Inflammatory response to viral airway infections and secondary bacterial complications
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Non-typable *Haemophilus influenzae* binds influenza A virus and reduces viral outgrowth *in vitro* and *in vivo*
Bacterial complications during influenza infections are a much more common cause of pneumonia than primary influenza infection. Increased colonization by bacteria, enhanced virulence of influenza virus and impaired host defense have been shown to account for the increased risk to develop pneumonia. However, the interaction between influenza virus and bacteria has never been studied. In the present study we show that influenza virus is able to bind to non-typable *Haemophilus influenzae* (NTHI). Moreover, NTHI infection reduced viral outgrowth in airway epithelial-like cells. Combined infection with NTHI and influenza virus was associated with synergistic IL-8 production. Combined infection in mice resulted in strongly diminished viral loads in the lung on day 2 and day 8 and reduced inflammation on day 8 post-infection. These data indicate that NTHI protects against influenza virus infection *in vitro* and *in vivo*. NTHI, which belongs to the commensal flora that persists in the upper airways, may serve as a natural scavenger of potentially pathogenic viruses.
Introduction

Influenza infections usually cause only mild symptoms such as fever, headache, sore throat, sneezing and nausea, accompanied by decreased activity and food intake [1]. Although influenza alone may lead to pneumonia, secondary bacterial infections during and shortly after recovery from influenza infections are much more common causes of pneumonia [2]. *Streptococcus pneumoniae, Staphylococcus aureus* and *Haemophilus influenzae* are the most frequently isolated during combined infection with influenza virus [1-3]. *H. influenzae* is one of the bacteria comprising the commensal flora of the human upper respiratory tract [4]. Under normal circumstances *H. influenzae* persists on mucosal surfaces as a consequence of biofilm formation [5]. At present, the function of persisting bacteria in the nasopharynx is poorly understood.

Acute or chronic inflammation causes *H. influenzae* to become pathogenic, leading to local or invasive infection [6]. Concurrent infection with influenza virus induces an inflammatory response, which may increase colonization by *H. influenzae* [7, 8]. Similarly, exacerbations in patients with chronic obstructive pulmonary disease (COPD) are often associated with *H. influenzae* infection [9, 10]. Several studies indicate that viral airway infections predispose COPD patients to *H. influenzae*-induced exacerbations [7, 11-13]. Both in healthy humans and COPD patients, an inflamed micro-environment enhances bacterial outgrowth of *H. influenzae* [6-8]. An alternative explanation for increased colonization by *H. influenzae* during influenza virus infection may be increased adherence to the airway mucosa after viral infection [13, 14]. Although several outer-membrane proteins and adhesins have been described to mediate binding to the airway mucosa, none of these factors have been implicated in the enhanced colonization by *H. influenzae* during viral respiratory tract infection [8, 15].

Although direct binding of bacteria and viruses has been described [16], a possible interaction of *H. influenzae* and influenza virus has never been studied. In the present study we show that influenza A binds to non-typable *H. influenzae* (NTHI). The capacity to bind influenza virus may interfere with the hosts’ response to influenza virus infection. To study the role of this interaction in host defense, we evaluated viral kinetics and pro-inflammatory cytokine production in airway epithelial-like cells and in a murine model for influenza virus infection.
Chapter 8

Materials and methods

Infectious agents
Influenza A/PR/8/34 (ATCC VR-95; Rockville, MD) was grown on LLC-MK2 cells (RIVM, Bilthoven, Netherlands). Virus was harvested by a freeze/thaw cycle, followed by centrifugation at 680g for 10 minutes. Supernatants were stored in aliquots at -80°C. Titration was performed in LLC-MK2 cells to calculate the median tissue culture infective dose (TCID\textsubscript{50}) of the viral stock [17]. A non-infected cell culture was used for preparation of the control inoculum. None of the stocks were contaminated by other respiratory viruses, i.e. influenza B, human parainfluenza type 1, 2, 3, 4A and 4B, Sendai virus, RSV A and B, rhinovirus, enterovirus, corona virus and adenovirus, as determined by PCR or cell culture. Viral stock and control stock were diluted just before use in phosphate-buffered saline (PBS, pH 7.4). Non-typable Haemophilus influenzae (NTHI, strain 12, patient isolate) was grown overnight on chocolate-agar plates at 37°C in a CO\textsubscript{2}-incubator and were subsequently suspended in BM1 medium supplemented with hemine and NAD\textsuperscript{+}. Bacteria were harvested by centrifugation at 2750 g for 10 minutes at 4°C and washed twice with ice-cold saline. After the second wash, the bacteria were resuspended in saline and diluted to a concentration of $2 \times 10^8$ colony forming units (CFU) per ml, which was determined by optical density at 530 nm and verified by plating out 10-fold dilutions onto chocolate-agar plates.

Cell culture
A human lung-derived mucoepidermoid adenocarcinoma cell-line NCI-H292 (ATCC no. CRL 1848, Rockville, MD) was maintained in RPMI medium (Gibco BRL, Paisley, UK) supplemented with 0.5 mM L-glutamine (Merck, Darmstadt, Germany), penicillin (100 U/ml), streptomycin (100 μg/ml) and 10% heat-inactivated fetal calf serum (FCS, Gibco BRL) at 37°C in a humidified atmosphere in a 5% CO\textsubscript{2}-incubator. During experiments, cell cultures were incubated with RPMI 1640 medium containing 0.5 mM L-glutamine and 10% FCS.

Mice
Pathogen-free 8 week-old female C57Bl/6 mice were obtained from Harlan-Sprague Dawley Inc. (Horst, Netherlands) and maintained at biosafety-level 2. All experiments were approved by the Animal Care and Use Committee of the Academic Medical Center, University of Amsterdam.

126
Non-typable *Haemophilus influenzae* interacts with influenza virus

**Binding assay**

10⁷ CFU NTHI and 10-fold dilutions of the original influenza virus stock were incubated at room temperature for 30 minutes in a final volume of 100 μl PBS. Bacteria were spun at low speed (100g for 10 minutes) and washed twice with sterile PBS. The pellet was resuspended in 500 μl Trizol reagent (Gibco BRL) to extract RNA. The number of viral particles present in the pellet was determined by real-time quantitative PCR as described before [18, 19]. In brief, RNA was resuspended in 10 μl DEPC-treated water. cDNA synthesis was performed using 1 μl of the RNA-suspension and a random hexamer cDNA synthesis kit (Applied, Foster City, CA). 5 μl out of 25 μl cDNA-suspension was used for amplification in a quantitative real-time PCR reaction (ABI PRISM 7700 Sequence Detector System). The viral load present in each sample was calculated using a standard curve of particle-counted influenza virus (virus particles were counted by electron microscopy), included in every assay. The following primers were used: 5'-GGACTGCAGCTGAGACGCT-3' (forward); 5'-CATCCTGTTGTATATGAGGCCCAT-3' (reverse) and 5'-CTCAGTTATTCTGCTGGTGCACTTGCC-3' (5'-FAM labelled probe).

**In vitro infection protocol**

Culture medium was removed and NCI-H292 cell cultures were washed twice with sterile PBS. Cells were incubated with 10⁷ CFU NTHI and 1.25 x 10⁴ TCID₅₀ influenza virus in a final volume of 200 μl PBS for 1 hour at room temperature. After 1 hour, culture medium (without penicillin and streptomycin) was added to a final volume of 1 mL and incubated at 37°C. Appropriate controls for both influenza virus infection and NTHI infection were included. After 4 hours, 24 hours and 48 hours medium was removed and stored at -20°C for cytokine measurements. The cells were washed with sterile PBS and treated with 500 μl Trizol reagent (Gibco BRL) to extract RNA and determine viral load as described above.

**In vivo infection protocol**

Mice were anesthetized by inhalation of isoflurane (Abbott Laboratories, Kent, UK) and intranasally inoculated with 10 TCID₅₀ influenza (1400 viral copies) and 10⁷ CFU NTHI in a final volume of 50 μl PBS. Alternatively, mice were intranasally inoculated with 10 TCID₅₀ influenza and one hour thereafter with 10⁷ CFU NTHI or vice versa. Appropriate controls for both influenza virus infection and NTHI infection were included. Forty-eight hours after
inoculation, mice (6 mice per group) were anesthetized using 0.3 ml FFM (fentanyl citrate 0.079 mg/ml, fluanisone 2.5 mg/ml, midazolam 1.25 mg/ml in H2O; of this mixture 7.0 ml/kg intraperitoneally) and sacrificed by bleeding out the vena cava inferior. Lungs were harvested and homogenized at 4°C in 4 volumes of sterile saline using a tissue homogenizer (Biospec Products, Bartlesville, OK). Fifty µl of lung homogenates were treated with 500 µl Trizol reagent to extract RNA and determine viral load as described above.

**Cytokine and chemokine measurements**

IL-6 and IL-8 in culture medium were quantified by enzyme-linked immunosorbent assay (ELISA) as described [20, 21]. Lung homogenates of mice were lysed with an equal volume of lysisbuffer (300 mM NaCl, 30 mM Tris, 2 mM MgCl2, 2 mM CaCl2, 1% (v/v) Triton X-100, 20 ng/ml Pepstatin A, 20 ng/ml Leupeptin, 20 ng/ml Aprotinin, pH 7.4) and incubated for 30 minutes on ice, followed by centrifugation at 680 g for 10 minutes. Supernatants were stored at -80°C until further use. Cytokines and chemokines in total lung lysates were measured by ELISA according to the manufacturer's protocol. Reagents for IL-6, KC and TNF-α measurements were obtained from R&D systems (Abingdon, UK).

**Histopathological analysis**

Lungs for histological examination were harvested 48 hours after infection, fixed in 10% formalin and embedded in paraffin. Four µm sections were stained with hematoxylin and eosin and analyzed by a pathologist who was blinded for the groups.

**Statistical analysis**

All data are expressed as mean ± SE, unless stated otherwise. Differences between groups were analysed by student's t-test (in vitro experiments) or Mann-Whitney U test (in vivo experiments). p < 0.05 was considered to represent a statistically significant difference.
Results

NTHI binds influenza virus
To determine direct interaction between NTHI and influenza, bacteria and virus were incubated for 30 minutes at room temperature. Bacteria were spun at low speed and washed twice with PBS. Incubation of NTHI with a 1/10-dilution of the original virus-stock resulted in approximately 25% recovery of the virus from the pellet (Figure 1). The 1/100 and 1/1000 dilutions showed approximately 100% recovery from the pellet. Influenza virus was also spun at low speed and washed twice to determine non-specific precipitation of the virus. No detectable numbers of the influenza virus were recovered, indicating influenza virus binds to NTHI.

Interaction between NTHI and influenza prevents viral infection in NCI-H292 cells
To investigate the effect of the interaction between NTHI and influenza, we determined viral load was determined in airway epithelial-like cells coinfected with NTHI. An increase in viral load was observed between 4 and 24 hours after infection in NCI-H292 cells, followed by a slight reduction thereafter (Figure 2). Combined infection with NTHI and influenza virus revealed a similar viral load after 4 hours. Viral load was trendwise lower 24 hours after combined infection (p = 0.13 vs influenza infection) and significantly lower 48 hours after combined infection (p = 0.0113 vs influenza infection). These data indicate that NTHI partially prevents influenza virus infection in NCI-H292 cells.
IL-6 and IL-8 production in NCI-H292 cells

To evaluate the inflammatory response by NCI-H292 we measured IL-6 and IL-8 levels in medium after 4, 24 and 48 hours after NTHI, influenza and combined infection. NTHI alone resulted in an increase in both IL-6 and IL-8 after 4 hours incubation. Both IL-6 and IL-8 levels increased only modestly thereafter. Influenza virus infection resulted in an increase in IL-6 and IL-8 production. Combined NTHI/influenza infection led to additional increase in IL-6 production after 48 hours. IL-8 was synergistically induced after combined NTHI/influenza infection as reflected by 2-fold increased IL-8 levels after 24 and 48 hours. These data indicate that combined infection of NTHI and influenza virus induced a more pronounced inflammatory response in airway epithelial like cells.

Figure 2: Viral outgrowth in NCI-H292 cells. Viral outgrowth in airway epithelial-like cells (NCI-H292) was determined by real-time PCR 4, 24 and 48 hours after infection. Viral load in NCI-H292 cells with primary influenza infection (filled bars) and combined NTHI/influenza infection (open bars) are expressed as viral RNA copies (mean ± SE). These data are representative for two independent experiments. * p < 0.05 vs influenza treated cells.

Figure 3: IL-6 and IL-8 production in NCI-H292 cells after influenza or combined infection. IL-6 and IL-8 levels in culture medium were measured by ELISA 4, 24 and 48 hours after infection. IL-6 and IL-8 levels are expressed in ng/mL (mean ± SE). These data are representative for two independent experiments. * p < 0.05 vs NTHI treated cells and influenza treated cells.
Non-typable *Haemophilus influenzae* interacts with influenza virus

**Figure 4:** Viral load in the lungs after primary influenza and combined infection. Viral load in the lungs was determined on day 2 and 8 after influenza virus infection (filled bars) and combined NTHI/influenza infection (open bars). Viral load is expressed as viral RNA copies/g lung tissue (mean ± SE, 8 mice per group). In control mice and NTHI infected mice influenza could not be detected (4 and 8 mice resp. per group, data not shown). *p < 0.05 vs mice infected with influenza alone.

*NTHI prevents viral outgrowth in vivo*

To further investigate the role of the interaction between NTHI and influenza, we inoculated C57Bl/6 mice with NTHI and influenza virus. Previous studies indicated that viral replication occurs between day 1 and day 4 [19, 22]. Combined NTHI and influenza infection revealed a 10,000-fold reduction in viral load (*p < 0.0001 vs influenza-infected mice, Figure 4*) on day 2 after infection. Viral loads on day 8 were 100,000 fold lower in mice with combined infection than in mice with influenza infection alone (*p < 0.0001, Figure 4*).

*Figure 5:* Cytokine and chemokine expression in the lungs after viral and/or bacterial infection. TNF-α (A), IL-6 (B) and KC (C) levels in total-lung homogenates were measured on day 2 and 8 after infection (8 mice per group). Data are expressed in pg/g lung tissue (mean ± SE). # p < 0.05 vs control mice. *p < 0.05 vs influenza infected mice.

**Cytokine and chemokine levels in total-lung-lysates**

Cytokine and chemokine levels were measured to evaluate the host response to combined infection with influenza and NTHI. Neither influenza, NTHI or combined infection resulted in detectable TNF-α and IL-6 concentrations in the lungs of mice after 48 hours (Figure 5). KC levels were increased in influenza, NTHI and combined infection. Influenza virus infected mice displayed increased levels of IL-6 and TNF-α on day 8, whereas NTHI infected mice
showed comparable IL-6 and TNF-α levels in the lungs. Combined infection resulted in significantly lower TNF-α and IL-6 levels than influenza virus infection alone (p < 0.05). Although KC levels in the lungs were still higher than in control mice, no differences were observed between influenza, NTHI or combined infection.

**Figure 6:** Histopathologic analysis. Histopathological analysis of the lungs of mice with combined infection (A) or with influenza infection alone (B). Lungs were isolated 8 days after infection and prepared for histopathological analysis. Slides (10x magnification) are representative for 8 mice per group. The area of inflamed lung tissue (C) is expressed as % of the total area (mean ± SE). * p < 0.05 vs influenza infected mice.

**Histopathologic analysis**

To identify differences in inflammation in the lungs between influenza, NTHI and combined infection in mice, histopathological analysis of H/E stained lung-slides from days 2 and 8 after infection was performed. Influenza virus infection was associated with interstitial inflammation, mild pleuritis and perivascular inflammation on day 2 (data not shown). The pleuritis, perivascular and interstitial inflammation were more pronounced on day 8 after viral infection and accompanied by bronchitis and edema (Figure 6), whereas NTHI infected mice showed only mild interstitial inflammation. Inflammation in mice with combined infection was characterized by pleuritis, bronchiolitis, edema, perivascular and interstitial inflammation as observed for influenza infected mice. However, the total inflamed area of the lungs was significantly lower in mice with combined NTHI/influenza infection (p = 0.02, Figure 6).

**Subsequent infection with NTHI and influenza does not prevent viral outgrowth in vivo**

Subsequent infections with influenza and NTHI were performed to investigate whether bacteria that are already present in the lungs could prevent viral outgrowth in mice. NTHI infection in mice followed by influenza after 1 hour did not prevent viral outgrowth as reflected by similar viral loads after 48 hours (Figure 7). Influenza infection in mice followed
Non-typable *Haemophilus influenzae* interacts with influenza virus

by NTHI infection after 1 hour did not reduce viral loads after 48 hours either. These data indicate that viral outgrowth is only prevented during combined viral/bacterial infection.

![Figure 7: Viral load on day 2 after NTHI infection followed by influenza infection and influenza infection followed by NTHI infection. Mice received influenza intranasally 1 hour before (A) or 1 hour after (B) NTHI infection. Viral load was determined by real-time PCR on day 2 after viral infection (6 mice per group). Data are expressed as viral RNA copies/g lung tissue (mean ± SE).](image)

**Discussion**

Superinfection with *H. influenzae* is a well known complication of influenza virus infection [1-3]. Mechanisms to explain this enhanced susceptibility to secondary bacterial infections include increased colonization of mucosal membranes [23, 24], enhanced virulence of influenza [25] and reduced host defense against bacteria as a consequence of viral infection [22, 26]. In the present study, we investigated the role of direct binding of influenza virus to NTHI during combined infection in vitro and in vivo. Our data indicate that combined infection with influenza and NTHI reduces viral outgrowth in airway epithelial-like cells. Moreover, influenza virus infection was almost completely prevented during combined/viral bacterial infection as reflected by 10,000-fold reduced viral loads in the lungs. Results from the in vitro experiments revealed that the interaction between influenza virus and NTHI impairs viral outgrowth in airway epithelial-like cells (NCI-H292). From our data it is not clear whether viral entry is inhibited in NCI-H292 cells or that enhanced responses to viral infection result in reduced viral outgrowth. The present study focused on viral replication and inflammatory responses, rather than antiviral mechanisms. IL-8 release, but not IL-6, was synergistically induced by influenza and NTHI. KC, the functional homologue of IL-8 in mice [27], was not further increased during combined viral/bacterial infection in mice. Although we did not measure granulocyte influx in these mice, the role of KC in enhanced viral clearance is limited. Likely, NTHI binds influenza virus and prevents, at least in part, infection of airway epithelial cells in vivo. In addition, influenza virus may be rapidly cleared.
as a consequence of direct binding to NTHI. Indeed, previous studies within our laboratory indicate that NTHI is cleared from the lungs within 24 hours after infection (unpublished results). Rapid clearance of bacteria requires opsonization and subsequent phagocytosis [28, 29]. Influenza has been shown to escape phagocytosis [30], despite opsonization of the virus [28, 31]. Moreover, macrophages are, besides epithelial cells, a primary target for replication of the influenza virus [32]. Bacteria are easily digested after opsonization. This additional mechanism may explain the 10,000-fold difference in viral load in mice with influenza infection alone and combined infection, while combined infection in vitro only resulted in 2-fold reduced viral loads. Further research is warranted to address the role of phagocytosis during rapid clearance of influenza during combined viral/bacterial infection.

Although our data indicate that influenza binds to NTHI, the molecules involved in this interaction remain to be identified. Group B streptococci (GBS) have been shown to bind influenza virus as well [16]. The virus-binding capacity of GBS largely depends on N-terminal sialic acid residues on the bacterial cell wall. Sialic acid residues on the outer membrane of NTHI [5, 33] may be responsible for the interaction between influenza and NTHI. Influenza virus is well known to bind sialic acid residues under highly different conditions. For instance, the capacity of influenza virus to agglutinate with red blood cells is largely dependent on sialic acid [34]. Furthermore, influenza virus has been shown to recognize sialyloligosaccharides on human respiratory epithelium, which enhances viral entry and subsequent replication of the virus [35]. Irrespective of the involvement of sialic acid, the virus-binding capacity of NTHI will interfere with the adherence to the airway epithelium. The importance of sialic acid residues has been reported by Reuter et al. who showed that sialic acid coated biopolymers prevented adhesion and infection of influenza virus in airway epithelial cells [36]. Further analysis revealed that these biopolymers prevented influenza-induced pneumonitis in mice [37]. Similarly, combined infection with NTHI and influenza prevented virus-induced pneumonia in mice. Besides binding to sialic acid residues, influenza virus may interact with specific proteins expressed on the cell wall of NTHI. In particular, adhesion molecules like p2 and p5 could be involved in the interaction between influenza and NTHI [15]. Binding studies with mutated NTHI strains as well as neuraminidase treatment of wildtype NTHI may provide insight into the molecular nature of the interaction between influenza and NTHI.
Non-typable *Haemophilus influenzae* interacts with influenza virus

Mice with combined NTHI and influenza infection were protected against influenza-induced pneumonia. Since NTHI belongs to the commensal flora of the nasopharynx, the interaction between influenza and NTHI may provide an additional mechanism to prevent viral airway infections in vivo. Van Eldere suggested a similar role for commensal inhabitants in the ear, nose and throat. This protective effect of bacteria involves reduced colonization and the induction of both innate and adaptive immune responses [38]. The fact that the protective effect is lost when NTHI was administered one hour before influenza virus was inoculated does not necessarily argue against this hypothesis. The NTHI strain used in this mouse-model does not persist in the respiratory tract as reflected by complete clearance within 24 hours (data not shown). Likely, the bacteria are easily opsonized after inoculation and may prevent binding of the influenza virus [28, 29]. Similarly, NTHI could not prevent viral infection when influenza was administered one our before NTHI, indicating that viral entry initiates within the first hour after infection. Further research is required to elucidate whether persistent bacteria protect against viral infection using several different intervals between bacterial and viral infection.

In the present study we show that influenza virus binds to NTHI and that coinfection with these pathogens reduces viral outgrowth *in vitro* and *in vivo*. These data indicate that complex interactions between viral and bacterial pathogens may take place in the airways of coinfected patients, which attenuates the outgrowth of these pathogens.

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**References**

Non-typable *Haemophilus influenzae* interacts with influenza virus
