Intercellular passage of epithelial cell layers: a pathogenic mechanism for Haemophilus Influenzae infections
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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. Encapsulated strains with a serotype b polysaccharide cause invasive *H. influenzae* disease such as meningitis. Infections by nonencapsulated (nontypeable) *H. influenzae* are mostly limited to respiratory mucosal surfaces and result from contiguous spread of *H. influenzae* from the nasopharynx. Nontypeable *H. influenzae* is a frequent cause of otitis media, and of lower respiratory tract infections, particularly in patients with chronic obstructive pulmonary disease (COPD) and cystic fibrosis (CF) (Chapter 1).

The first interaction of *H. influenzae* with its host is an interaction between the bacterium and the epithelial cell layer of the respiratory tract. *H. influenzae* is generally considered to be an invasive organism, since in the onset of systemic disease as well as during persistent lower respiratory tract infections *H. influenzae* bacteria pass through the respiratory tract epithelium. Once present in the lower respiratory tract of COPD and CF patients, *H. influenzae* is hardly eradicated by antibiotic treatment. Furthermore, the hypervariability of surface antigens impairs the eradication by host immune defense mechanisms. Knowledge of the process of invasion of *H. influenzae* through the epithelium is of importance to design strategies to block this process as well as to obtain insight into the invasion mechanisms used by various bacteria.

In Chapter 2 and 3 an *in vitro* model system consisting of human lung epithelial cells on permeable supports is described and used to study the passage of *H. influenzae* through epithelial cell layers. Microscopic examination revealed the presence of clusters of *H. influenzae* bacteria between the epithelial cells, indicating that bacterial passage was due to paracytosis. Based on morphological features, *H. influenzae* passage through these cell layers was representative for *in vivo* penetration. *H. influenzae* passed between the epithelial cells independent of the presence of capsule or fimbriae on *H. influenzae* or the ability of the bacteria to adhere to the epithelial cells (Chapter 2). However, highly adherent strains showed greater paracytosis than non-adherent *H. influenzae*, since a larger number of bacterial clusters of adherent strains between the epithelial cells was observed. Furthermore, *de novo* bacterial protein synthesis was needed for the bacteria to reach the intercellular space.

In Chapter 3 we showed that localization of *H. influenzae* between epithelial cell layers in the *in vitro* model system shielded the bacteria against the bactericidal activity of several antibiotics and against antibody-mediated killing. In addition, after prolonged incubation in the cell system in the presence of a specific bactericidal antibody against major outer membrane protein (MOMP) P2, antigenic variation occurred. The antigenic
variation was due to a point mutation in the MOMP P2 gene, similar as point mutations observed in vivo, indicating that it was possible to mimic immune pressure for mutation in this model.

From these experiments we concluded that (1) penetration of H. influenzae between lung epithelial cells may contribute to the persistence of this microorganism in COPD and CF patients and (2) our in vitro model could be used to study passage of H. influenzae through lung epithelial cells. The in vitro model was used to identify H. influenzae genes involved in passage of the epithelial cell layer, as described in Chapter 4, 5 and 6.

Since passage of H. influenzae through the cell layers was more efficient for adherent strains, the adherence of nontypeable H. influenzae isolates obtained from clinical samples and from the throat of healthy individuals to two human epithelial cell lines was compared in the study described in Chapter 4. In contrast to otitis media isolates, COPD isolates did not adhere significantly more to the Chang and NCI-H292 epithelial cells than throat isolates. Perhaps, adherence to the epithelial cells is of less significance for COPD isolates than for otitis media isolates because the mucociliary clearance in the lower respiratory tract of COPD patients is impaired. In addition, nonadhering COPD isolates may adhere to the lung epithelial cells through neutrophil defensins that are present in high amounts in the lungs of COPD patients. A majority of the adherent isolates expressed high molecular weight proteins (HMW), indicating that this is an important adhesins for the adherent strains of all three groups. Screening of the isolates with HMW-specific antibodies showed that the HMW proteins of COPD isolates and carrier isolates were antigenically distinct from the HMW proteins from otitis media isolates. Despite the antigenic heterogeneity of the HMW proteins the adherence patterns found for the various H. influenzae isolates are conserved.

We characterized genes of nontypeable H. influenzae expressed upon interaction with the human lung epithelial cells in Chapter 5. A library of 8,000 clones was constructed in H. influenzae Rd (recF), by cloning chromosomal fragments upstream of a promoterless cat gene encoding chloramphenicol acetyl transferase. Clones that were specifically resistant to chloramphenicol in the presence of epithelial cells but not on agar plates were selected. The induced expression from the cloned promoter sequences in the presence of the epithelial cells indicated that these genes are important for interaction with the cells or for survival in the cell culture. Some of these genes, including four novel genes, encoded proteins of unknown function. These may play a role in the interaction with epithelial cells. The group of genes encoding proteins with assigned functions gave some insight into the bacterial processes involved in bacterial interaction with epithelial cells. It seems that in the presence of the epithelial cells, growth and protein synthesis of H. influenzae is promoted, and that the bacteria have to be able to survive anaerobic conditions and high salt concentrations.

In Chapter 6 we describe the cloning and characterization of H. influenzae genes involved in penetration into the epithelial cell layer. A chromosomal library of a
nonencapsulated *H. influenzae* isolate in *Escherichia coli* DH5α was constructed in order to identify bacterial genes contributing to paracytosis. Two open reading frames contributing to an increased penetration of *E. coli* in the epithelial cell layers were identified. These two open reading frames were similar to HI0636 and HI0638 of *H. influenzae* Rd, that encode for two small proteins of unknown functions. Disruption of HI0638 by kanamycin box insertion in *H. influenzae* strain A960053 resulted in loss of penetration into the epithelial cell layers. Disruption of HI0636 had no effect on penetration in this model system. We concluded that the protein encoded by ORF HI0638 may function as a paracytin, and HI0636 may have an auxiliary function for paracytosis of the *E. coli* clones.

The identification of bacterial components involved in interaction and passage of human respiratory epithelial cells, may lead to novel approaches in the treatment of lower respiratory tract infections due to *H. influenzae*, as discussed in Chapter 7. Alternatively, a vaccination strategy may be considered for individuals at risk for *H. influenzae* infections. Furthermore, the *H. influenzae* paracytin may be a valuable tool to study the mechanisms by which epithelial cells maintain intercellular contacts.