteria are present in the subepithelial layers of the lower respiratory tract of these patients (Moller et al., 1998; Hers and Mulder, 1953), probably forming the niche from where H. influenzae may provoke reinfection.

In vitro model systems consisting of lung epithelial cells on permeable supports were developed to mimic the first steps of the infection process (Chapter 2 and 3). We observed that localization of H. influenzae between the epithelial cells was associated with a reduced effect of various antibiotics and bactericidal antibodies in the presence of complement on the bacteria. This indicated that the intercellular localization shielded the bacteria against the antibacterial activity of antibiotics and host components (Chapter 3). In other studies it has been suggested that uptake of H. influenzae in epithelial cells or in subepithelial macrophages provides such a reservoir (Ketterer et al., 1999; Holmes and Bakaleitz, 1997; Forsgren et al., 1994). Uptake of H. influenzae into epithelial cells is initiated by binding of the bacteria to the host cell surface (Ketterer et al., 1999; Holmes and Bakaleitz, 1997), which promotes reorganization of the cytoskeleton. Uptake of bacteria by the epithelial cells was also observed in our study but intracellular H. influenzae seemed to be degraded (chapter 2 and 3). It is unclear which factors are involved in active uptake of the bacteria by the epithelial cells or endocytosis. Adherence of H. influenzae to the epithelial cell surface in our model was not related to an enhanced uptake into the epithelial cells. Endocytosis probably involves bacterial factors as well as cellular factors, since only specific cells in the cell layer were ingesting bacteria. Although H. influenzae was cultured from macrophages of the upper respiratory tract of children (Forsgren et al., 1994) it is not known how long bacteria survive in these macrophages. In in vitro experiments, internalization of H. influenzae by human phagocytes is generally very poor and requires additional opsonization with specific antibodies (Troelstra et al., 1994; Burnett et al., 1993). In the absence of serum opsonisation macrophage-bound H. influenzae remained largely extracellular and viable (Noel et al., 1994). Once internalized by macrophages or polymorhuclear leukocytes H. influenzae is rapidly killed (Noel et al., 1994; Vogel et al., 1994). Thus, in contrast to
bacteria such as *Mycobacterium* spp., *Bordetella pertussis*, *Shigella flexneri*, *Salmonella* spp., and *Escherichia coli* that have been shown to survive or even replicate inside host cells (reviewed by Rosenshine and Finlay, 1993). *H. influenzae* inside in the epithelial cells or macrophages seems not to be able to resist the intracellular host environment or to direct its cellular processing. In lung tissue sections from CF or COPD patients *H. influenzae* was mostly present extracellularly (Moller et al., 1998; Hers and Mulder, 1953). Therefore, an intracellular location providing a reservoir for *H. influenzae* infections is much more unlikely than that the intercellular presence of *H. influenzae* forms such a reservoir.

**Mechanisms of paracellular passage**

The passage of *H. influenzae* between the lung epithelial cells seems to involve selective disclosure of intercellular junctions (Chapter 3). The mechanisms by which *H. influenzae* is able to disclose these junctions are unknown. Other bacteria employing paracytosis or interjuntional passage are e.g. the spirochetes *Borrelia burgdorferi* and *Treponema pallidum* (Haake and Lovett, 1994). The mechanisms underlying their passage are also unknown.

Various intestinal pathogens perturb the paracellular barrier by production of toxins, such as the zonula occludens toxin (ZOT) and the hemagglutinin/protease of *Vibrio cholerae* (Fasano et al., 1995; Baudry et al., 1992) (Wu et al., 1996), toxin A and toxin B of *Clostridium difficile* (Feltis et al., 1999), and VacA of *Helicobacter pylori* (Papini et al., 1998). The increase in permeability of the epithelial cell layer induced by these toxins mostly coincides with a decrease in transepithelial resistance and with a rearrangement of (F)-actin. However, each of the toxins exerts a different effect on the paracellular barrier. VacA increased the paracellular epithelial permeability only to low molecular mass (<350-440) molecules without affecting the structure of the intercellular junctions (Papini et al., 1998). ZOT has been shown to alter the structure of intercellular junctions (Fasano et al., 1995). A major change in permeability leading to paracellular transmigration of different bacterial species through epithelial cell layers was observed due to toxins A and B. However, these toxins exert a pathologic effect on epithelial cells, which was never observed during passage of *H. influenzae* between the epithelial cells.

By which mechanisms *H. influenzae* may influence the intercellular permeability of epithelial cells in a subtle, reversible way which permits whole bacteria to protrude is currently unknown. The mechanisms may resemble the mechanism by which leukocytes penetrate through epithelial cell layers. Leukocytes penetrate the cell layers after upregulation of the intercellular adhesion molecule ICAM1 by TNFα. In addition, TNFα affects the tight junctional region between epithelial cells, which coincides with lower transepithelial resistance and increased flow of solute between cells and across the epithelium (Mullin and Snock, 1990). The increased paracellular permeability of brain endothelial cells induced by TNFα increased virus penetration of HIV by the paracellular
route (Fiala et al., 1997). Infection of epithelial cells with *Mycobacterium tuberculosis* led to the production of TNFα by the cells which was associated with an increased permeability of the epithelial cell layer (Zhang et al., 1997). Interaction of *H. influenzae* (Bresser et al., 1997) or *H. influenzae* LPS (Khair et al., 1994) with different lung epithelial cell lines also induced the production of TNFα as well as other inflammatory mediators such as IL6 and IL8 by the epithelial cells and the expression of ICAM1. However, the epithelial permeability in our model was unaltered, and decreased in the study using HBEC lung epithelial cells (Khair et al., 1994). In addition, it is unknown whether *H. influenzae* can interact with the ICAM1 molecules.

The paracytin gene of *H. influenzae* involved in paracytosis was identified (Chapter 6). This allows us to characterize the paracytin and how it regulates the intercellular permeability. Knowledge of the modification of the function of the intercellular junctions by the *H. influenzae* paracytin may enlarge our understanding of the normal physiologic regulation of intercellular junctions and the intercellular permeability.

The role of adhesins in bacterial colonization of the respiratory tract

Adherence of bacteria to human epithelial cells is considered to be the first step in colonization and subsequent infection (Foxwell et al., 1998). Adherence of *H. influenzae* resulted in enhanced numbers of bacteria passing the NCI-H292 epithelial cell layers. However, nonadherent *H. influenzae* strains also passed (Chapter 2 and 3). Therefore, adherence and entrance of nontypeable *H. influenzae* into the cell layer are two separate processes, although adherence may promote the bacterial passage. Adherence of *H. influenzae* isolates from patients with respiratory tract infections such as otitis media and COPD was compared to adherence of isolates from the throat of healthy individuals (Chapter 4). Adherence to the cell lines correlated strongly with HMW adherence proteins, indicating that the HMW1 or HMW2 are important adhesins. However, significantly more otitis media isolates adhered to the two cell lines used than throat isolates from healthy individuals. Also more COPD isolates adhered to both cell lines than isolates from healthy individuals, but the difference was not significant. Of the 19 isolates from COPD patients 37% did not adhere to either cell line. Therefore, the ability of *H. influenzae* to adhere may be more relevant for the onset of acute upper respiratory tract infections such as otitis media than for lower respiratory tract infection in COPD patients. Since *H. influenzae* strains that are nonadherent are able to adhere to these cell lines in the presence of neutrophil defensins (Gorter et al., 1998), it may be that such an adherence mechanism operates in COPD patients. In the lower respiratory tract of these patients neutrophil defensins are present continuously due to the low-level inflammatory reaction in the bronchial tree (Gorter et al., 1998).
Implications for the treatment of *H. influenzae* infections

The localization of *H. influenzae* bacteria between epithelial cells resulted in shielding against antibiotics and antibody-mediated defense mechanisms (Chapter 3). Therefore, penetration of *H. influenzae* between epithelial cells may contribute to bacterial persistence in the lower respiratory tract of COPD and CF patients, where bacterial eradication does not occur despite the antibiotic treatment of the patients or the presence of specific antibodies in the sputum and serum of the patients (Groeneveld et al., 1990b). Persistence of nontypeable *H. influenzae* strains in the lower respiratory tract of COPD and CF patients is associated with antigenic variation of major outer membrane proteins (MOMP), being the targets for antibodies against such *H. influenzae* strains (Groeneveld et al., 1988; Duim et al., 1997). Immunological pressure induced by vaccination with the infecting strain in rabbit cages persistently infected with *H. influenzae*, promoted the selection of MOMP variants of *H. influenzae*, instead of leading to eradication (Vogel et al., 1996). These results indicate that a vaccination strategy against infections due to nontypeable *H. influenzae* may be useful only in an early stage so that colonization of the respiratory tract might be prevented.

Vaccination with certain bacterial components may elicit antibodies interfering with the mechanisms involved in the colonization of the upper respiratory tract by these bacteria, like vaccination of large groups of children with *H. influenzae* type b conjugate vaccine reduced oropharyngeal carriage of *H. influenzae* type b (Garpenholt et al., 1996; Mohle-Boetani et al., 1993; Murphy et al., 1993). Antibodies to *H. influenzae* type b most likely reduce colonization by a direct effect on the bacterial growth or on the bacterial adherence to the nasopharyngeal mucosa (Kauppi-Korkela et al., 1996; van Alphen et al., 1996). Similarly, prevention of carriage of nontypeable *H. influenzae* in the upper respiratory tract may provide protection against persistent infections with *H. influenzae* in patients with COPD and CF, and against otitis media in children. Immunization with different outer membrane proteins of nontypeable *H. influenzae* including OMP26, OMP P6, HMW adhesins, and HtrA, enhanced bacterial clearance in animal protection models (Suzuki et al., 1998; Kyd and Cripps, 1998; Barenkamp, 1996; Loosmore et al., 1998). In addition, OMP P6 and HMW adhesins have been considered vaccine antigens to prevent nasopharyngeal colonization by *H. influenzae* (Yang et al., 1998; Hotomi et al., 1998; Kyd et al., 1995). OMP P6 is a highly conserved antigen on the outer membrane of nontypeable *H. influenzae* (Murphy et al., 1986) and the HMW protein is expressed by the majority of the isolates obtained from patients with respiratory tract infection, especially otitis media (Virkola et al., 1996) (Chapter 4). However, HMW proteins are very antigenically divers (St.Geme et al., 1998; Barenkamp and Leininger, 1992) (Chapter 4) which makes them unsuitable as vaccines.

The *H. influenzae* paracytin, identified in chapter 6 and involved in penetration between the epithelial cell, has also to be considered as a vaccine-candidate, since it is a conserved protein. Blocking of the function of the paracytin by antibodies may impair paracytosis and thus the persistence of *H. influenzae* in the epithelial cell layers. Other
genes of *H. influenzae* that may be involved in colonization of the respiratory epithelium were identified in chapter 5. In this chapter we identified a group of genes that were specifically induced upon interaction with the epithelial cell. Several genes encoded proteins of unknown function of which some may play a role in the interaction with epithelial cells, like the gene encoding the conserved D15 surface antigen, which has been shown to be immunogenic and protective in animal models (Loosmore et al., 1997).

**In conclusion**

Several aspects of the interaction of *H. influenzae* with human lung epithelial cells were studied. Using an *in vitro* model with immortalized human lung epithelial cells we identified bacterial components that may be important for the persistent colonization of the respiratory tract *in vivo*. The function of these components can be studied in other models, such as the chinchilla model for nasopharyngeal colonization (Yang et al., 1998) to obtain data of the role of these components in colonization *in vivo* and to investigate whether these components are useful vaccine candidates to prevent *H. influenzae* infections.
References


GENERAL DISCUSSION


GENEAL DISCUSSION


