Pathogenesis of Haemophilus influenzae. Respiratory infection in COPD patients
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STIMULATION OF THE ADHERENCE OF

HAEMOPHILUS INFLUENZAE TO

HUMAN LUNG EPITHELIAL CELLS BY

ANTIMICROBIAL NEUTROPHIL DEFENSINS

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Abstract

Patients with chronic obstructive pulmonary disease (COPD) frequently have recurrent lower respiratory tract infections with nonencapsulated *Haemophilus influenzae*. The infected mucosa of these patients is infiltrated with neutrophils, which upon activation may release antimicrobial peptides, including defensins. In this study we show that defensins isolated from neutrophils or from sputum samples of COPD patients, did not kill *H. influenzae* from these patients, but stimulated their adherence to human bronchial epithelial cells, in a time and dose dependent way. Maximal stimulation was observed after 3 hr in the presence of 10 μg/ml or more defensins, resulting in 65 ± 36 CFU/cell (61-fold increase). The enhanced adherence was not solely due to charge effects and was specifically blocked by α1-proteinase inhibitor. Because adherence is the first step in the onset of respiratory tract infections, our findings indicate that neutrophil defensins likely contribute to the pathogenesis of *H. influenzae* infection in the lower respiratory tract.
Introduction

Patients with chronic obstructive pulmonary disease (COPD) frequently have recurrent lower respiratory tract infections with *Haemophilus influenzae* as the most frequently isolated pathogen [13,14]. A characteristic feature of the lower respiratory tract of COPD patients is the elevated state of bronchial inflammation due to various noxious agents assaulting the epithelial cells [26]. Leukocytes, including neutrophils, are abundantly present in the bronchial lavage fluid [28,31]. Activated neutrophils release a variety of antimicrobial peptides, including neutrophil defensins (human neutrophil peptides [HNP1-4]) [9]. Defensins are small (29-34 amino acid residues), cationic, antimicrobial, and cytotoxic polypeptides lacking enzymatic activity [10,11,22]. They constitute 5-7% of the total protein content of human mature neutrophils and 30-50% of the total protein content of the azurophilic granules [21]. The elevated state of inflammation in the lower respiratory tract of COPD patients leads to high levels of neutrophil defensins in sputum [27]. Since defensins have profound effects on epithelial cells as well as on bacteria [10], we determined the effect of neutrophil defensins on the interaction of *H. influenzae* to epithelial cells, an important first step in the onset of respiratory tract infections.

Materials and methods

**Bacterial strains**

In total 15 *H. influenzae* isolates cultured from sputum samples of 15 COPD patients were used in the studies. COPD is defined as a disorder characterized by abnormal tests of expiratory flow that do not change markedly over periods of several months of observation [1]. *H. influenzae* was isolated from the clinical materials according to standard procedures [14]. Of the 15 COPD isolates, 10 were persistent strains, isolated at least twice in a period longer than 6 months, and 4 other strains designated as acute strains, were isolated only once from sputum samples collected sequentially in a period of more than 6 months. It is not known whether the remaining strain was a persistent or an acute strain. Additionally, 15 non-encapsulated *H. influenzae* strains were isolated from the throat of 15 healthy subjects (carrier strains). Strain d1 [7], the nonfimbriated, nonencapsulated 770235 (t^b^5) and the encapsulated variant 770235 f^b^+ [16,33] were described before. All isolates were serotyped by coagglutination [34]. Most of the experiments were done with the nonencapsulated *H. influenzae* strain A850048 isolated from a persistently infected COPD patient.

All *H. influenzae* strains were cultured overnight on chocolate agar plates at 37°C in a humidified atmosphere containing 5% CO₂. For the adherence assays, the bacteria were resuspended in phosphate-buffered saline (PBS) (10 mM sodium phosphate, 140 mM NaCl,
DEFENSIN-ENHANCED BACTERIAL ADHERENCE

pH 7.4) resulting in a bacterial suspension containing $10^9$ colony forming units (CFU) per ml (OD$_{530nm}$ = 1).

**Epithelial cell culture**

Cells of the epithelial cell line NCI-H292 (ATCC CRL 1848) [3], originating from a human lung mucoepidermoid carcinoma, were grown in 25 cm$^2$ culture flasks (Costar, Cambridge) at 37°C in a humidified atmosphere containing 5% CO$_2$. NCI-H292 cells were maintained in RPMI 1640 medium with 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid) buffer (Gibco Life Technologies Ltd, Paisley, Scotland) supplemented with 10% fetal calf serum (Boehringer, Mannheim, Germany) without antibiotics. The cells were passaged twice weekly in a split ratio of 1:6 after trypsinization with 0.05% (w/v) trypsin (Difco) plus 0.02% (w/v) EDTA in Dulbecco’s PBS (137 mM NaCl, 8 mM Na$_2$PO$_4$, 1.5 mM KH$_2$PO$_4$, 2.6 mM KCl, pH 7.3).

For the adherence assay, a cell suspension containing $1.5 \times 10^5$ cells/ml was added to 24-well plates (1 ml per well; Falcon, Becton Dickinson Labware, New Jersey) containing round coverslips with a diameter of 12 mm (Menzelglaser, Germany). To obtain semiconfluent cell layers, the tissue cell cultures were incubated at 37°C for approximately 42 hr, resulting in $3.4 \pm 0.9 \times 10^4$ cells/well.

**Culture of primary bronchial epithelial cells**

Subcultures of primary bronchial epithelial cells were obtained from lung tissue with a macroscopically normal appearance. Lung tissue was derived from lungs of patients who underwent a thoracotomy or lobectomy because of lung cancer. Bronchial epithelial cells were obtained after proteinase digestion of the tissue and were cultured on a fibronectin/collagen matrix [4,24] in serum-free keratinocyte low calcium (0.09 mM) medium (KSFM) (Gibco Life Technologies Ltd) supplemented with epidermal growth factor (EGF), bovine pituitary extract (BPE), and isoproterenol. After the cultures had reached near-confluency, this medium was replaced by KSFM containing 1 mM CaCl$_2$, but without isoproterenol, and incubated for another 36 hr to allow differentiation of the cells [20]. The cultures were devoid of fibroblasts and leukocytes, and the epithelial origin of the cultured cells was confirmed by cell-specific staining for vimentine, desmine, and cytokeratine.

**Isolation of defensins**

Defensins (HNP1-3) were isolated either from an acetic acid extract of purulent sputum from COPD patients or from human neutrophil granules using gel filtration chromatography on Sephacryl S-200 HR (2.5 x 100 cm) (Pharmacia, Fine Chemicals AB, Uppsala, Sweden) as described [17,36]. HNP-1 was further purified from the fractionated granule extract by reversed phase high-performance liquid chromatography (RP-HPLC) on a C18 column (4.6 x 250 mm) (Vydac, The Separations group, Hesperia, CA). The isolated defensins were characterized using polyacrylamide gel electrophoresis and mass spectrometry as described [36] and quantified using the BCA protein system (Pierce Chemical Company, Rockford, Illinois).
Adherence assay
Routinely, the medium covering the NCI-H292 epithelial cells grown on glass coverslips was replaced by RPMI 1640 without fetal calf serum, with a final volume of 500 μl. Subsequently, the bacterial suspension, prepared as described above, at a final concentration of 10⁸ CFU/ml and 20 μg/ml HNP1-3 (isolated from sputum samples) were added, followed by incubation at 37°C for 4 hr. Then, the coverslips were transferred to other 24-wells plates and washed 3 times with PBS to remove the nonadherent bacteria. The cells were treated with 500 μl of 1% saponin in PBS to release the cells from the coverslips. Serial dilutions of the cell suspensions were plated on chocolate agar plates to determine the number of bacteria associated with the cells as CFU/ml [35]. The number of CFU bound per epithelial cell was calculated by dividing the number of CFU/ml by the number of epithelial cells per ml (3.4 ± 0.9 x 10⁵ cells/ml). The number of bacteria adherent on cells was also determined by light microscopy examination after overnight fixation in 1 ml of 4% paraformaldehyde (PFA) (Merck, Darmstadt, Germany) plus 1% glutaraldehyde (GA) (Merck), and subsequent staining with 0.007% crystal violet for at least 30 min. Approximately 50 cells were examined to calculate the number of adherent bacteria per epithelial cell [35]. All experiments were performed at least two times in triplicate.

To examine the best conditions for the routine adherence assay, various incubation times, and defensin concentrations were tested. The differences in the stimulatory effect of the defensins isolated from different sources was examined with various concentrations of HNP1-3 isolated from sputum, HNP1-3 isolated from neutrophils and HNP-1. To study the specificity of the enhanced effect of defensins on the bacterial adherence, the cationic defensins were replaced by the positively charged peptides poly-L-lysine (20 μg/ml) (Sigma, St. Louis), or protamine (20 μg/ml) (Sigma). To determine whether negatively charged molecules neutralized the enhancing effect of defensins the negatively charged poly-L-aspartic acid (20 μg/ml) (Sigma) or polysaccharide of H. influenzae type b (20 μg/ml) (polysaccharide was a generous gift from Dr. R. Tiesjema RIVM, Bilthoven, The Netherlands) were added in a rapid succession with defensins and bacteria. The specificity of the stimulation of bacterial adherence was analyzed by preincubating 20 μg/ml HNP1-3 with 270 μg/ml α1-proteinase inhibitor (α1-PI) (Cutter Biological, Berkeley, Ca) for 1 hr. The requirement for metabolic activity of the bacteria for the stimulatory effect of defensins was determined by chloramphenicol treatment (10 μg/ml) (Sigma) for 30 min and the effect of killing of the bacteria was studied by heating at 56°C for 30 min prior to the adherence. To assess the requirement for epithelial cell viability, the NCI-H292 cells were killed by heating at 56°C for 30 min or treatment with 1% PFA for 30 min. Finally, the standard adherence assay was performed with primary bronchial epithelial cells instead of the immortalized NCI-H292 cell line.

Adherence to pharyngeal epithelial cells
The adherence to pharyngeal epithelial cells was performed as described [32] with some adaptations. Briefly, pharyngeal epithelial cells were harvested from healthy donors by scraping the posterior of the oral cavity. The cells were collected in 10 ml of PBS, centrifuged
at 190 x g for 5 min, and resuspended in 1 ml of RPMI 1640 without fetal calf serum to obtain concentration of $10^5$ cells per ml. One hundred microliters of the epithelial cell suspension was mixed with 10 µl of the bacterial suspension described above (end concentration $10^8$ CFU/ml). To examine the effect of defensins, 20 µg/ml of HNP1-3, isolated from human neutrophil granules, were added to the mixture of epithelial cells and bacteria. The mixtures were incubated at 37°C for 1 hr. The nonadherent bacteria were washed 4 times with PBS by repeated centrifugation at 80 x g for 5 min. The cells were resuspended in 50 µl of PBS and preparations were made on glass slides. After drying, the cells were fixed in methanol for 15 min. The bacteria were detected by immunoperoxidase staining as described using the monoclonal antibody 8BD9, directed against outer membrane protein P6 of *H. influenzae* [15]. The epithelial cells were counterstained with 0.5% methylene blue in water. The number of adherent bacteria on 20 cells was counted under the light microscope to calculate the mean number of adherent bacteria per cell.

**Scanning electron microscopy**

Glass slides prepared for light microscopy were washed with destilled water, dehydrated in graded alcohol followed by drying for 10 min in hexamethyl disilizane. The dried preparations were sputter coated with gold in a Balzers SCD 040. Scanning electron microscopy was performed using a Philips SEM 525.

**Results**

**Adherence of *H. influenzae* to NCI-H292 cells in the presence of defensins**

The adherence of nonencapsulated *H. influenzae* isolates (n=15) from chronically infected COPD patients to the human lung epithelial cell line NCI-H292 was determined in the absence or presence of 20 µg/ml defensins purified as a mixture of HNP1-3 from sputum samples from COPD patients. The adherence of *H. influenzae* strain A850048 to these cells was enhanced over time in the presence of defensins (figure 1). The colony counting revealed that defensin-stimulated adherence was maximal after 3 hr, resulting in 65 ± 36 colony forming units (CFU) bound per epithelial cell compared to 1.1 ± 0.7 CFU/cell in the absence of defensins. The results from examination by light microscopy (LM) were in agreement with those obtained by colony counting (figure 1). The bacteria were distributed equally on the cell surface and no aggregation of the bacteria was observed. Since LM examination was less sensitive than colony counting, LM examination was only used for screening and morphologic confirmation of adherence. Adherence increased with increasing concentration of the defensins (figure 2), and a maximal stimulatory effect of defensins on the adherence was observed for defensin concentrations of 10 µg/ml or
Figure 1. Adherence of the *H. influenzae* strain A850048 to lung epithelial cell line NCI-H292 in the presence (open squares) or in the absence (closed squares) of 20 μg/ml HNP1-3 over time. Results of light microscopy (LM) examination are indicated with symbols: -, no adherence; +, 5-10 bacteria bound per epithelial cell; ++, 10-50 bacteria per cell; ++++, 50-100 bacteria per cell; ++++, more than 100 bacteria per cell. The figure represents the results of three independent experiments, two performed in duplicate and one in triplicate.

Higher. Similar concentration-dependent enhancement of adherence was observed for defensins (both HNP1-3 and HNP-1) isolated from neutrophil granules of blood from healthy individuals. At the concentrations tested, defensins did not kill *H. influenzae* under these assay conditions, since the survival was 91%. In addition, at the concentrations tested the defensins were not cytotoxic for the NCI-H292 cell line as determined by a ^51^Cr release assay [27]. From the results of these studies, a 4 hr incubation time, and 20 μg/ml HNP1-3 purified from sputum samples were selected as standard conditions for the subsequent experiments.
Specificity of the defensin-enhanced adherence

Because defensins are cationic peptides, the effect of various charged molecules on adherence was tested to analyze if the stimulatory effect of the defensins on the adherence was due to charge effects. Whereas defensins markedly increased the adherence of *H. influenzae*, the positively charged polypeptides poly-L-lysine (20 μg/ml), or protamine (20 μg/ml) which is similar in size and charge as defensins, had no stimulatory effect (0.20 ± 0.13 CFU/cell and 0.24 ± 0.15 CFU/cell, respectively). In addition, the enhanced adherence by defensins was not affected after addition of the negatively charged poly-L-aspartic acid (20 μg/ml) or capsular polysaccharide of encapsulated *H. influenzae* type b (20 μg/ml) (50 ± 30 CFU/cell and 50 ± 20 CFU/cell, respectively). Addition of the divalent cations Mg$^{2+}$ (up to 20 mM) or Ca$^{2+}$ (up to 5 mM) to the medium had also no effect on the enhancement of adherence by defensins (data not shown). These results indicate that the cationic character of defensins was not responsible for the enhancement of adherence. To test the specificity of the defensin-enhanced adherence, we performed inhibition experiments with α1-proteinase inhibitor (α1-PI), a member of the serine proteinase
inhibitor (serpin) family. Defensins form complexes with α1-PI, which neutralizes the cytotoxic activity of defensins [27] and IL-8 production [36]. Preincubation of defensins with equimolar amounts of α1-PI for 1 hr, resulted in a 90% inhibition of the stimulatory effect of defensins on the adherence of *H. influenzae* to NCI-H292 cells after 4 hr incubation (table 1). α1-PI did not influence the adherence of *H. influenzae* to NCI-H292 cells in the absence of defensins (0.62 ± 0.33 CFU/cell).

### Table 1. Inhibition of the defensin-enhanced adherence of *H. influenzae* strain A850048 to NCI-H292 cells by α1-proteinase inhibitor (α1-PI).

<table>
<thead>
<tr>
<th></th>
<th>Adherence (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>1.4</td>
</tr>
<tr>
<td>HNP1-3</td>
<td>100</td>
</tr>
<tr>
<td>HNP1-3 + α1-PI</td>
<td>10.4</td>
</tr>
</tbody>
</table>

*a* Adherence of strain A850048 in the presence of 20 μg/ml defensins was set at 100%.

This corresponded with 82 ± 54 CFU/cell.

*b* The activity of the defensins (20 μg/ml) was inhibited by a preincubation with equimolar concentrations of α1-PI (270 μg/ml) at 37°C for 1 hr, prior to the addition to NCI-H292 cells and *H. influenzae* strain A850048 for 4 hr.

So far, the effect of neutrophil defensins on the adherence of *H. influenzae* was studied by adding defensins and bacteria directly to the epithelial cells. We next investigated the effect of preincubation of defensins with either the bacteria or epithelial cells alone on the adherence. Preincubation of bacteria with defensins for 10 min followed by washing, reduced, but did not abrogate the enhancement (6.5 ± 3.6 CFU/cell). A similar effect was found for preincubation of the epithelial cells with defensins for 10 min, followed by washing and incubation with bacteria (10 ± 5.6 CFU/cell).

### Defensin-enhanced adherence to primary bronchial epithelial cells

Since the aforementioned results were obtained with a cell line, we determined the effect of defensins on the adherence of *H. influenzae* to subcultures (third passage) of human primary bronchial epithelial cells. Adherence of *H. influenzae* strain A850048 to these cells increased by addition of 20 μg/ml defensins (12.9 ± 10.2 CFU/cell) compared to the adherence in the absence of defensins (0.82 ± 0.41 CFU/cell). The morphology of the enhanced adherence is shown in figure 3. The bacteria were
randomly distributed over the cell surface, did not aggregate and were not associated with microvilli. Figure 3 also shows that the morphology of the adherence in the presence of defensins was very similar for the cell line NCI-H292 and the primary cells, indicating that defensins stimulated not only the adherence of *H. influenzae* to the NCI-H292 cell line but also to human primary bronchial epithelial cells.

![Figure 3](image)

**Figure 3.** Scanning electron microscopy of adherence of *H. influenzae* strain A850048 to cell line NCI-H292 (A,B) and subcultures of primary bronchial epithelial cells (C,D) in the absence (A,C) or presence (B,D) of 20 µg/ml HNP1-3, isolated from neutrophils from the blood of healthy donors. This experiment was repeated in triplicate with cells from a different donor with similar results. The bar represents 10 µm.

**Adherence to pharyngeal epithelial cells**

We next examined the effect of defensins on the adherence of *H. influenzae* to human pharyngeal epithelial cells. These cells were directly used after harvesting, and therefore, the mucus is still present. Table 2 shows that the adherence of the nonfimbriated, nonencapsulated *H. influenzae* strain 770235 (Fhb^0^) was increased 22-fold and the adherence of strain d1 was enhanced 5-fold in the presence of defensins. Strain A850048, which was used in most of the experiments, adhered to the pharyngeal cells in the absence and in the presence of defensins. From this result, we
concluded that defensins stimulated adherence of *H. influenzae* to freshly obtained cells from which mucus was not removed.

**Table 2.** The effect of 20 μg/ml defensins (HNP1-3) on the adherence nonencapsulated *Haemophilus influenzae* strains to pharyngeal epithelial cells.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Bacterial adherencea</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>control</td>
<td>HNP1-3</td>
<td></td>
</tr>
<tr>
<td>No added bacteria</td>
<td>1.0 ± 1.1</td>
<td>ndb</td>
<td></td>
</tr>
<tr>
<td>A850048</td>
<td>82 ± 36</td>
<td>82 ± 40</td>
<td></td>
</tr>
<tr>
<td>d1</td>
<td>4.2 ± 3.1</td>
<td>22 ± 13</td>
<td></td>
</tr>
<tr>
<td>770235 f°b°</td>
<td>1.6 ± 1.6</td>
<td>36 ± 17</td>
<td></td>
</tr>
</tbody>
</table>

a  The adherence is expressed as adherent bacteria per epithelial cell (mean ± SD), as counted by light microscopy. The results from this experiment were repeated with 2 other donors with similar results.

b  nd: not done.

**Adherence of various patient isolates and carrier strains**

We hypothesized that if defensin-enhanced adherence is important for the occurrence of the frequently observed chronic *H. influenzae* infections in COPD patients, the adherence of *H. influenzae* isolates from these patients to epithelial cells is stimulated by defensins. In the absence of defensins, 8/15 of the COPD isolates bound to NCI-H292 cells (table 3). The number of adherent bacteria per epithelial cell ranged from 10 to 100, 53 on average. The adherence of all 15 COPD isolates to NCI-H292 cells was enhanced by defensins, resulting in over 100 bacteria per cell. Of 15 nonencapsulated *H. influenzae* isolates from the throat, the natural habitat of pathogenic and non-pathogenic *H. influenzae*, 13 bound to NCI-H292 cells in the absence of defensins (range 10 to 100, 37 on average) (table 3). Enhancement of the adherence by defensins occurred in 9 of these adherent carrier strains, resulting in 89 bacteria per cell on average (range 10 to over 100). The adherence of the 2 nonadherent carrier strains was stimulated to 19 bacteria per cell (range 5 to 50). The adherence of nonfimbriated, encapsulated *H. influenzae* type b, strain 770235 (f°b°), that causes invasive disease, was not stimulated by defensins, in contrast to its nonencapsulated variant (770235 f°b°), indicating that the capsule prevented the adherence enhancing effect of defensins (data not shown).
Table 3. The effect of 20 μg/ml defensins on the adherence of various nonencapsulated *H. influenzae* strains, isolated from COPD patients and controls, to NCI-H292 cells.

<table>
<thead>
<tr>
<th>Source of strain, type</th>
<th>Number of strains with enhanced adherence by HNP1-3&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Source of strain</th>
<th>n</th>
<th>Persistent</th>
<th>Acute</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sputum of COPD patients</td>
<td>8&lt;sup&gt;c&lt;/sup&gt;</td>
<td>- adherent&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>7</td>
<td>- nonadherent</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Throat of healthy carriers</td>
<td>-</td>
<td>- adherent</td>
<td>13</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>- nonadherent</td>
<td>2</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Stimulation of adherence by at least one “+” according to the definition of figure 1.

<sup>b</sup> The adherence was determined by LM examination.

<sup>c</sup> One strain could not be classified as persistent or acute.

**Discussion**

In this study we showed that in the presence of 20 μg/ml neutrophil defensins, nonencapsulated *H. influenzae* were not killed during interaction with bronchial epithelial cells, but that the adherence of *H. influenzae* to the NCI-H292 cell line and primary bronchial epithelial cells was enhanced. Furthermore, the defensins stimulated also the adherence of *H. influenzae* to the mucus containing human pharyngeal epithelial cells. Therefore, the presence of mucus did not inhibit the activity of defensins. In line with this observation, defensins also stimulated bacterial adherence to mucus-covered bronchial tissue obtained from autopsy procedures (data not shown). Since neutrophil defensins are released upon neutrophil degranulation [9], are present in high concentrations in purulent airway secretions from patients with inflammatory lung disease such as chronic bronchitis [27] and cystic fibrosis [30], and have recently been reported to be present at the epithelial surface in lung tissue from patients with diffuse panbronchiolitis [2], the *in vitro* stimulatory effect of defensins on bacterial adherence observed in the present study are likely relevant to the *in vivo* situation.

Defensins are members of a family of cationic antimicrobial peptides, which can bind in a nonspecific way to the negatively charged surfaces of bacteria and cells [22].
Such a non-specific stimulatory effect by defensins was observed for interaction with macrophages resulting in phagocytosis [8]. In addition, various cationic polypeptides, including poly-L-lysine and protamine, have been shown to increase the uptake of particles by leukocytes [6]. Since we observed that ionic interactions did not mediate the increased adherence of *H. influenzae* to epithelial cells, the mechanisms involved in defensin-enhanced adherence to epithelial cells are distinct from those involving defensin-dependent adherence of bacteria to leukocytes. This was further supported by the observation that preincubation of defensins with either *H. influenzae* or epithelial cells, followed by washing, reduced but did not abolish enhanced adherence. In contrast, defensin-stimulated phagocytosis by the macrophages was previously reported not to reach above basal levels after preincubation followed by washing [8]. The specific involvement of the defensins in the stimulation of adherence was demonstrated by a 90% inhibition with α1-PI, a member of the serpin family. In moderately inflamed tissues defensins are probably complexed due to abundance of α1-PI and other defensin binding substances, resulting in prevention of tissue damage [27]. However, neutrophils, attracted to the site of inflammation [28,31], release high concentrations of defensins in the lumen of the lung [9,27,30]. Since during inflammation, the high concentrations of defensins may overwhelm the defensin-binding components [27], defensins may contribute to stimulation of bacterial adherence. In patients with α1-PI deficiency, it is tempting to speculate that the uncomplexed defensins contribute to increased bacterial adherence and thereby to the recurrent infections frequently occurring in these patients. An alternative explanation is that defensin-stimulated adherence of *H. influenzae* to bronchial epithelial cells results in the exposure of the adherent bacteria to high concentrations of epithelial cell-derived antimicrobial molecules, including human β-defensins [37].

*H. influenzae* was not killed during the interaction with the bronchial epithelial cells in the presence of defensins. Since the antimicrobial activity of neutrophil defensins is abolished when NaCl concentrations are as high as in the cell culture medium [23,29], bacterial killing may have been prevented. Also in the bronchial secretions of COPD patients, especially those suffering from infections, similar salt concentrations are present [12,18]. Therefore, the extracellular released neutrophil defensins are probably also not bactericidal *in vivo*, whilst they may exert the enhancing effect of *H. influenzae* adherence.
Although the bacterial and cellular components involved in the interaction with the defensins have not been identified, the binding characteristics may give clues to the significance of this type of interaction for the pathogenesis of chronic *H. influenzae* infections in COPD patients. The defensin-increased binding did not require viable or growing bacteria, since light microscopic examination revealed that heat-killed (56°C for 30 min) and chloramphenicol-treated (10 μg/ml for 30 min) bacteria adhered in similar numbers to the NCI-H292 cells as viable or growing bacteria. This result indicates that the bacterial component is not induced during the interaction, and that bacterial metabolic processes are not involved. Epithelial cell viability on the other hand was essential for the stimulation of adherence of *H. influenzae* to these cells in the presence of defensins, since no bacteria were seen on heat-killed (56°C for 30 min) and 1% paraformaldehyde killed (30 min) cells. This suggests that active cellular processes are required for defensin-stimulated adherence.

The interaction of defensins with bronchial epithelial cells results in cellular activation, since the cells start to produce IL-8 [36]. Therefore, the expression and or activity of a cellular receptor might be increased by defensins. Increased expression of a cellular receptor necessary for adherence of *Pseudomonas aeruginosa* to the asialo ganglioside M1 receptor in repairing epithelial cells from cystic fibrosis patients has been described [5]. Alternatively, redistribution and unmasking of a cellular receptor required for adherence may explain defensin-increased adherence. Redistribution of receptors has been observed for β1 integrins that are normally exposed to the basolateral surface. Upon neutrophil migration through epithelial cell layers, β1 integrins were transferred to the apical site of epithelial cells, facilitating interaction with the *inv* gene product (invasin) of *Yersinia pseudotuberculosis* [25].

We conclude that neutrophil defensins, in concentrations present in bronchial secretions from COPD patients, did not kill *H. influenzae* isolates during interaction with human airway epithelial cells, but defensins stimulated specific adherence to these cells. Since adherence to epithelial cells is an important step in the onset of mucosal infections [19], our findings indicate that defensins may contribute to the occurrence of *H. influenzae* infections in the lower respiratory tract. Because defensins are released during inflammation and infection, defensin-stimulated bacterial adherence to epithelium may be an important factor in the recurrent infections in the airways of COPD patients. It is tempting to speculate that this mechanism is general for recurrent infections.
Acknowledgments

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