Pathogenesis of Haemophilus influenzae. Respiratory infection in COPD patients
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STIMULATION OF BACTERIAL ADHERENCE BY NEUTROPHIL DEFENSINS VARIES AMONG BACTERIAL SPECIES BUT NOT AMONG HOST CELL TYPES

Annelies D. Gorter, Pieter S. Hiemstra, Sophie de Bentzmann, Sandra van Wetering, Jacob Dankert, and Loek van Alphen

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Abstract

Adherence of *Haemophilus influenzae* to bronchial epithelial cells is enhanced by neutrophil defensins, which are released from activated neutrophils during inflammation [Gorter et al., (1998) J. Infect. Dis. 178, 1067-1078]. In this study, we showed that the adherence of *H. influenzae* to various epithelial, fibroblast-like, and endothelial cell types was significantly enhanced by defensins (20 μg/ml). Defensins stimulated also the adherence of *Moraxella catarrhalis*, *Neisseria meningitidis*, and nonencapsulated *Streptococcus pneumoniae* to the NCI-H292 cell line. In contrast, defensins did not affect the adherence of *Pseudomonas aeruginosa*, encapsulated *S. pneumoniae*, *Escherichia coli*, and *Staphylococcus epidermidis*. These results suggest that the defensin-enhanced adherence might support the adherence and possibly persistence of the selected bacterial species using the respiratory tract as port of entry.
Introduction

Neutrophils accumulate at sites of inflammation and may release a variety of mediators upon stimulation. These neutrophil products include neutrophil defensins (human neutrophil peptide HNP1-4) [20], that have been characterized as small (29 to 34 amino acid residues), cationic peptides that display broad spectrum antimicrobial activity [10,20]. Defensins are active against Gram-negative as well as Gram-positive bacteria, fungi, some enveloped viruses and they are cytotoxic for mammalian cells [20]. Defensins can permeabilize target cell membranes, an event for which active metabolism of the target cells is required [14,17,22]. Approximately 5-7% of the total protein contents of mature neutrophils and 30-50% of the content of the azurophilic granules consists of defensins [18]. Upon stimulation of the neutrophil, defensins are released from the granules [9]. High concentrations of defensins have been found in purulent airway secretions from patients with chronic obstructive pulmonary disease (COPD) (100 μg/ml) [24] and cystic fibrosis patients (>300 μg/ml) [28]. Furthermore, defensins have been found to be present at the epithelial surface in lung tissue from patients with diffuse panbronchiolitis [1].

Recently, we have shown that neutrophil defensins (at 20 μg/ml) stimulate the adherence of Haemophilus influenzae (COPD isolates and carrier strains) to human bronchial epithelial cells in a time and concentration dependent way [12]. This may indicate that defensins that are released during inflammation and infection, contribute to the recurrent infections in COPD patients by stimulating bacterial adherence. In the present study, we determined the cellular and bacterial specificity of the defensin-enhanced adherence. We tested whether defensins stimulate the adherence of H. influenzae to various epithelial and non-epithelial cell types. In addition, we determined whether defensins stimulate the adherence of a variety of bacterial species, both respiratory pathogens and controls, to the bronchial epithelial cell line NCI-H292.

Materials and methods

Bacterial strains and growth conditions

H. influenzae, Moraxella (Branhamella) catarrhalis, Pseudomonas aeruginosa, and Escherichia coli were grown on chocolate agar plates (Oxoid, Haarlem, The Netherlands), Neisseria meningitidis on gonococcus (GC) agar plates (SVM, Bilthoven, The Netherlands), and Streptococcus pneumoniae and Staphylococcus epidermidis on blood agar plates (Oxoid,
Haarlem, The Netherlands). All strains were grown overnight at 37°C in a humidified atmosphere containing 5% CO₂. For the adherence assays, bacterial suspensions were prepared in phosphate buffered saline (PBS) at an optical density (OD₆₀₀ₙ₉) of 1. *N. meningitidis* was resuspended in RPMI 1640 (Gibco Life Technologies Ltd, Paisley, Scotland).

Trypan blue exclusion (500 µg/ml) demonstrated that *H. influenzae* strains A850048 and A950006, *N. meningitidis* strain H44/76 (provided by Dr. P. van der Ley, RIVM, Bilthoven, The Netherlands), *E. coli* strain DH5α, and *S. epidermidis* strain B1 (a gift from Dr. L. Vogel, Department of Medical Microbiology, Leiden, The Netherlands) were not toxic for the NCI-H292 cell line during the 4 hr incubation in the adherence assay. Since *P. aeruginosa* and *S. pneumoniae* produce toxins causing cell lysis, mutants lacking these toxins were included. *P. aeruginosa* strain PAO-R1 (a gift from Dr. B. Iglewsky, Department of Microbiology and Immunology, Rochester, New York, USA) has a mutation in the lasR gene, a positive regulator of virulence factors, and is unable to produce toxic elastase [8]. *S. pneumoniae* T3/PL, D39/PLN-A, and Rx1/Ply (provided by Dr. J. Paton, Department of Microbiology, Adelaide, Australia) are pneumolysin mutants of strains T3 (type 3), D39 (type 2) and Rx1 (non-encapsulated variant of D39) and have strongly reduced pneumolysin activity [5]. Trypan blue exclusion revealed that the NCI-H292 cells were more viable after incubation with the mutants than with the parent strains. Because *M. catarrhalis* easily forms aggregates, which make it difficult to analyze the adherence, the non-clumping variant of strain 4223, strain 4223NC [15] (provided by Dr. T.F. Murphy, Division of Infectious Diseases, Buffalo, USA), was included. *E. coli* strain 780401 I was also cytotoxic for the NCI-H292 cell line, and therefore, adherence assays with viable bacteria were performed with the non-pathogenic strain DH5α. The pathogenic parent strains PAO-1 (*P. aeruginosa*) and T3, D39, and Rx1 (*S. pneumoniae*), 4223 (*M. catarrhalis*), and strain 780401 I (*E. coli*) were included for reference after heat-inactivation (56°C for 30 min) and showed the same adherence patterns as the mutants.

**Culture of various cell lines**

The bronchial epithelial cell line NCI-H292 (ATCC CCL-1848) (originating from a human lung epidermoid carcinoma) [4], the alveolar epithelial cell line A549 (ATCC CCL-185), and the cervical carcinoma cell line HeLa (ATCC CCL-2) were maintained in RPMI 1640 medium with 25 mM HEPEs buffer (Gibco, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS). The Chang conjunctival epithelial cell line (ATCC CCL-20.2) was maintained in RPMI 1640 supplemented with 5% FCS, the laryngeal epithelial cell line HEP-2 (ATCC CCL-23) in DMEM (Gibco, Paisley, Scotland) supplemented with 10% FCS, and the colon carcinoma cell line Caco-2 (ATCC HTB-37) in DMEM supplemented with 0.1% (v/v) non-essential amino acids and 10% FCS. The human embryonic lung (HEL) (ATCC CCL-137) fibroblast like cells were maintained in MEM with 25 mM HEPEs (Gibco, Paisley, Scotland) supplemented with 10% FCS. All cell lines used were of human origin. For the adherence assay, the cells were grown to semiconfluency in 24-wells plates on glass coverslips (12-mm diameter) (Menzelgläser, Germany) [12].
Subcultures of primary cells

Subcultures of the human primary bronchial epithelial cells (PBEC) were obtained from lung tissue with a macroscopically normal appearance and maintained in keratinocyte serum free medium with low Ca²⁺ concentrations (0.09 mM) (keratinocyte-SFM, Gibco, Paisley, Scotland) [12]. For the adherence assay, the PBEC were placed in medium containing 1 mM CaCl₂ to allow differentiation [16]. The proximal tubular epithelial cells from human kidney (PTEC) were cultured in FCS (10%) coated tissue culture flasks in serum-free DMEM-HAM’s F12 medium in a 1:1 ratio, supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), selenium (5 ng/ml), hydrocortisone (36 ng/ml), triiodothyronine (40 pg/ml) and EGF (10 ng/ml) [11]. The human umbilical vein endothelial cells (HUVEC) were cultured on gelatin-coated tissue culture plates in M199 medium containing Earle’s salts and glutamine, supplemented with 10% FCS, 5% endothelial cell growth factor, and 7.5 U/ml heparin [2].

Isolation of defensins

Human neutrophil defensins (HNP1-3) were isolated from an acetic acid extract obtained from human neutrophil granules using gel filtration chromatography on Sephacryl S-200 HR (2.5 x 100 cm) (Pharmacia, Uppsala, Sweden) as described [13,30]. The purity of the defensin preparations was assessed by SDS-PAGE, and acid urea-PAGE.

Adherence assay

The adherence assays were performed in 24-wells plates as described [12,29]. Briefly, the medium covering the cells grown on glass coverslips was replaced by the appropriate medium lacking FCS to a final volume of 500 μl. Subsequently, the bacterial suspension prepared as described above (1:10 diluted) and HNP1-3 (final concentration 20 μg/ml) were added and incubated at 37°C for 4 hr. After washing, the remaining bacteria were released by incubation with 1% saponin at 37°C for 15 min. The number of adherent bacteria was determined after serial dilution and plating (colony counting method) [12,29]. Alternatively, the adherence was analyzed by light microscopy (LM) examination after fixation in 500 μl of 4% paraformaldehyde (Merck, Darmstadt, Germany) plus 1% glutaraldehyde (Merck) and subsequent staining with 0.07% crystal violet [12,29]. The potential antimicrobial activity of defensins during the assay was determined by colony counting the total amount of bacteria without washing after the 4 hr incubation with the cells in the presence and absence of defensins. In order to test the adherence of heat-inactivated bacteria in the presence or absence of defensins, the bacteria were treated at 56°C for 30 min prior the adherence assay. All adherence assays were performed at least 2 times in duplicate.

Scanning electron microscopy (SEM)

For SEM, the cells were fixed after the adherence assay in 4% paraformaldehyde (Merck) plus 1% glutaraldehyde (Merck) and washed with PBS. The cells were dehydrated in graded ethanol, critical point dried with CO₂, and coated with gold-palladium beads with a diameter of 15 nm. The cells were analyzed with a computer assisted SEM (Philips XL 30) with a biocom 500 image analyzer.
SPECIFICITY OF THE DEFENSIN-ENHANCED ADHERENCE

Statistical analysis
The data were analyzed for statistical significance by a two-tailed student's t-test for unpaired data. When p < 0.05, the data were considered significant.

Results

Adherence of *H. influenzae* to various non-inflammatory cells
The host cell specificity of the defensin-enhanced adherence was investigated by analyzing the adherence of *H. influenzae* to epithelial cell lines of the upper (HEp-2) and lower (NCI-H292, PBEC, and A549) respiratory tract. As controls, various non-respiratory epithelial cells (Chang, Caco-2, HeLa, and PTEC) and non-epithelial cells (HEL and HUVEC) were used. Irrespective of cell lines and subcultures of primary cells, the adherence of *H. influenzae* strain A850048 in the presence of defensins increased significantly (table 1). Using light microscopy (LM) analysis, similar results were obtained as with the colony counting method (table 1). The adherence of three other *H. influenzae* COPD isolates to the various cell types was also enhanced in the presence of defensins, as determined by colony counting and LM examination. The adherence of one of the *H. influenzae* strains, strain A950006, was also analyzed by scanning electron microscopy (SEM). The SEM revealed that in the absence of defensins A950006 adhered less to NCI-H292 cells (figure 1A) than in the presence of defensins (figure 1B). Apparently, the defensin-enhanced adherence was not associated with adhesins of *H. influenzae*.

Adherence of various bacterial species to NCI-H292 cells
The adherence of Gram-negative as well as the Gram-positive respiratory tract pathogens to the NCI-H292 cell line was determined in the presence and absence of defensins. The non-respiratory Gram-negative *E. coli* and the Gram-positive *S. epidermidis* were controls. In the presence of defensins, a significant increase of the bacterial adherence was observed for *H. influenzae*, *M. catarrhalis*, nonencapsulated *S. pneumoniae*, and *N. meningitidis* as determined by the colony counting method and by examination by LM (table 2). The stimulation of the adherence occurred with viable as well as heat-inactivated bacteria. The adherence of *P. aeruginosa*, encapsulated *S. pneumoniae*, *E. coli*, and *S. epidermidis* was not enhanced in the presence of defensins. Although *P. aeruginosa* adhered to the NCI-H292 cell line
Table 1. Adherence of *H. influenzae* strain A850048 to various cell lines in the presence or absence of defensins (HNP1-3, 20 μg/ml)

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Origin</th>
<th>Control</th>
<th>HNP1-3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CFU/ml</td>
<td>LM</td>
<td>CFU/ml</td>
</tr>
<tr>
<td><strong>Respiratory epithelial cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NCI-H292</td>
<td>Bronchus</td>
<td>3.6 ± 2.3 x 10^5</td>
<td>-</td>
</tr>
<tr>
<td>PBEC</td>
<td>Bronchus</td>
<td>3.0 ± 1.1 x 10^5</td>
<td>-</td>
</tr>
<tr>
<td>A549</td>
<td>Alveoli</td>
<td>2.8 ± 2.4 x 10^5</td>
<td>-</td>
</tr>
<tr>
<td>HEp-2</td>
<td>Larynx</td>
<td>1.3 ± 0.5 x 10^5</td>
<td>-</td>
</tr>
<tr>
<td><strong>Other epithelial cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chang</td>
<td>Conjunctiva</td>
<td>2.2 ± 1.7 x 10^5</td>
<td>-</td>
</tr>
<tr>
<td>Caco-2</td>
<td>Colon</td>
<td>2.8 ± 2.5 x 10^5</td>
<td>-</td>
</tr>
<tr>
<td>HeLa</td>
<td>Cervix</td>
<td>1.7 ± 0.9 x 10^5</td>
<td>-</td>
</tr>
<tr>
<td>PTEC</td>
<td>Kidney</td>
<td>2.0 ± 1.5 x 10^5</td>
<td>-</td>
</tr>
<tr>
<td><strong>Non-epithelial cells</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HEL</td>
<td>Fibroblast</td>
<td>2.2 ± 1.7 x 10^6</td>
<td>-</td>
</tr>
<tr>
<td>HUVEC</td>
<td>Endothelial</td>
<td>8.3 ± 1.4 x 10^5</td>
<td>-</td>
</tr>
</tbody>
</table>

* Average number of colony forming units (CFU)/ml ± SD as determined by colony counting.

b Adherence as determined by light microscopy (LM). Symbols: -, no adherence; ++, 10-50 bacteria/cell; ++++, 50-100 bacteria/cell; ++++, > 100 bacteria per cell.

c F.I, fold increase in colony counts.

*p < 0.05.

With rather high numbers of CFU/ml (table 2), the LM results clearly showed that the *P. aeruginosa* bacteria only adhered to the glass coverslips between the epithelial cells and not to the cells themselves. The adherence of *P. aeruginosa* to the NCI-H292 cell line in the absence and presence of defensins as determined by LM was 2.3 bacteria per cell and 3.3 bacteria per cell, respectively. All bacterial species survived the adherence assay in the presence and absence of defensins. During the 4 hr incubation, most of the bacterial strains grew, except for *P. aeruginosa* PAO-R1 and *N. meningitidis* H44/76, of which only 80% of the inoculum was recovered. These results indicated that defensins stimulated the adherence of selected bacterial species that have as common feature an association with colonization and infections of the respiratory tract.
Table 2. Adherence of various bacteria to the NCI-H292 cell line in the presence or absence of defensins (HNP1-3, 20 μg/ml)

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>Adherence control (CFU/ml)</th>
<th>LM</th>
<th>Adherence HNP1-3 (CFU/ml)</th>
<th>LM</th>
<th>FI</th>
</tr>
</thead>
<tbody>
<tr>
<td>H. influenzae</td>
<td>A850048</td>
<td>3.6 ± 2.3 x 10^5</td>
<td>-</td>
<td>2.2 ± 1.1 x 10^7</td>
<td>++++</td>
<td>61*</td>
</tr>
<tr>
<td>M. catarrhalis</td>
<td>4223NC</td>
<td>5.7 ± 3.8 x 10^6</td>
<td>-</td>
<td>4.3 ± 1.2 x 10^7</td>
<td>++++</td>
<td>7.6*</td>
</tr>
<tr>
<td>P. aeruginosa</td>
<td>PAO-R1</td>
<td>1.8 ± 1.0 x 10^3</td>
<td>-</td>
<td>1.1 ± 0.7 x 10^7</td>
<td>-</td>
<td>0.6</td>
</tr>
<tr>
<td>N. meningitidis</td>
<td>H44/76</td>
<td>7.9 ± 9.0 x 10^4</td>
<td>-</td>
<td>3.4 ± 1.6 x 10^6</td>
<td>++++</td>
<td>4.3*</td>
</tr>
<tr>
<td>S. pneumoniae</td>
<td>T3/PL</td>
<td>1.1 ± 0.9 x 10^4</td>
<td>-</td>
<td>3.3 ± 3.3 x 10^2</td>
<td>-</td>
<td>0.3</td>
</tr>
<tr>
<td></td>
<td>D39/PLN-A</td>
<td>1.7 ± 1.0 x 10^5</td>
<td>-</td>
<td>2.1 ± 0.8 x 10^3</td>
<td>-</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Rx1/Ply</td>
<td>1.5 ± 1.4 x 10^6</td>
<td>-</td>
<td>4.7 ± 1.9 x 10^6</td>
<td>+</td>
<td>3.1*</td>
</tr>
<tr>
<td>E. coli</td>
<td>DH5α</td>
<td>1.1 ± 0.5 x 10^6</td>
<td>-</td>
<td>7.8 ± 5.6 x 10^5</td>
<td>-</td>
<td>0.7</td>
</tr>
<tr>
<td>S. epidermidis</td>
<td>B1</td>
<td>4.5 ± 3.9 x 10^6</td>
<td>-</td>
<td>6.5 ± 1.6 x 10^6</td>
<td>-</td>
<td>1.5</td>
</tr>
</tbody>
</table>

* Average number of colony forming units (CFU) per ml ± SD as determined by colony counting.

b Adherence analyzed by light microscopy (LM). Symbols: -, no adherence; +, 5-10 bacteria per cell; ++++, > 100 bacteria per cell.

c FI, fold increase in colony counts.

d LM examination revealed that P. aeruginosa adhered only between the epithelial cells.
The adherence as demonstrated by LM was 2.3 bacteria per cell in the absence of defensins and 3.3 bacteria per cell in the presence of defensins.

* p < 0.05.

Discussion

In this study we showed that the defensin-enhanced adherence of H. influenzae was similar for various epithelial cell types, fibroblast and endothelial cells. It has been shown that defensins interact with cellular membranes of the target cells such as E. coli, Candida albicans, and mammalian cells [17,19,23]. Defensins permeabilize membranes by formation of multimeric pores [31] followed by cytoskeletal- and energy-dependent internalization of defensins [23]. It is possible that defensins in the pores or the internalized defensins change the cellular surface, resulting in exposure of receptors for adhesins of H. influenzae. The aspecific nature of the interaction of defensins with cellular membranes might explain the defensin-stimulated adherence of H. influenzae on all tested cells, irrespective of their type or source.
The mechanism by which defensins stimulate the bacterial adherence is not known. Since the enhanced adherence was observed with all cell types used in this study, it is likely that defensins bind to a common receptor. It is also possible that defensins increase the expression of a cellular receptor by activating the cells. Alternatively, the defensins might redistribute the cell surface, resulting in unmasking of a cellular receptor.

In COPD patients, cigarette smoking, inflammation and infectious processes are thought to contribute to damage of the epithelium. *H. influenzae* have been found in association with damaged epithelial cells in vitro [27]. Furthermore, *P. aeruginosa* adheres specifically to cells at sites of epithelial cell injury [7]. The concentrations of neutrophil defensins found in e.g. COPD or CF patients, are higher than the concentrations used in this study. Previously, we have shown that α1-proteinase inhibitor (α1-PI), a member from the serpin family of proteinase inhibitors, blocks the defensin-enhanced adherence of *H. influenzae* to bronchial epithelial cells [12]. Some activities of defensins are neutralized by components present in purulent secretions, such as α1-PI and possibly other substances such as mucins. Therefore, it is likely that only a part of the defensins in purulent secretions are available for stimulation of bacterial adherence. Although the activity of defensins might be affected by mucins, we have showed that defensins stimulated also the adherence of *H. influenzae* to mucus-containing human pharyngeal epithelial cells and to mucus-covered bronchial tissue obtained from autopsy procedures [12]. Therefore, the enhancement of bacterial adherence by defensins is likely to be relevant in vivo.

Defensins stimulated the adherence of *H. influenzae*, *M. catarrhalis*, *N. meningitidis*, and nonencapsulated *S. pneumoniae*. The adherence of

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**Figure 1.** Scanning electron microscopy examination of the adherence of *H. influenzae* strain A950006 to the NCI-H292 cell line in the absence (A) and presence (B) of neutrophil defensins (HNP1-3, 20 μg/ml). Bar represents 5 μm.
P. aeruginosa, encapsulated S. pneumoniae, E. coli and S. epidermidis was not enhanced by defensins. These results were found with colony counting as well as LM and with viable as well as heat-inactivated bacteria. The adherence patterns of the mutants were confirmed for the heat-inactivated parent strains. Since encapsulated S. pneumoniae was not stimulated in its adherence by defensins in contrast to the nonencapsulated S. pneumoniae, the capsule of S. pneumoniae might shield the bacterial component that interacts with defensins, as found for H. influenzae type b [12]. In contrast, adherence of encapsulated N. meningitidis type B was stimulated, indicating that the capsule of N. meningitidis did not shield the bacterial component involved in the defensins-enhanced adherence. Since there are many different capsule types, it is possible that some capsule types shield the bacterial component involved in the defensin-enhanced adherence, whereas other capsule types do not shield the bacterial component.

Defensins interact with lipopolysaccharide (LPS), a component of the outer membrane of Gram-negative bacteria [21]. In addition, bacteria expressing a rough LPS type by lacking the O-specific side chains are more sensitive to the antimicrobial activities of defensins [3,26]. The Gram-negative bacteria H. influenzae, M. catarrhalis, and N. meningitidis which showed an enhanced adherence in the presence of defensins, have similar LPS structures, lacking O-specific side chains, and share conserved LPS epitopes [6]. Interestingly, the adherence of E. coli strain DH5α, which also lacks O-specific side chains, is not stimulated by defensins. Therefore, even among the rough LPS types, specific LPS structures might be involved in the defensin-enhanced adherence.

A cell wall component of Gram-positive bacteria that might be involved in the interaction with defensins is teichoic acid. Recently, it was shown that mutations in teichoic acid in Staphylococcus aureus result in an increased negative surface charge of the cell that is associated with an increased sensitivity to the antimicrobial action of human defensins HNP1-3 [25]. If similar mechanisms are involved in sensitivity and adherence, this result suggests that teichoic acid may be the receptor for defensins in their stimulation of the adherence of some Gram-positive bacteria including S. pneumoniae.

In conclusion, neutrophil defensins stimulated the adherence of H. influenzae similarly to various cell lines and primary cells. However, the defensin-enhanced adherence is specific for selected bacterial species, using the respiratory tract as port of entry. Since adherence is an important step in the onset of the infection process, the
defensin-enhanced adherence may contribute to the persistence of (airway) infections when defensins are present, such as in COPD patients.

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