Pathogenesis of Haemophilus influenzae. Respiratory infection in COPD patients

van Leeuwen-Gorter, A.D.

Link to publication

Citation for published version (APA):

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CHAPTER 6

GENERAL DISCUSSION
**H. influenzae** infection of the lower respiratory tract in COPD patients

In healthy individuals, bacteria do not colonize on the epithelial cell layer of the lower respiratory tract. Bacteria reaching the deeper airways by inhalation and aspiration will be cleared from the airways by the mucociliary elevator and by killing by antimicrobial polypeptides such as β-defensins and secretory leukocyte protease inhibitor (SLPI) produced by the airway epithelial cells (figure 1). The epithelial cell layer of the airways from patients with chronic obstructive pulmonary disease (COPD) is damaged by cigarette smoke and by the inflammatory processes, in which neutrophils are involved (figure 2). The neutrophils contain a variety of antimicrobial peptides, including the neutrophil defensins that belong to the family of α-defensins [13,23]. Based on clinical observations, it appears that the chronically inflamed airways of COPD patients are more susceptible to bacterial infection. Therefore, we hypothesized that bacteria, such as *Haemophilus influenzae*, exploit the inflammatory process and their components like neutrophil defensins, to enhance their adherence [chapter 2]. Since bacterial adherence to epithelial cells is an important step in the pathogenesis of *H. influenzae* infection, it is most likely that the defensin-enhanced bacterial adherence results in colonization and infection.

![Figure 1](image)

**Figure 1.** Schematic representation of the airways from a healthy individual. The airway surface liquid (ASL) contains antimicrobial polypeptides produced by the respiratory epithelial cells, such as β-defensins and secretory leukocyte proteinase inhibitor (SLPI). If bacteria enter the lower respiratory tract, they are cleared from the airways by the antimicrobial polypeptides as well as the mucociliary clearance.
Mechanism of the defensin-enhanced bacterial adherence

The defensin-enhanced bacterial adherence occurred in conditions where the defensins did not display their antimicrobial activities [chapter 2], indicating that both mechanisms are distinct. For the understanding of the mechanism involved in the defensin-enhanced adherence, it is important to know by which bacterial component or components defensins are bound to initiate the enhanced bacterial adherence. Originally, we constructed a gene bank of *H. influenzae* in *Escherichia coli* to identify this bacterial component. However, in the presence of neutrophil defensins the adherence of none of the *E. coli* clones to the human epithelial cell line NCI-H292 was enhanced. Therefore, we hypothesized that a complex molecule of which the expression is regulated by multiple genes is involved. Such a molecule may be the lipooligosaccharide (LOS) of *H. influenzae*. In addition, heat-inactivated bacteria still showed the defensin-enhanced adherence [chapter 2 and 3]. The three Gram-negative bacterial species showing the defensin-enhanced adherence, *H. influenzae, Moraxella*

![Figure 2. Schematic representation of the airways from a COPD patient. The epithelium is damaged and there is excessive mucus production, preventing the mucociliary clearance. Neutrophils are attracted and they release their granule peptides upon activation, among which the neutrophil defensins. Under these conditions, bacteria such as *H. influenzae* may colonize the respiratory tract, which is facilitated by defensins through enhancing bacterial adherence.](image_url)
catarrhalis, and Neisseria meningitidis [chapter 3], have LOS in their outer membrane [8], in contrast to E. coli and Pseudomonas aeruginosa, of which the adherence is not enhanced by defensins [chapter 3] and which have lipopolysaccharide. Using LOS mutants of H. influenzae and N. meningitidis, we concluded that LOS, especially the lipid A part of LOS, mediated the defensin-enhanced adherence [chapter 4]. The fact that for the defensin-enhanced adherence the presence of bacteria, defensins, and epithelial cells are needed simultaneously [chapter 2], suggested that defensins form a bridge between the bacteria and the epithelial cells, probably via an interaction with the fatty acids of the lipid A of LOS (figure 3). The bacterial species not showing the defensin-enhanced bacterial adherence, E. coli and Pseudomonas aeruginosa [chapter 3], have LPS in their outer membrane, which is larger than LOS. Since close contact between the defensins and the lipid A part is likely necessary for the defensin-enhanced adherence (figure 3), the defensins might not be able to form a bridge between lipid A of the large LPS molecules and the epithelial cell membrane. This could explain the fact that the defensin-enhanced adherence was only observed for bacterial species with LOS in their outer membrane.

Although our data clearly demonstrate that LOS is involved in the defensin-enhanced bacterial adherence [chapter 4], the mechanism is yet unknown. A problem with defining the role of LOS in the defensin-enhanced adherence is that the configuration of purified LOS differs from that of LOS in the bacterial membrane [40]. To circumvent this problem, it would be interesting to analyze the adherence of liposomes in which H. influenzae wild type and mutated LOS is incorporated. In this way, the role of LOS in the defensin-enhanced adherence of H. influenzae can be analyzed without the interference of other bacterial components.

**Effects of defensins and bacteria on epithelial cells**

In the airways of COPD patients, the epithelial cell layer may be damaged [2]. It was shown previously that H. influenzae preferably adhered to damaged epithelial cells [43]. In response to the injury, epithelial cells start to migrate and a repair process is initiated [44,45]. Since dr. E. Puchelle and colleagues (INSERM U314, Reims, France) showed that P. aeruginosa adhered to the migrating epithelial cells [10,11], the effect of defensins on the adherence of H. influenzae to migrating epithelial cells was determined in collaboration with this group. Unpublished experiments revealed that H. influenzae adhered preferably to the migrating epithelial cells. In the presence
of defensins the adherence of *H. influenzae* to the migrating epithelial cells was enhanced. These results indicated that the defensin-enhanced adherence occurred not only with epithelial cells in intact epithelium, but also to migrating epithelial cells.

![Figure 3. Proposed model describing the interactions of neutrophil defensins with epithelial and bacterial membranes, leading to the defensin-enhanced adherence. In this model, the neutrophil defensins form a bridge between the lipid A of the LOS of the bacteria and the epithelial cell membrane.](image)

*H. influenzae* as well as neutrophil defensins may contribute to airway inflammation in COPD patients by inducing the production of pro-inflammatory cytokines [5,20,38,42]. We have found that *H. influenzae* plus neutrophil defensins synergistically increased the release of pro-inflammatory cytokines [chapter 5]. Therefore, it is likely that the inflammatory process in COPD patients is exaggerated if *H. influenzae* is present in the lower respiratory tract. The production of cytokines is regulated by transcription factors, which are activated by a signal transduction cascade originating from the binding of ligands to a receptor. The epithelial receptors for bacteria and bacterial products include the Toll like receptor (TLR) family [18,39]. Since activation of TLRs may result in the activation of NF-κB and NF-κB is involved in the induction of pro-inflammatory cytokines, it would be interesting to analyze the role of TLR in the cytokine release by epithelial cells upon stimulation with *H. influenzae* plus defensins.
Clinical relevance of the effects of defensins on the interaction of *H. influenzae* with epithelial cells

During inflammation neutrophils and thus also neutrophil defensins are present in the airways of COPD patients (figure 4). Stimulated neutrophils may release high amounts of defensins, resulting in high concentrations of defensins in the surroundings of the neutrophil, especially in the sequestered areas between the neutrophil and the target cell to which it is adhering [12]. As demonstrated by sputum analysis, high concentrations of neutrophil defensins are present in the airway secretions of COPD patients [31,42]. In patients with airway infection and chronic bronchitis, high salt concentrations have also been found [14,17]. This could be due to the fact that in dedifferentiating and remodeling airway epithelium, the expression of the cystic fibrosis transmembrane regulator (CFTR) protein decreases, irrespective of underlying disease or mutations in the CFTR gene [6,7]. Since the antimicrobial activity of the neutrophil defensins is dependent on the salt concentrations [24,32], it is likely that the neutrophil defensins cannot display their optimal antimicrobial activity in the airway surface liquid from COPD patients. This suggests that bacteria in the lower respiratory tract of these patients most likely are not killed. However, we demonstrated that in the presence of high salt concentrations defensins enhanced the adherence of *H. influenzae*. This enhancement of the bacterial adherence may also be relevant in other diseases, such as cystic fibrosis (CF). It has been shown that patients with CF have bacterial infection, with *H. influenzae* as one of the important pathogens [29]. Furthermore, high concentrations (300-1600 µg/ml) of neutrophil defensins have been isolated from patients with CF [36]. Studies in CF patients have indicated that the antimicrobial activity of epithelial antimicrobial peptides such as β-defensins may be impaired as a result of the increased salt content found in the airway surface liquid secreted by cultured epithelial cells [15]. Whether this impaired effect of β-defensins is a factor by which the susceptibility of CF patients to respiratory infections is increased is not yet clear.

Since the IL-8 production probably results in an influx of neutrophils, the process of infection and inflammation in the COPD patients is likely maintained by the defensin-enhanced adherence and subsequently increased cytokine production (figure 4). This is supported by several studies showing that patients with stable COPD which are colonized with bacteria have more inflammation, as shown by the amount of
**Figure 4.** Contribution of neutrophil defensins to the chronic inflammation and infection as observed in COPD patients. Defensins enhance the bacterial adherence, which may lead to enhanced bacterial colonization and infection. Subsequently, the inflammation is continued by the enhanced production of pro-inflammatory cytokines. Note that normally, the effects of neutrophil defensins and neutrophil elastase are counteracted by α1-AT.

Neutrophils and the production of pro-inflammatory cytokines such as TNFα and IL-8, than stable COPD patients which are not colonized [4,16,35]. In addition, during exacerbations COPD patients with *H. influenzae* and *M. catarrhalis* infections had significantly higher sputum concentrations of inflammatory markers compared to pathogen-negative exacerbations [34]. Since the combination of *H. influenzae* with neutrophil defensins synergistically increased the production of pro-inflammatory cytokines [chapter 5], it is likely that the defensin-enhanced cytokine production is involved in the increased inflammation observed in the *H. influenzae* and *M. catarrhalis* exacerbations.
Normally, the effects of neutrophil defensins are counteracted by components in the mucus, such as serine protease inhibitors like α1-AT [31]. We hypothesize that in the sequestered areas, the high concentrations of defensins are not directly inhibited and in those areas, defensins probably enhance the bacterial adherence and subsequently enhance the production of pro-inflammatory cytokines by epithelial cells (figure 4). Whereas at least 100 μg/ml neutrophil defensins may be found in sputum [31], it is not clear what percentage is present in a free form, not complexed to inhibitors. With respect to the study described in this thesis, it would be interesting to analyze whether the concentrations of defensins in sputum differs between COPD patients with and without bacterial infection.

The deficiency of α1-AT is a genetic risk factor in the development of COPD. The patients with α1-AT deficiency have more bacterial infections than patients without this deficiency [25]. Also the inflammatory state, as measured by the presence of MPO, IL-8 and LTB₄, is higher in patients with α1-AT deficiency [16]. Since most of the activities of neutrophil defensins are inhibited by α1-AT [31], including the defensin-enhanced adherence [chapter 2], it is tempting to speculate that the defensin-enhanced adherence as well as the increased cytokine production are important in the development of bacterial infection and inflammation in COPD patients with α1-AT deficiency (figure 4). Further support for this hypothesis that α1-AT decreases bacterial infection and inflammation by inhibition of the defensin-enhanced adherence and the defensin-increased cytokine production, comes from a study in which α1-AT deficient COPD patients were treated with α1-proteinase inhibitor (α1-PI). This treatment resulted in a reduction in the bacterial infection as well as a reduction in the inflammatory state of the airways of these patients [25].

In our in vitro assays, the defensin-enhanced adherence and cytokine production was analyzed on epithelial cells that were not stimulated in advance. This differs from the in vivo situation in COPD patients, where epithelial cells are activated by pro-inflammatory cytokines, such as TNFα. Therefore, it will be interesting to determine the effect of stimulation of the epithelial cells, e.g. with TNFα, before the addition of H. influenzae and defensins. Nevertheless, the effects of defensins we observed in vitro are likely relevant for the patients, since COPD patients with bacterial colonization have more inflammation [4,16,35].

At present the only effective strategy that has been shown to reduce progression of COPD is smoking cessation. Since only one third of the COPD patients are able to
give up smoking, even with support [3], other therapies are required. Since COPD patients have chronic airway inflammation, these patients are treated with anti-inflammatory drugs, such as inhaled corticosteroids. In patients with asthma, an airway disease which is also characterized by chronic inflammation, these corticosteroids are very effective in reducing inflammation [19]. In contrast, in COPD patients there is little evidence for a beneficial effect of the corticosteroids [9,19,28]. Neither inhaled nor oral steroids had any significant effect on the neutrophil counts, neutrophil granule proteins, or pro-inflammatory cytokines in sputum [19], which is in agreement with the lack of effect of steroids on the disease progression. The bacteria alone induce the release of pro-inflammatory cytokines by epithelial cells, a process which is synergized in the presence of neutrophil defensins [chapter 5]. This may suggest that the effect of the corticosteroids in COPD patients with lower respiratory tract \(H. influenzae\) infection will be diminished. The finding that \(H. influenzae\) as well as defensins increased the half life of IL-6 and IL-8 mRNA [chapter 5], could also be an explanation for the fact that corticosteroids are not able to inhibit the ongoing inflammation and infection in COPD patients.

Another approach to circumvent the persisting \(H. influenzae\) infection in COPD patients may be vaccination. Immunization with different outer membrane proteins of \(H. influenzae\), including P6, OMP26, D15, HtrA and HMW, enhanced the bacterial clearance in animal models [1,21,22,26,27,30]. The HMW proteins, however, are highly variable [37] and therefore not suitable as vaccine candidates. Another vaccine candidate is the \(H. influenzae\) paracyn protein, since the gene encoding paracyn is conserved [41]. Blocking paracyn might impair the paracytosis and therefore decrease the persistence of \(H. influenzae\).

**Concluding remarks**

In this thesis, we described that neutrophil defensins, which are present in high amounts in purulent sputum, enhance bacterial adherence. In combination with \(H. influenzae\), neutrophil defensins synergistically enhanced the production of pro-inflammatory cytokines. COPD patients which are colonized by bacteria have more inflammation than COPD patients without colonization [4,35] and this difference in inflammatory state is even more evident in COPD patients with \(\alpha1\)-AT deficiency, who have also more infection than COPD patients with normal \(\alpha1\)-AT levels [16]. Therefore, it is tempting to speculate that defensins enhance the bacterial
adherence and colonization in vivo. The subsequent induction of the pro-inflammatory cytokines will lead to increased amounts of neutrophils in the COPD patients, subsequently leading to increased concentrations of neutrophil defensins in the airway lumen, maintaining inflammation and infection as observed in COPD patients (figure 4). Our findings are in line with the vicious circle hypothesis which has been proposed for COPD patients [33], which states that inflammation will lead to bacterial infection and this infection will lead to a further increase in inflammation. Further understanding of the mechanisms by which defensins affect the interaction of H. influenzae to airway epithelial cells is needed to be able to break through this vicious circle in COPD patients.

References


species and may represent a universal protective antigen against invasive disease. 


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