Haemophilus influenzae and airway inflammation in chronic bronchitis
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General discussion
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Recurrent and chronic infections of the lower respiratory tract with bacteria like *Haemophilus influenzae* are prominent in chronic bronchitis patients. These infections are considered to be the consequence of altered local conditions induced by chronic airway inflammation [1]. Bacteria like *H. influenzae*, however, may also stimulate local inflammatory processes by inducing inflammatory mediators [2] and by causing epithelial damage [3,4]. About the role of *H. influenzae* in airway inflammation, however, relatively little is known. Therefore, in our studies, we employed both ex vivo and in vitro approaches to study various aspects of *H. influenzae* in relation to airway inflammation.

In Chapter 2 we studied the presence of *H. influenzae* strains within the lower respiratory tract of chronic bronchitis patients with a documented history of recurrent *H. influenzae* infections. For most of these patients, chronic infection of the airways with either a persisting strain (with or without antigenic variation), consecutive unrelated strains, or a combination of both was observed. These findings are consistent with a previous study from our group in patients with chronic obstructive pulmonary disease (COPD) who also had a documented history of lower respiratory tract infections with *H. influenzae* [5]. In this earlier study, however, a substantial number of patients were non-smokers living in a home for the elderly, and thus we cannot exclude that these patients represented a selected population with relatively advanced airway disease. In the present study, most of the patients were (ex-)smokers, and all were selected from the outpatient clinic of our hospital. In addition, we ruled out the presence of bronchiectasis or a humoral immunodeficiency in these patients. Therefore, our findings indicate that long-term persistence also occurs in airways from typical CB patients. Also, we showed that distinct bacterial strains could be demonstrated despite antibiotic treatment, consistent with our earlier reports [5,6].

Our long-term follow-up of *H. influenzae* strains in the airways of these patients also revealed a difference with the earlier study. In the earlier study [5], exacerbations were demonstrated to coincide with endogenous and exogenous reinfections with *H. influenzae*. This suggested that bacterial changes were causally linked to exacerbations observed in these patients. In the present study, however, we found that in the airways of a number of patients distinct strains were demonstrated to be present both before and after an exacerbation. Although this does not exclude that *H. influenzae* is involved in the pathogenesis of exacerbations, it indicates at least that exacerbations in chronic bronchitis patients are not always associated with a change in the bacterial phenotype.
In view of the concept that bacterial infections in chronic bronchitis patients are the consequence of altered local conditions caused by chronic airway inflammation [1], we argued that reduction of the local inflammatory process might reduce bacterial infection. However, we could not demonstrate an anti-inflammatory effect of treatment with either budesonide or N-acetylcysteine (NAC) for a three months period. In line with the current concept, we did not find an effect on bacterial persistence or antigenic variation by either treatment. Reduction of airway inflammation upon treatment with inhaled corticosteroids has been reported in non-infected obstructive chronic bronchitis, also in relatively small numbers of patients [7-9]. However, considering the relatively low number of patients in the present study, it is difficult to draw conclusions about more subtle effects of treatment with either budesonide or NAC on airway inflammation and bacterial persistence.

The mechanism of bacterial persistence is still far from clear. Possible explanations for persistence include antigenic variation [10-14], and the ability of *H. influenzae* to persist intercellularly [15-17] as well as intracellularly [18]. In the studies described in Chapter 3, we found that *H. influenzae* isolates that were effectively cleared from the airways induced the release of high levels of the pro-inflammatory mediators interleukin(IL)-6 and IL-8 in H292 airway epithelial cells. In contrast, strains that were shown to persist locally in the airways of chronic bronchitis patients for at least six months, induced a much lower IL-6 and IL-8 response in the airway epithelial cells. We propose that this may be an additional mechanism in local bacterial persistence of *H. influenzae* in chronic bronchitis and COPD.

By the induction of only a low inflammatory response in airway epithelial cells in vivo, bacteria descending into the lower airways may gain time to establish themselves.

The reason for the difference in IL-6 and IL-8 induction by nonpersisting and persisting strains is unknown. Previously, lipopolysaccharide (LPS) isolated from *H. influenzae* was demonstrated to induce the production of inflammatory mediators in airway epithelial cells *in vitro* [2]. In our studies, IL-6 and IL-8 production in our studies was demonstrated to be induced by factors released by the bacteria into the bacterial culture supernatant. By removing LPS from the culture supernatant by polymyxin-B immobilized on beads, we lost the capacity to induce IL-6 and IL-8 in H292 cells by bacterial culture supernatant. These results pointed to LPS as the factor responsible for the mediator release. However, LPS isolated from *Escherichia coli* and from *H. influenzae*, induced only low amounts of IL-6 and IL-8 in H292 cells even after exposure to relatively high concentrations of LPS (unpublished results). We cannot exclude conformational changes in LPS due to its isolation which could make LPS a less potent stimulus. An alternative explanation is that either a component bound to LPS, or a synergism between two factors, LPS and an as yet unknown
factor are involved in the induction of the mediator release by the H292 cells. Peptidoglycans might be candidates in this respect, since the induction of IL-6 and IL-8 by culture supernatants was potentiated with a β-lactam antibiotic (ampicillin) in the medium during bacterial growth. Results of a representative experiment are shown in Figure 1.

![Figure 1](image-url)

**Figure 1.** Interleukin-6 production by H292 epithelial cells upon exposure for 6 hr to 10% (v/v) bacterial culture supernatant of persisting strain 5324 (P), nonpersisting strain 6173 (NP), or without bacteria (C). Values are expressed as mean of duplicate experiments. Bacteria were grown in Brain Heart Infusion broth, supplemented with hemin and NAD (10 mg/L), with or without ampicillin (A; 20 mg/ml) for 6 hrs. Ampicillin was added after 3 h, while the culture showed exponential growth of the bacteria, as assessed by optical densitometry (OD530) of the culture broth.

Bacteria grown in the presence of β-lactam antibiotics, by which transpeptidation and incorporation of peptidoglycans into the cell wall by peptide-crosslinking are inhibited, typically secrete soluble polymeric peptidoglycan fragments. The peptidoglycan composition of the cell wall of *H. influenzae* was demonstrated to be strain dependent and, in addition, distinct fragments were demonstrated to exert different biological activities both *in vitro* and *in vivo* [19-21]. Therefore, our studies warrant further studies into possible differences in peptidoglycan composition of persisting and nonpersisting *H. influenzae* isolates.

Binding of *H. influenzae* to extracellular matrix (ECM) components may be another factor involved in bacterial persistence [4,22,23]. Our results presented in Chapter 4 indicate that this is most probably a factor of limited importance. Although clinical isolates of *H. influenzae* were demonstrated to adhere to various ECM proteins, we could not demonstrate a difference with respect to adherence between persisting and non-persisting strains.
Little was known about airway inflammation in chronically infected chronic bronchitis patients, and about the contribution of chronic infections to airway inflammation in COPD. Our analyses on airway inflammation in COPD patients described in Chapter 5, indicate that chronic infection with *H. influenzae* in COPD patients is associated with a more marked airway inflammation. As compared to non-infected COPD patients, in chronically infected COPD patients significantly higher levels of tumor necrosis factor-α (TNF-α), IL-8 and myeloperoxidase (MPO) were detected in sputum. Furthermore, chronic infection in COPD patients was associated with a more pronounced plasma protein leakage, indicating a more marked local inflammatory damage. Based on these findings it is tempting to suggest that the observed difference is caused by the chronic bacterial infection. Since these patients often experience airway infections with other bacterial pathogens [24], it might be of interest to study whether bacterial infections other than *H. influenzae* are also associated with a more marked airway inflammation.

The observed differences in airway inflammation between infected and non-infected COPD patients, however, may also represent a primary difference between the two groups of patients. It could be envisaged that COPD patients with an exaggerated TNF-α production have greater airway inflammation, as a result of which bacteria like *H. influenzae* can more easily colonize the airways of these patients [1,24,25]. This observation should form an incentive for a study on TNF-α gene promoter polymorphism in these groups of patients, especially since the TNF2 allele has been implicated in greater TNF-α production [26,27]. Furthermore, since chronic airway obstruction in COPD is considered the consequence of airway inflammation, it may be suggested that chronic bacterial infections in these patients will be associated with a more rapid decline in lung function. The contribution of acute infections associated with exacerbations to the decline in lung function in COPD seems limited [24]. About the contribution of recurrent and chronic bacterial infections, however, little if anything is known. Therefore, our results also warrant a large, prospective multi-center study on the impact of recurrent and chronic bacterial infections on airway inflammation and the decline in lung function in COPD patients.

Our findings on airway inflammation in chronically infected obstructive and non-obstructive CB patients points to differences in pathophysiology. In this context, the high levels of TNF-α in the chronically infected obstructive chronic bronchitis patients, as compared with the levels in non-obstructive chronic bronchitis patients, are of particular interest. TNF-α has been implicated in the pathogenesis of chronic airway obstruction in smokers [28,29]. In particular, in a Taiwanese population, the TNF2 allele has been found
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at a higher frequency in smokers with obstructive chronic bronchitis [29]. Recently, two studies in Caucasian populations have been published that could not confirm the previously observed association between the TNF2 allele and the development of chronic airway obstruction [30,31]. Also our findings are not in line with a role of TNF-α in the development of chronic airway obstruction. TNF-α levels were much lower in the non-infected COPD patients as compared with the chronically infected COPD patients, whereas airway obstruction was similar in both groups. We can not exclude, however, that TNF-α together with other factors play a major role in the pathogenesis of chronic airway obstruction in smokers.

Exacerbations in chronic bronchitis patients were demonstrated to be associated with bacterial [24,32-35], as well as viral respiratory tract infections [5,6,36-38]. About the role of viral infections in the pathogenesis of exacerbations, however, relatively little is known. In Chapter 6, we showed that exposure of H292 airway epithelial cells to parainfluenza type IV virus resulted in a dose-dependent production of IL-6 and IL-8. The induction was biphasic in that there was a rapid transient expression of IL-6 and IL-8 mRNA, followed by a second peak of mRNA expression. This second peak was due to a reduced degradation of IL-6 and IL-8 mRNA. Previously, we have shown that H292 airway epithelial cells in which mRNA degradation is reduced, show exaggerated IL-6 and IL-8 responses, exemplified by leftward shifted and steeper dose-response curves [39]. Indeed we found an exaggerated IL-8 response to TNF-α. Of further interest is that the effect on the IL-6 response was less than that of IL-8. As IL-6 also has anti-inflammatory effects, the effect of a viral infection may promote inflammatory processes. Based on our findings, it may be hypothesized that during a viral respiratory tract infection the IL-8 response by epithelial cells will become more responsive to secondary stimuli such as components of H. influenzae. As a consequence, there will be an increased production of IL-8 which may exacerbate the inflammatory process.

References

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