Stimulation of the adherence of Haemophilus influenzae tu human lung epithelial cells by antimicrobial neutrophil defensins

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Stimulation of the Adherence of *Haemophilus influenzae* to Human Lung Epithelial Cells by Antimicrobial Neutrophil Defensins

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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. It was shown that defensins isolated from neutrophils or from sputum samples of COPD patients did not kill *H. influenzae* from these patients, but they did stimulate its adherence to human bronchial epithelial cells in a time- and dose-dependent manner. Maximal stimulation was observed after 3 h in the presence of ≥10 μg/mL 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.

Materials and Methods

**Bacterial strains.** Fifteen *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 [12]. *H. influenzae* was isolated from the clinical materials according to standard procedures [2]. Of the 15 COPD isolates, 10 were persistent strains (isolated at least twice in a period of >6 months) and 4 were acute strains (isolated only once from sputum samples collected sequentially in a period of >6 months). It is not known whether the remaining strain was a persistent or an acute strain. Additionally, 15 nonencapsulated *H. influenzae* strains were isolated from the throat of 15 healthy subjects (carrier strains). Three of the strains have been previously described [13–15]: d1; 770235 (f*b*), the nonfimbriated, nonencapsulated strain; and 770235 f*b*+, the encapsulated variant. All isolates were serotyped by coagglutination [16]. Most of the experiments were done with the nonencapsulated strain 850048 of *H. influenzae* 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 PBS (10 mM sodium phosphate, 140 mM NaCl, pH 7.4), resulting in a bacterial suspension containing 10⁷ cfu/mL (the optical density at 530 nm = 1).

**Epithelial cell culture.** Cells of the epithelial cell line NCI-H292 (ATCC CRL 1848) [17], originating from a human lung mucoepidermoid carcinoma, were grown in 25-cm² culture flasks (Costar, Cambridge, MA) at 37°C in a humidified atmosphere containing 5% CO₂. NCI-H292 cells were maintained in RPMI 1640 medium with 25 mM HEPES buffer (Life Technologies Gibco BRL, Paisley, Scotland) supplemented with 10% fetal calf serum (Boehringer Mannheim, Mannheim, Germany) without antibiotics. The cells were passaged twice weekly in a split ratio of 1:6 after trypsinization with 0.05% (wt/vol) trypsin (Difco, Detroit) plus 0.02% (wt/vol) EDTA in Dulbecco’s PBS (137 mM NaCl, 8 mM Na₂HPO₄, 1.5 mM KH₂PO₄, 2.6 mM KCl, pH 7.3).
For the adherence assay, a cell suspension containing $1.5 \times 10^6$ cells/mL was added to 24-well plates (1 mL/well; Falcon, Becton Dickinson Labware, Lincoln Park, NJ) containing round coverslips (12-mm diameter; Menzelgläser, Braunschweitz, Germany). To obtain semiconfluent cell layers, we incubated the tissue cell cultures at 37°C for ~42 h, resulting in $3.4 \pm 0.9 \times 10^3$ 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 [18, 19] in serum-free, low calcium (0.09 mM) keratinocyte medium (Keratinocyte-SFM [1×]; Life Technologies) supplemented with epidermal growth factor, bovine pituitary extract, and isoproterenol. After the cultures reached near confluency, the medium was replaced by keratinocyte medium containing 1 mM CaCl$_2$; without isoproterenol and incubated for another 36 h 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 vimentin, desmin, and cytokeratin.

**Isolation of defensins.** Defensins (HNP-1–3) 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 × 100 cm; Pharmacia Fine Chemicals, Uppsala, Sweden) as described [21, 22]. HNP-1 was further purified from the fractionated granule extract by reversed-phase high-performance liquid chromatography on a C18 column (4.6 × 250 mm, Vydac; The Separations Group, Hesperia, CA). The isolated defensins were characterized by use of polyacrylamide gel electrophoresis and mass spectrometry as described [21] and quantified by use of the BCA protein system (Pierce Chemical, Rockford, IL).

**Adherence assay.** The medium covering the NCI-H292 epithelial cells grown on glass coverslips was replaced routinely by RPMI 1640 without fetal calf serum to a final volume of 500 μL. Subsequently, the bacterial suspension at a final concentration of $10^7$ cfu/mL (prepared as described above) and 20 μg/mL HNP-1–3 (isolated from sputum samples) were added and incubated at 37°C for 4 h. The coverslips were then transferred to other 24-well plates and washed three 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 colony-forming units [cfu] per milliliter) [23]. The number of cfu bound per epithelial cell was calculated by dividing the number of cfu per milliliter by the number of epithelial cells per milliliter (3.4 ± 0.9 × 10³ 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 (Merck, Darmstadt, Germany) plus 1% glutaraldehyde (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 [23]. All experiments were done 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 were examined with various concentrations of HNP-1–3 isolated from sputum, HNP-1–3 isolated from neutrophils, and HNP-1. To study the specificity of the enhanced effect of defensins on the bacterial adherence, we replaced the cationic defensins by one of 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 polypeptide poly-L-aspartic acid (20 μg/mL; Sigma) or polysaccharide of *H. influenzae* type b (20 μg/mL; gift of R. Tiesjema, National Institute for Public Health and the Environment, Bilthoven, The Netherlands) was added in rapid succession with defensins and bacteria. The specificity of the stimulation of bacterial adherence was analyzed by preincubating 20 μg/mL HNP-1–3 with 270 μg/mL α1-proteinase inhibitor (α1-PI; Cutter Biological, Berkeley, CA) for 1 h. The requirement for metabolic activity of the bacteria for the stimulatory effect of defensins was determined by treatment with chloramphenicol (10 μg/mL; Sigma) for 30 min; 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% paraformaldehyde for 30 min. Finally, the standard adherence assay was done 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 done as described [24] with some adaptations. In brief, 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 g for 5 min, and resuspended in 1 mL of RPMI 1640 without fetal calf serum to obtain $10^5$ cells/mL. Epithelial cell suspension (100 μL) was mixed with 10 μL of the bacterial suspension described above (end concentration, $10^8$ cfu/mL).

To examine the effect of defensins, we added 20 μg/mL HNP-1–3 (isolated from human neutrophil granules) to the mixture of epithelial cells and bacteria. The mixture was incubated at 37°C for 1 h. The nonadherent bacteria were washed 4 times with PBS by repeated centrifugation at 80 g for 5 min. The cells were resuspended in 50 μL of PBS, and preparations were made on glass slides. After being dried, 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* [25]. The epithelial cells were counterstained with 0.5% methylene blue in water. The number of adherent bacteria on 20 cells was counted by use of a light microscope to calculate the mean number of adherent bacteria per cell.

**Scanning electron microscopy.** Glass slides prepared for light microscopy were washed with distilled water, dehydrated in graded alcohol, and dried for 10 min in hexamethyldisilazane. The dried preparations were sputter coated with gold in a Balzers SCD 040 (Balzers Union, Vaduz, Liechtenstein). A Philips scanning electron microscope 525 was used.

**Results**

**Adherence of *H. influenzae* to NCI-H292 cells in the presence of defensins.** The adherence of nonencapsulated *H. in-
*H. influenzae* isolates (*n* = 15) from chronically infected COPD patients to the human lung epithelial cell line NCI-H292 was determined in the absence and presence of defensins (20 μg/mL) purified as a mixture of HNP-1–3 from sputum samples from COPD patients. The adherence of *H. influenzae* A850048 to these cells was enhanced over time in the presence of defensins (figure 1). Colony counting revealed that defensin-stimulated adherence was maximal after 3 h, resulting in 65 ± 36 cfu bound per epithelial cell compared with 1.1 ± 0.7 cfu/cell in the absence of defensins.

The results from examination by light microscopy 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 light microscopy examination was less sensitive than colony counting, it was used only 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 ≥10 μg/mL. Similar concentration-dependent enhancement of adherence was observed for defensins (both HNP-1–3 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 51Cr release assay [11]. From the results of these studies, a 4-h incubation time and 20 μg/mL HNP-1–3 purified from sputum samples were selected as standard conditions for the subsequent experiments.

**Specificity of defensin-enhanced adherence.** Because defensins are cationic peptides, the effect of various charged molecules on adherence was tested to determine 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) and protamine (20 μg/mL), which are 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 Mg2+/Ca2+ (up to 20 mM) or Ca2+ (up to 5 mM) to the medium also had 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 did inhibition experiments with α1-proteinase inhibitor (α1-PI), a member of the serine proteinase inhibitor (serpin) family.

**Figure 1.** Adherence of *H. influenzae* A850048 to lung epithelial cell line NCI-H292 in presence (open squares) and absence (closed squares) of 20 μg/mL defensins (human neutrophil peptides [HNP] 1–3) over time. Results of light microscopy (LM) examination are indicated with symbols: −, no adherence; +, 5–10 bacteria bound/epithelial cell; ++, 10–50 bacteria/cell; ++++, 50–100 bacteria/cell; ++++, >100 bacteria/cell. Figure represents results of 3 independent experiments, 2 done in duplicate and 1 in triplicate. min = minutes.
Defensins form complexes with α1-PI, which neutralizes the cytotoxic activity of defensins [11] and interleukin-8 production [21]. Preincubation of defensins with equimolar amounts of α1-PI for 1 h resulted in a 90% inhibition of the stimulatory effect of defensins on the adherence of *H. influenzae* to NCI-H292 cells after 4 h of 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).

Up to this point, we had only studied the effect of neutrophil defensins on the adherence of *H. influenzae* 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 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* A850048 to these cells increased with the addition of 20 μg/mL defensins (12.9 ± 10.2 cfu/cell), compared with 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 2.** Concentration dependence of stimulatory effect of defensins on adherence. Adherence of *H. influenzae* A850048 to NCI-H292 cells after 4-h incubation in presence of various concentrations of human neutrophil peptides (HNP)-1 to HNP-3 isolated from sputum samples (squares) or HNP-1–3 (circles) or HNP-1 (triangles) isolated from blood neutrophils. Figure represents mean ± SD from 2 independent experiments done in triplicate.

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**Table 1.** Adherence of *H. influenzae* A850048 to NCI-H292 epithelial cells in the presence and absence of defensins (human neutrophil peptides [HNP]-1 to -3) with and without α1-proteinase inhibitor (α1-PI).

<table>
<thead>
<tr>
<th></th>
<th>Adherence</th>
</tr>
</thead>
<tbody>
<tr>
<td>A850048</td>
<td>1.4%</td>
</tr>
<tr>
<td>A850048 + HNP-1–3</td>
<td>100.0%</td>
</tr>
<tr>
<td>A850048 + HNP-1–3*</td>
<td>10.4%</td>
</tr>
</tbody>
</table>

*NOTE.* Adherence of strain A850048 in presence of 20 μg/mL defensins was set at 100%, which corresponded with 82 ± 54 cfu/cell.

*Activity of defensins was inhibited by preincubation with equimolar concentrations of α1-PI (270 μg/mL) at 37°C for 1 h prior to addition to NCI-H292 cells and *H. influenzae* A850048 for 4 h.

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Figure 3. Scanning electron microscopy of adherence of *H. influenzae* A850048 to epithelial cell line NCI-H292 (A, B) and subcultures of primary bronchial epithelial cells (C, D) in absence (A, C) and presence (B, D) of defensins (20 µg/mL human neutrophil peptides-1 to -3), isolated from neutrophils from blood of healthy donors. Experiment was repeated in triplicate with cells from different donor with similar results. Bar represents 10 µm.
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 of the 15 COPD isolates bound to NCI-H292 cells (table 3). The number of adherent bacteria per epithelial cell ranged from 10 to 100 (average, 53). The adherence of all 15 COPD isolates to NCI-H292 cells was enhanced by defensins, resulting in >100 bacteria per cell. Of 15 nonencapsulated *H. influenzae* isolates from the throat, the natural habitat of pathogenic and nonpathogenic *H. influenzae*, 13 bound to NCI-H292 cells in the absence of defensins (range, 10–100; average, 37) (table 3). Enhancement of adherence by defensins occurred in 9 of these adenvariant carrier strains, resulting in an average of 89 bacteria/cell (range, 10 to >100). The adherence of the 2 non-adherent carrier strains was stimulated to 19 bacteria/cell (range, 5–50). The adherence of 770235 (Φb−), the nonfimbriated, encapsulated *H. influenzae* type b strain that causes invasive disease, was not stimulated by defensins, in contrast to its nonencapsulated variant, strain 770235 (Φb+), indicating that the capsule prevented the adherence-enhancing effect of defensins (data not shown).

### Discussion

In this study we showed that in the presence of 20 μg/mL of neutrophil defensins, nonencapsulated *H. influenzae* was not killed during interaction with bronchial epithelial cells, but the adherence of *H. influenzae* to the NCI-H292 cell line and primary bronchial epithelial cells was enhanced. Furthermore, the defensins also stimulated 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. Since neutrophil defensins are released upon neutrophil degranulation, present in high concentrations in purulent airway secretions from patients with inflammatory lung disease (e.g., chronic bronchitis and cystic fibrosis), and recently have been reported to be present at the epithelial surface in lung tissue from patients with diffuse panbronchiolitis, 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 that can bind in a nonspecific way to the negatively charged surfaces of bacteria and cells. Such a nonspecific stimulatory effect by defensins was observed for the interaction with macrophages resulting in phagocytosis. In addition, various cationic polypeptides, including poly-l-lysine and protamine, increase the uptake of particles by leukocytes. 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 and subsequent washing.

The specific involvement of the defensins in the stimulation of adherence was demonstrated by a 90% inhibition with α1-

### Table 2. Adherence of 3 nonencapsulated *H. influenzae* strains to pharyngeal epithelial cells in the presence and absence of 20 μg/mL defensins (human neutrophil peptides [HNP]-1 to -3).

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Without HNP-1−3</th>
<th>With HNP-1−3</th>
</tr>
</thead>
<tbody>
<tr>
<td>No added bacteria</td>
<td>1.0 ± 1.1</td>
<td>ND</td>
</tr>
<tr>
<td>A850048</td>
<td>82 ± 36</td>
<td>82 ± 40</td>
</tr>
<tr>
<td>d1</td>
<td>4.2 ± 3.1</td>
<td>22 ± 13</td>
</tr>
<tr>
<td>770235 Φb+</td>
<td>1.6 ± 1.6</td>
<td>36 ± 17</td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. of adherent bacteria (mean ± SD) per epithelial cell, as determined by light microscopy. Experiment was repeated for 2 other donors, with similar results. ND = not done.
PI, a member of the serpin family. In moderately inflamed tissues, defensins are probably complexed due to an abundance of α1-PI and other defensin-binding substances, resulting in prevention of tissue damage [11]. However, neutrophils attracted to the site of inflammation [4, 5] release high concentrations of defensins in the lumen of the lung [6, 11, 26]. Since during inflammation, the high concentrations of defensins may overwhelm the defensin-binding components [11], 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 seen 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 [30].

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 [31, 32], bacterial killing may have been prevented. Also in the bronchial secretions of COPD patients, especially those with infections, similar salt concentrations are present [33, 34]. Therefore, the extracellularly released neutrophil defensins are probably also not bactericidal in vivo, although they may exert the enhancing effect of H. influenzae adherence.

Although the bacterial and cellular components involved in the interaction with 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 examination by light microscopy revealed that heat-killed (56°C for 30 min) and chloramphenicol-treated (10 μg/mL for 30 min) bacteria adhered to NCI-H292 cells in numbers similar to those for 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. On the other hand, epithelial cell viability was essential for the stimulation of adherence of H. influenzae to these cells in the presence of defensins because 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 interleukin-8 [21]. Therefore, the expression and 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 [35]. 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 [36].

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 did stimulate specific adherence to these cells. Since adherence to epithelial cells is an important step in the onset of mucosal infections [37], 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 is a general mechanism for recurrent infections.

Acknowledgments

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References


