Pathogenesis of Haemophilus influenzae. Respiratoy infection in COPD patients
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INVolVEMENT OF LIPOOLIGOSACCHARIDES OF Haemophilus influenzae AND Neisseria meninGitidis IN DEFENsIN-ENHANCED BACTERIAL ADHERENCE TO EPITHELIAL CELLS

Annelies D. Gorter, Jaap Oostrik, Peter van der Ley, Pieter S. Hiemstra, Jacob Dankert, and Loek van Alphen

Submitted for publication
Abstract

Stimulated neutrophils release a variety of antimicrobial peptides, including neutrophil defensins (HNP1-4). We have previously reported that neutrophil defensins enhanced the adherence of *Haemophilus influenzae* and *Neisseria meningitidis* to cultured respiratory epithelial cells. In this study, the effect of defensins on the adherence of *H. influenzae* and *N. meningitidis* lipooligosaccharide (LOS) mutants to epithelial cells was tested. Neutrophil defensins enhanced the adherence of the oligosaccharide mutants of *H. influenzae* and *N. meningitidis*, whilst the lipid A mutants B29 of *H. influenzae* and *lpxL1* and *lpxL2* of *N. meningitidis* were not or only moderately stimulated by neutrophil defensins. The adherence of the *N. meningitidis* LOS negative mutant *lpxA* was not enhanced by defensins. These findings suggested that the secondary fatty acids of lipid A were involved in the defensin-enhanced adherence. Using biotinylated HNP-1, we demonstrated that defensins bound to whole bacterial cells of *H. influenzae* and *N. meningitidis*, irrespective the presence of LOS. Binding of biotinylated defensins to purified LOS from *H. influenzae* strain 2019 and its *htrB* mutant B29 was also observed. Wild type *N. meningitidis* H44/76 LOS and *rfaC* LOS did bind to defensins, whereas *lpxL1* mutated LOS did not. Purified LOS from strain H44/76 or HNP-LOS complexes did not inhibit or stimulate the adherence of *N. meningitidis*, although the defensin-enhanced adherence is specific for certain bacterial species having LOS in their outer membrane. Defensin-enhanced adherence requires therefore most likely simultaneously binding of defensins to LOS and epithelial cells.
**Introduction**

Neutrophils are often present in substantial numbers at sites of inflammation and infection and these cells contain a variety of antimicrobial peptides, including neutrophil defensins. These defensins (also referred to as human neutrophil peptide [HNP]) are small cationic peptides (29-33 amino acid residues) lacking enzymatic activity [9,26,46]. In the human mature neutrophil, defensins constitute 5-7% of the total protein content and in azurophilic granules 30-50% [25]. Upon activation of the neutrophil, defensins are released from the granules [8]. High concentrations of neutrophil defensins have been found in plasma from patients with septicemia and bacterial meningitis [31] and in purulent airway secretions from chronic obstructive pulmonary disease (COPD) patients [30], and cystic fibrosis (CF) patients [35]. Neutrophil defensins are also present on the epithelial cell surface in lung tissue from patients with diffuse panbronchiolitis [1]. Defensins display a broad spectrum of antimicrobial activity as they can kill Gram-negative as well as Gram-positive bacteria, fungi, and some enveloped viruses [26]. Besides being antimicrobial, defensins are cytotoxic for mammalian cells and play a role in inflammation, wound repair and regulation of the specific immune response [5,46].

Previously we have shown in *in vitro* studies that in the presence of neutrophil defensins, the adherence of various bacterial species to human epithelial cells was enhanced [10,11]. Particularly, the adherence of bacteria present in the upper respiratory tract, such as *Haemophilus influenzae* and *Neisseria meningitidis*, was increased whereas adherence of *Escherichia coli* was not affected [11]. By which mechanism defensins increase adherence of certain bacterial species is unknown. Defensins have been shown to interact with the lipopolysaccharide (LPS) from Gram-negative bacteria, since endotoxic activities of LPS was decreased in the presence of defensins [27]. Further evidence for an interaction between defensins and LPS comes from the observation that modifications in the lipid A of *Salmonella typhimurium* LPS resulted into bacterial resistance to various antimicrobial peptides, including neutrophil defensins [13,14,16]. We have found that the defensin-enhanced bacterial adherence occurred also with heat-killed *H. influenzae* and *N. meningitidis*, indicating that the bacterial component involved was heat-resistant [10,11]. LPS is a heat-resistant cell wall component [47]. Viable and heat-killed *E. coli* showed no increased adherence in the presence of defensins. In contrast to *E. coli*, both *H. influenzae* and *N. meningitidis* have a rough type of LPS, lacking O-specific side chains, which is
also referred to as lipoooligosaccharide (LOS) [4]. Therefore, we hypothesized that LOS was involved in the defensin-enhanced adherence of *H. influenzae* and *N. meningitidis*. In this study, we evaluated the effect of various LOS mutations of *H. influenzae* and *N. meningitidis* on their defensin-enhanced adherence to epithelial cells. In addition, we analyzed the binding characteristics of neutrophil defensins to whole bacterial cells and purified LOS.

**Materials and Methods**

**Bacterial strains and culture**

*H. influenzae* and *N. meningitidis* strains used are listed in table 1. Bacteria were cultured on chocolate agar plates (Oxoid, Haarlem, The Netherlands) at 37°C in a humidified atmosphere containing 5% CO₂. For the adherence assays, *H. influenzae* was resuspended in phosphate buffered saline (PBS) and *N. meningitidis* was resuspended in the DMEM culture medium (Gibco Life Technologies Ltd, Paisley, Scotland) lacking fetal calf serum (FCS), at a density of OD₅₅₀ of 1 (~10⁷ CFU/ml, as determined by colony counting).

**Cell culture**

Cells from the epithelial cell line NCI-H292 (ATCC CRL 1848), originating from a lung mucoepidermoid carcinoma [3], were maintained in RPMI1640 culture medium (Gibco Life Technologies) supplemented with 10% FCS. HEp-2 laryngeal epithelial cells (ATCC CCL-23) were maintained in DMEM supplemented with 10% FCS. The cells were cultured in the absence of antibiotics and grown in 25 cm² culture flasks (Corning Costar Corporation, Cambridge, MA) at 37°C in a humidified atmosphere containing 5% CO₂. NCI-H292 cells were used for the adherence assays with *H. influenzae* and HEp-2 cells were used for *N. meningitidis*. The epithelial cells were grown to semiconfluency (~3x10⁵ cells) in 24-well plates (Greiner BV, Alphen a/d Rijn, The Netherlands) on round glass coverslips (12 mm diameter) (Menzelgläser, Braunschweitz, Germany).

**Purification and biotinylation of defensins**

The neutrophil defensins were isolated from an acetic acid extract obtained from human neutrophil granules, derived from the blood of healthy donors, using gel filtration chromatography on Sephacryl S-200 HR (2.5x100 cm) (Pharmacia, Uppsala, Sweden) as described [45]. The purity of the defensins was assessed by SDS-PAGE and acid urea-PAGE, and mass spectrometry [15,45]. Neutrophil defensins were used as a mixture of HNP1-3 in the adherence experiments.

HNP-1 was further purified from the fractionated granule extract by reverse-phase high-performance liquid chromatography on a C18 column (4.6x250 mm) (Vydac, The SeparationsGroup, Hesperia, CA) as described previously [45]. This HNP-1 was biotinylated with biotin-N-Hydroxysuccinimide ester (HNP¹⁷⁵[¹⁷⁵]) [41] according to the manufacturer’s protocol (Zymed Laboratories, Inc., San Fransisco, CA).
### Table 1. LOS or LPS composition of the bacterial strains used in this study.

<table>
<thead>
<tr>
<th>Species</th>
<th>Strain</th>
<th>LOS / LPS composition</th>
<th>Ref</th>
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<tr>
<td></td>
<td></td>
<td>Gal – Hep</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hep</td>
<td></td>
</tr>
<tr>
<td>FK1, <em>rfaF</em> mutant</td>
<td></td>
<td>Hep – Kdo – lipidA</td>
<td>[29]</td>
</tr>
<tr>
<td>DK1, <em>rfaD</em> mutant</td>
<td></td>
<td>ddHep – Kdo – lipidA</td>
<td>[29]</td>
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<tr>
<td>B29, <em>htrB</em> mutant</td>
<td></td>
<td>lacks one or both secondary fatty acids (myristate, C14)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>GlcNac – Hep – Kdo</td>
<td>[32]</td>
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<tr>
<td></td>
<td></td>
<td>GlcNac – Hep – Kdo</td>
<td></td>
</tr>
<tr>
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<td>GlcNac – Hep – Kdo</td>
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</tr>
<tr>
<td>gcslE mutant</td>
<td></td>
<td>Hep – Kdo – lipidA</td>
<td>[42]</td>
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<td></td>
<td></td>
<td>GlcNac – Hep – Kdo</td>
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</tr>
<tr>
<td>icsB mutant</td>
<td></td>
<td>Hep – Kdo – lipidA</td>
<td>[42]</td>
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<td>GlcNac – Hep – Kdo</td>
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</tr>
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<td></td>
<td>Hep – Kdo</td>
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</tr>
<tr>
<td>rfasF mutant</td>
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<td>Hep – Kdo – lipidA</td>
<td>[38]</td>
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<tr>
<td></td>
<td></td>
<td>Kdo</td>
<td></td>
</tr>
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<td>rfasC mutant</td>
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<td>Kdo – lipidA</td>
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<tr>
<td>lpsL1 mutant</td>
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<td>lacks one secondary fatty acid (laurate, C12)</td>
<td>[43]</td>
</tr>
<tr>
<td>lpsL2 mutant</td>
<td></td>
<td>lacks two secondary fatty acids (laurate, C12)</td>
<td>[43]</td>
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<tr>
<td>lpsA mutant</td>
<td></td>
<td>lacks LOS completely</td>
<td>[37]</td>
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**Generalized structure of LPS**

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</tbody>
</table>
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*Abbreviations: Gal, galactose; GlcNac, N-acetylglucosamine; Glc, glucose; Hep, L-glycero-D-manno-heptose; Kdo, 3-deoxy-D-manno-octulosonic acid; DDHep, D-glycero-D-manno-heptose; Hex, hexose; Ra, Re, position of the Ra and Re types of *S. minnesota* LPS, respectively. Dashed line indicate non-stoichiometric substitution.*
Isolation and purification of LOS

LOS from *H. influenzae* strains 760705, 2019 and B29 and *N. meningitidis* strains H44/76, rfaC and ipxL1 was isolated using hot-phenol extraction as described [47]. Briefly, *H. influenzae* was grown overnight in BHI medium (Oxoid) and *N. meningitidis* in meningococcal medium. The suspensions were heat-inactivated at 56°C for 30 min. An equal volume of pre-heated phenol (68°C) was added to the bacterial suspensions and incubated at 68°C for 30 min. After centrifugation, the liquid phase was collected and the hot-phenol extraction was repeated. The phenol was removed from the extract by dialyzing against tapwater at 20°C overnight. After treatment with DNase (50 μg/ml) (Sigma Chemical Co, St Louis, MO), with RNase (50 μg/ml) (Sigma Chemical Co) and with proteinase K (50 μg/ml) (Sigma Chemical Co), the LOS preparation of each strain was collected, lyophilized and stored at -20°C. The purity of the LOS preparations was analyzed on a tricine-SDS-PAGE using silver staining.

LPS from *Salmonella minnesota* wild type, R60 (Ra type) and R595 (Re type) was obtained from List Biological Laboratories, Inc. (Campbell, CA).

Binding of defensins

The binding of defensins to whole bacterial cells or the purified LOS was determined by ELISA using HNP<sup>biotin</sup>. The assay was adapted from van den Berg *et al.* [41]. All incubation steps were performed in a volume of 100 μl. Immunolon 1 microtiter plates (Dynatech Laboratories, Inc., Chantilly, Virginia), were coated at 37°C overnight with whole bacterial cells, heat-inactivated at 56°C for 30 min, from a bacterial suspension with an OD<sub>625nm</sub>~1 diluted 160 times in PBS (~ 6.3x10<sup>6</sup> CFU/ml). In other experiments, 10 μg/ml purified LOS in PBS was coated on Immunolon 1 microtiter plates at 37°C overnight. After coating with either whole bacterial cells or LOS, the wells were washed with PBS-0.1%Tween-80 and then HNP<sup>biotin</sup> was added in various concentrations diluted in PBS-0.1%Tween-80 followed by incubation at 37°C for 1 hr. The biotin was detected by incubation for 30 min with streptavidin-poly horse radish peroxidase (HRP) (CLB, Amsterdam, The Netherlands) diluted 1:10,000 in PBS-0.1%Tween-80 at 37°C. The HRP was detected with tetramethylbenzidine (TMB) (Sigma Chemical Co) substrate and the reaction was stopped with 2 M H<sub>2</sub>SO<sub>4</sub> after 10 min. The OD was measured at 450 nm using a microtiter biokinetics reader (Biotek Instruments, Inc., Winooski, VT).

Inhibition experiments were performed with non-labeled HNP-1. For this purpose, the microtiter plates coated with the whole cells were incubated with various concentrations of non-labeled HNP-1 in 50 μl per well and incubated at 37°C for 1 hr. Next, 50 μl HNP<sup>biotin</sup> with a final concentration of 62.5 ng/ml was added without washing the wells and the plates were incubated at 37°C for another hour.

Adherence assay

The adherence assays were performed as previously described [10,11,44]. Briefly, the medium covering the epithelial cells grown on glass coverslips was replaced with the appropriate culture medium lacking FCS. Bacteria and HNP1-3 were added at final concentrations of 10<sup>8</sup> CFU/ml and 20 μg/ml respectively. After 4 hr of incubation, the
NCI-H292 cells were washed with PBS and the HEP-2 cells with DMEM. Then the cells were fixed in 4% paraformaldehyde (Merck, Darmstadt, Germany) and 1% glutaraldehyde (Merck) and subsequently stained in 0.007% (w/v) crystal violet. The number of adhering bacteria per cell was counted by light microscopic examination. In total the number of adhering bacteria of 10 cells per well was determined and expressed as the mean number of adhering bacteria per cell [10,44]. Alternatively, after washing the cells were treated with 1% saponin in PBS for 15 min and 10-fold serial dilutions were prepared in PBS for *H. influenzae* and in DMEM for *N. meningitidis*. From these dilutions aliquots of 10 or 100 μl were cultured on chocolate agar plates. The number of CFU/cell was calculated by dividing the number of CFU/ml, counted on the chocolate agar plates, by the number of epithelial cells (~ 3x10^5) [10,44]. In selected experiments, 20 μg/ml HNP1-3 and 100 μg/ml purified LOS of *N. meningitidis* strain H44/76 were mixed in DMEM and incubated at 37°C for 1 hr, prior to addition to the cell layer. The HNP-LOS mixtures were added to the epithelial cells simultaneously with the bacteria. In some experiments, the epithelial cells were pre-exposed to the HNP-LOS mixtures at 37°C for 1 hr prior to the addition of bacteria.

**Statistics**

The data were analyzed for significance using a students t-test. Differences were considered significant at p-values < 0.05.

**Results**

**Adherence of LOS mutants of *H. influenzae* to NCI-H292 epithelial cells**

Initially, the contribution of LOS in the defensin-enhanced adherence was determined by testing several LOS mutants of *H. influenzae*, since we had analyzed the defensin-enhanced adherence most extensively for this bacterium. The nontypeable *H. influenzae* clinical isolate 2019 and its isogenic LOS mutants FK-1, DK-1 and B29, with mutations in the *rfaF*, *rfaD* and *htrB* gene, respectively (table 1) were used

<table>
<thead>
<tr>
<th>Strain</th>
<th>Control (range)</th>
<th>HNP1-3 (range)</th>
<th>Increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>2019</td>
<td>48 (16-88)</td>
<td>150 (106-180)</td>
<td>3.1</td>
</tr>
<tr>
<td>FK1</td>
<td>53 (30-81)</td>
<td>110 (77-136)</td>
<td>2.1</td>
</tr>
<tr>
<td>DK1</td>
<td>60 (34-103)</td>
<td>107 (54-155)</td>
<td>1.8</td>
</tr>
<tr>
<td>B29</td>
<td>71 (32-121)</td>
<td>93 (61-125)</td>
<td>1.3</td>
</tr>
</tbody>
</table>

*a Adherence is expressed as adherent bacteria per epithelial cell (range) as determined by light microscopy.*
since their LOS structures have been resolved [23,29,33]. The *H. influenzae* wild type and LOS mutants survived the 4 hr exposure to neutrophil defensins during the adherence assay, since the numbers of CFU in the presence of defensins and controls were similar (data not shown). In the absence of defensins, all strains adhered to the NCI-H292 cells (table 2). The adherence of the wild type strain and LOS mutants increased in the presence of defensins (table 2). However, the increase by which defensins enhanced the adherence of the LOS mutants was less than the increase of the wild type strain 2019 (table 2), suggesting that *H. influenzae* LOS was involved in the defensin-enhanced bacterial adherence to epithelial cells.

**Adherence of *N. meningitidis* to HEp-2 epithelial cells**
The effect of defensins on the adherence for *N. meningitidis* strain H44/76 and its isogenic LOS mutants to HEp-2 cells was determined. *N. meningitidis* LOS mutants, including the LOS deficient mutant survived the 4 hr exposure to neutrophil defensins under the conditions of the adherence assay (data not shown). The adherence of *N. meningitidis* LOS mutants truncated in their oligosaccharide chain was enhanced by neutrophil defensins, but their adherence was less than that of the wild type strain

![Figure 1](image)

**Figure 1.** Adherence of *N. meningitidis* wild type and LOS mutants to HEp-2 epithelial cells in the presence and absence of neutrophil defensins. The adherence was analyzed using light microscopy. The results are the average and standard error of the mean (SEM) of three different experiments each performed in duplicate. *, p < 0.001 (HNP1-3 versus control); #, p < 0.001 (LOS mutant + HNP1-3 versus H44/76 + HNP1-3).
H44/76 (figure 1). The adherence of the lipid A mutants lpxL1 and lpxL2 was not stimulated by neutrophil defensins (figure 1). Similar results were obtained using the colony counting method (data not shown). These findings suggested that LOS, especially the secondary fatty acid chains of the lipid A were involved in the defensin-enhanced bacterial adherence to the epithelial cells. The adherence of the LOS deficient mutant lpxA was determined by light microscopy. Most of the epithelial cells did not show adherent bacteria in the presence of neutrophil defensins. However, on some epithelial cells bacterial aggregates were observed. When the adherence was assessed by the colony counting method, no increased adherence was observed for the lpxA mutant (figure 2B). Therefore we concluded that the adherence of the lpxA mutant was not enhanced by defensins.

![Figure 2](image)

**Figure 2.** Defensin-enhanced adherence of *N. meningitidis* H44/76 (A) and lpxA mutant (B) in the presence and absence of purified LOS of *N. meningitidis* strain H44/76 (100 μg/ml). HEp-2 epithelial cells were pre-exposed with LOS of *N. meningitidis* strain H44/76 at 37°C for 1 hr, prior the addition of the bacteria and defensins. The adherence was analyzed by the colony counting method. The results are the average and SEM of three different experiments each performed in duplicate. *, p < 0.001 (HNP1-3 versus control).
**Binding of defensins to whole bacterial cells**

The binding of defensins to bacteria was examined by whole cell ELISA. The binding of biotinylated HNP-1 (HNP\textsuperscript{biotin}) to wild type and LOS mutants of *H. influenzae* and *N. meningitidis* was observed. Approximately $6 \times 10^6$ CFU/ml of bacteria in PBS were coated on Immunolon 1 microtiter plates. The binding of HNP\textsuperscript{biotin} was detected with streptavidin-poly-HRP and TMB as substrate. Control, only PBS present. The results are the average of two different experiments each performed in duplicate.

**Figure 3.** Binding of HNP\textsuperscript{biotin} to whole bacterial cells of wild type strains and LOS mutants from *H. influenzae* (A) and *N. meningitidis* (B) and the controls *E. coli* DH5α and *S. typhimurium* SL1344 (C). Approximately $6 \times 10^6$ CFU/ml of bacteria in PBS were coated on Immunolon 1 microtiter plates. The binding of HNP\textsuperscript{biotin} was detected with streptavidin-poly-HRP and TMB as substrate. Control, only PBS present. The results are the average of two different experiments each performed in duplicate.
N. meningitidis coated on microtiter plates was determined. Controls included E. coli strain DH5α, since the adherence of this strain was not stimulated by neutrophil defensins [11], and S. typhimurium strain SL1344 [12], since this strain has a LPS containing an O-specific side chain. Bacterial adherence of both H. influenzae and N. meningitidis in the presence of HNP\textsuperscript{biotin} did not differ from the adherence using unlabeled HNP1-3 (data not shown), indicating that the HNP\textsuperscript{biotin} was functional in the defensin-enhanced adherence. The HNP\textsuperscript{biotin} bound similarly to the whole cells of H. influenzae (figure 3A) and N. meningitidis (figure 3B), wild type and LOS mutants, including the B29 htrB mutant of H. influenzae (figure 3A) and the lpxL1 and the LOS negative lpxA mutants of N. meningitidis (figure 3B). Also for E. coli strain DH5α and S. typhimurium strain SL1344 binding of HNP\textsuperscript{biotin} was observed (figure 3C). These results indicated that the binding of defensins to bacteria was not dependent on the type of LOS or LPS. The specificity of the binding of HNP\textsuperscript{biotin} to whole cells of N. meningitidis strain H44/76 was determined by competition experiments with non-labeled HNP-1. The binding of 62.5 ng/ml HNP\textsuperscript{biotin} to whole cells of N. meningitidis strain H44/76 was not inhibited by non-labeled HNP-1, even not in a 80-fold excess (figure 4). Some background was observed in the control

![Graph](https://example.com/graph.png)

**Figure 4.** Effect of non-labeled HNP-1 on the binding of HNP\textsuperscript{biotin} to whole cells of N. meningitidis. Approximately 6\times10^6 CFU/ml of bacteria in PBS were coated on Immunolon 1 microtiter plates. The binding of 62.5 ng/ml HNP\textsuperscript{biotin} was competed with various concentrations of unlabeled HNP-1. HNP\textsuperscript{biotin} binding was detected with streptavidin-poly-HRP and TMB substrate. Control, only PBS present. The results of a representative experiment are shown. For H. influenzae and E. coli similar results were found.
well, without coated bacteria, with increasing concentrations of non-labeled HNP-1. Non-labeled defensins also did not inhibit the binding of HNP\textsuperscript{biotin} to whole cells of *H. influenzae* strain 2019 and *E. coli* strain DH5α (data not shown).

**Binding of defensins to purified LOS**

To determine whether LOS could be the ligand for binding to defensins, the binding of purified LOS to HNP\textsuperscript{biotin} was analyzed. HNP\textsuperscript{biotin} bound to all *H. influenzae* LOS types used, although binding to LOS from strain 2019 and its B29 *htrB* mutant required higher concentrations of defensins than the binding to LOS from strain 760705 (figure 5A). Binding of HNP\textsuperscript{biotin} was observed to purified LOS of *N. meningitidis* wt H44/76 and the deep rough *rfaC* LOS (figure 5B). The binding of HNP\textsuperscript{biotin} to purified *lpxl* LOS was not significantly higher compared to the control wells in which only PBS was present (figure 5B). As a control, the binding of HNP\textsuperscript{biotin} to purified LPS of *S. minnesota* was determined. No binding above background was observed for *S. minnesota* wt LPS and the Ra mutant, lacking the O-specific side chains. The deep rough Re mutant showed a moderate binding to defensins (figure 5C), which required higher concentrations of HNP\textsuperscript{biotin} than the binding to *H. influenzae* and *N. meningitidis* LOS. These data indicated that neutrophil defensins preferably bound to LOS of *H. influenzae* and *N. meningitidis* and to rough type LPS of *S. minnesota*.

**The effect of purified LOS on the defensin-enhanced adherence**

To determine whether the presence of purified LOS could inhibit the defensin-enhanced adherence of *N. meningitidis*, neutrophil defensins (20 µg/ml) and purified LOS of *N. meningitidis* wild type strain H44/76 (100 µg/ml) were preincubated for 1 hr prior to the addition to HEp-2 cells in the adherence assays. The adherence of *N. meningitidis* strain H44/76 in the presence of these HNP-LOS mixtures was similar to the adherence of *N. meningitidis* in the presence of neutrophil defensins (figure 6), indicating that the purified LOS bound to defensins did not reduce the enhanced adherence phenomenon of the defensins. Pre-exposure of the HEp-2 cells to the HNP-LOS mixtures for 1 hr before the addition of *N. meningitidis* H44/76 bacteria, reduced the effect of the neutrophil defensins on the adherence (figure 6). Addition of fresh defensins simultaneously with the bacteria restored the enhanced adherence phenomenon (figure 6). Pre-exposure of the HEp-2 cells to neutrophil defensins alone
Figure 5. Binding of HNP\textsuperscript{biotin} to purified wild type LOS and mutated LOS of *H. influenzae* (A) and *N. meningitidis* (B) and for control wild type and mutated LPS of *S. minnesota* (C). Ten μg/ml of purified LOS in PBS was coated on Immunolon 1 microtiter plates. The binding of HNP\textsuperscript{biotin} was detected with streptavidin-poly-HRP and TMB substrate. Control, only PBS present. The results are the average and SEM of three different experiments each performed in duplicate. The binding of HNP\textsuperscript{biotin} to *H. influenzae* LOS types 760705, 2019 and B29 was significant compared to the control wells for concentrations of HNP\textsuperscript{biotin} of 39 ng/ml or more (p < 0.05) For *N. meningitidis* H44/76 and rfaC LOS, the binding of HNP\textsuperscript{biotin} was significant for concentrations HNP\textsuperscript{biotin} of 78 ng/ml or more (p < 0.05). HNP\textsuperscript{biotin} bound significantly to *S. minnesota* Re LPS at concentrations of 2.5 μg/ml or more (p < 0.05).
resulted in a lower bacterial adherence compared to the adherence occurring when bacteria and defensins were added simultaneously (figure 6). These experiments showed that pre-exposure of defensins as well as the HNP-LOS mixtures did increase the adherence of *N. meningitidis* strain H44/76 above baseline levels, but this adherence was lower than that observed when defensins were added simultaneously with the bacteria. This inhibiting effect is most likely due to the interaction of defensins with the epithelial cells rather than due to a direct inhibitory effect by defensins bound to the purified LOS, since subsequent addition of bacteria and defensins restored the defensin-enhanced adherence.

To determine whether purified LOS influenced epithelial cells, resulting into the defensin-enhanced adherence, 100 µg/ml of purified LOS from *N. meningitidis* strain H44/76 was added to the HEp-2 epithelial cells for 1 hr prior the addition of

![Figure 6](image)

*Figure 6.* Effect of mixtures of purified LOS and defensins on the defensin-enhanced adherence of *N. meningitidis* H44/76. LOS (100 µg/ml) of *N. meningitidis* strain H44/76 was preincubated at 37°C with HNP1-3 (20 µg/ml) for 1 hr. These HNP-LOS mixtures were used directly in the adherence assays or pre-exposed to the HEp-2 cells at 37°C for one additional hr. As a control, defensins were pre-exposed to the HEp-2 cells at 37°C for 1 hr. The adherence was analyzed by light microscopy. The results are the average and SEM of three different experiments each performed in duplicate. *, p < 0.001 compared to the adherence of H44/76 in the absence of defensins; #, p < 0.001 compared to the adherence of H44/76+HNP1-3.
N. meningitidis H44/76 bacteria. Using the colony counting method, the (defensin-enhanced) adherence of strain H44/76 after the pre-exposure of HEp-2 cells to LOS was similar to the adherence in the absence of LOS, with 4.1-4.6 CFU/cell for the adherence in the absence of defensins and 31-44 CFU/cell in the presence of defensins (figure 2A). The adherence of the LOS deficient mutant lpxA to HEp-2 cells in the presence and absence of LOS and defensins did not differ and was low being only approximately 0.01 CFU/cell (figure 2B). These data suggested that LOS pretreatment of epithelial cells did not affect the adherence of N. meningitidis in the absence and presence of neutrophil defensins.

Discussion

The results from the present study demonstrated that neutrophil defensins enhanced the adherence of H. influenzae and N. meningitidis wild type strains to respiratory epithelial cells, whilst the adherence of the LOS negative mutant lpxA of N. meningitidis was not enhanced. Various LOS mutants with mutations in the oligosaccharide chain showed a much lower increase in adherence than the wild type strains. This observation indicated that LOS was involved in the defensin-enhanced adherence.

Subsequent binding studies of defensins to LOS showed binding of HNP\textsuperscript{biotin} to purified LOS of H. influenzae and N. meningitidis. The results of these experiments were in agreement with the results as observed for the adherence studies, except for H. influenzae htrB mutant B29. The adherence of this mutant was poorly enhanced by defensins, whereas HNP\textsuperscript{biotin} bound to LOS of this H. influenzae htrB mutant. Interactions of defensins with LOS and LPS have been reported. Although toxic effects of the LPS of Gram-negative bacteria were neutralized by the interaction with various antimicrobial peptides, including defensins, defensins preferably neutralized the toxic effects of rough types of LPS, lacking the O-specific side chains [27]. Bordetella pertussis, a bacterial species characterized by the presence of LOS, was more susceptible to antimicrobial peptides than Bordetella bronchiseptica, having LPS containing O-specific side chains [2]. LOS of H. influenzae, N. meningitidis and B. pertussis is characterized by the lack of the O-specific side chains [4]. These data suggested that defensins bind functionally preferably to bacteria with LOS. Therefore, it is tempting to speculate that the longer O-specific side chains mask the binding site for defensins to LPS. Such a binding site may be the lipid A part of the LOS structure.
Our lipid A deficient *N. meningitidis* mutants *lpxL1*, *lpxL2* and *lpxA*, showed no adherence in the presence of defensins, and the increase in adherence of *H. influenzae* *htrB* mutant B29 was slight. These mutants lacked one or two secondary fatty acid chains in their lipid A, leading to a penta- or hexa-acylated lipid A structure. This finding suggested that defensins enhanced the bacterial adherence by binding to the secondary fatty acid chains in the lipid A. Previously, it has been reported that resistance to antimicrobial peptides was associated with modifications in the lipid A. For *S. typhimurium*, the resistant *pmrA* and *phoP-phoQ* mutants have an addition of aminoarabinose to lipid A phosphate groups and addition of a palmitate resulting in a hepta-acylated lipid A [13,14,16]. Furthermore, *Pseudomonas aeruginosa* isolates with specific lipid A, containing aminoarabinose and palmitate, have been cultured from patients with CF and these isolates were resistant to antimicrobial peptides [6]. These observations might indicate that defensins preferably interact with hexa-acylated lipid A.

Despite the binding of defensins to purified LOS, the defensin-enhanced adherence of *N. meningitidis* was not inhibited by purified LOS of *N. meningitidis*, indicating that binding of defensins to LOS is not the only requirement for enhanced bacterial adherence. This conclusion is supported by the finding that the adherence of latex beads coated with purified LOS from *N. meningitidis* or *H. influenzae* to epithelial cells was not stimulated by defensins (Gorter *et al.*, unpublished observations). However, it may be that the binding of defensins to LOS depends on the configuration of the LOS, since the configuration of purified LOS is different from the LOS configuration in bacterial membranes [40]. Another explanation for the finding that purified LOS did not inhibit the defensin-enhanced adherence may be that other components of the bacterial outer membrane than LOS are involved in the defensin-enhanced adherence. A major role of outer membrane protein is not very likely, since the defensin-enhanced adherence of the various isogenic LOS mutants differed. In addition, defensins can interact with phospholipids in bacterial membranes [7,48]. Therefore, multiple bindings sites for defensins are available on the surface of these bacteria. Light microscopy revealed the presence of bacterial aggregates on some of the epithelial cells, in particular for the *lpxA* mutant of *N. meningitidis*. This mutant has more negatively charged phospholipids in its outer membrane compared to the other LOS mutants [36]. Therefore, the aggregates might be the result of an interaction of defensins with the bacterial phospholipids. Binding of HNP<sup>biotin</sup> to whole cells of *E. coli* and *S. typhimurium*, both having LPS, was
observed, whereas no binding of HNP$^{\text{biotin}}$ to purified LPS of S. minnesota occurred. These observations suggested that the binding of defensins to the whole cells could be the result of binding of defensins to phospholipids. Since the adherence of E. coli is not enhanced by defensins [11], it is not likely that phospholipids are involved in the defensin-enhanced adherence.

What could be the mechanism by which defensins stimulate the adherence of certain bacterial species? Neutrophil defensins interact with negatively charged membranes in general [7]. Upon binding, defensins form multimers which are inserted in the membranes to form voltage dependent channels, which are suggested to contribute to the antimicrobial properties of defensins [22,48]. Although defensins did not enhance the adherence of E. coli [11], they are capable of killing E. coli in vitro [24]. This apparent discrepancy in the antimicrobial effects of defensins and the defensin-enhanced adherence for E. coli, suggests that different mechanisms are involved in the antimicrobial activity of defensins and the stimulatory effects on bacterial adherence. This is further supported by our observation that defensin-enhanced bacterial adherence occurred in culture medium under conditions that clearly restrict antibacterial activity of defensins due to the salt concentration in the culture medium.

Our adherence studies revealed that the defensins may stimulate the adherence through binding to the secondary fatty acid chains in the lipid A of H. influenzae and N. meningitidis LOS. Defensins bound to purified LOS from N. meningitidis and H. influenzae, whereas this purified LOS did not inhibit their defensin-enhanced adherence. This suggested that the conformation of the LOS is probably important in the defensin-enhanced adherence. If defensins are allowed to bind to the epithelial cells or bacteria separately, the enhancing effect of defensins on the bacterial adherence was much lower compared to the defensin-enhanced adherence if bacteria and epithelial cells are exposed to the defensins at the same time [10]. This suggested that defensins may form a bridge between the exposed lipid A part of the bacteria and the epithelial cells.

We have shown that the epithelial cells must be metabolic active to observe the defensin-enhanced bacterial adherence [10]. In this study, pre-exposure of the epithelial cells with defensins alone or the mixtures of defensin and purified LOS reduced the enhanced bacterial adherence phenomenon of the defensins. Since defensins can be internalized into the epithelial cells upon binding [28], it is likely that in our pre-exposure experiments the defensins and the HNP-LOS mixtures are
internalized, thus preventing them for enhancing the bacterial adherence. This is supported by the observation that the enhanced adherence phenomenon was restored after addition of fresh defensins together with the bacteria. Stimulation of the epithelial cells with purified LOS did not influence the defensin-enhanced adherence, suggesting that the stimulation with purified LOS did not e.g. increase a receptor of the epithelial cells by which defensins enhanced the bacterial adherence. However, the differences in configuration of purified LOS compared to LOS in the bacterial membrane could also be important in these experiments.

In conclusion, the data in this study showed that the structure of the lipid A part of LOS, including the secondary fatty acid chains, is important for the defensin-enhanced adherence of *N. meningitidis* and *H. influenzae*. The mechanism by which defensins and LOS interact with epithelial cells to promote bacterial adherence remains to be resolved.

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**References**


