Pathogenesis of Haemophilus influenzae. Respiratory infection in COPD patients
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Citation for published version (APA):

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CHAPTER 5

STABILIZATION OF IL-8 mRNA IS INVOLVED IN SYNERGISTICALLY INCREASED IL-8 RELEASE BY NCI-H292 EPITHELIAL CELLS UPON EXPOSURE TO HAEMOPHILUS INFLUENZAE AND NEUTROPHIL DEFENSINS

Annelies D. Gorter, Arjen van den Berg, Mieke Snoek, René Lutter, Pieter S. Hicmstra, Jacob Dankert, and Lock van Alphen

_in preparation_
Abstract

Neutrophils are involved in inflammatory process in patients with chronic bronchitis and with chronic obstructive pulmonary disease (COPD). Neutrophils contain a variety of antimicrobial peptides, such as neutrophil defensins, that kill microorganisms and may contribute to the airway inflammation by induction of the release of pro-inflammatory cytokines by epithelial cells. Bacteria such as *H. influenzae* colonize such inflamed airways and cause recurrent infections in COPD patients. In this study, we showed that *H. influenzae* and neutrophil defensins synergized in increasing the release of IL-6 and IL-8 by the airway epithelial cell line NCI-H292, by increasing the IL-6 and IL-8 release more than 3-fold (*p < 0.02*). The synergy in the IL-6 and IL-8 release was mainly observed using relatively low bacterial load (10^5 and 10^6 CFU/ml) and low concentrations of defensins (20 μg/ml) after 24 hr of exposure. IL-6 and IL-8 mRNA in NCI-H292 cells was also increased upon exposure to *H. influenzae* and defensins. Exposure to *H. influenzae* and neutrophil defensins compared to both stimuli alone had no detectable effect on the activity of the transcription factor NF-κB, and only a moderate effect on AP-1 and C/EBP. In the presence of *H. influenzae* and defensins the stability of the IL-6 and IL-8 mRNA was increased, suggesting that the synergism was mainly post-transcriptionally regulated. Since COPD patients, especially those with bacterial infections, have chronically inflamed airways, it is likely that the observed IL-6 and IL-8 release contributes to the inflammation in the COPD patients.
Introduction

The airways of patients with chronic bronchitis and chronic obstructive pulmonary disease (COPD) are chronically inflamed [3]. This inflammatory process may predispose to bacterial colonization and (recurrent) infection of the lower respiratory tract, with *Haemophilus influenzae* as one of the most frequently isolated pathogens [13]. *H. influenzae* persists in the airways of these patients, despite the abundant presence of bactericidal antibodies and neutrophils [13]. Neutrophils contain a variety of antimicrobial peptides, including neutrophil defensins. Defensins (also referred to as human neutrophil peptide [HNP]) are small cationic peptides with a broad spectrum of antimicrobial activities [9,10,18] and toxic activity for mammalian cells [17,26].

Previously, we have shown that defensins affect the interaction of *H. influenzae* with epithelial cells by enhancing bacterial adherence, a process for which metabolically active epithelial cells are required [11,12]. Since both *H. influenzae* [4,16,23] and neutrophil defensins [27,28] induce the release of the pro-inflammatory cytokines interleukin-6 (IL-6) and IL-8, we determined whether defensins affect the IL-6 and IL-8 release induced by *H. influenzae*. This is of particular interest because both IL-6 [15,22] and IL-8 [1] are key mediators in neutrophilic inflammation. The lung mucoepidermoid carcinoma derived epithelial cell line NCI-H292 was used as a model to analyze this interaction, since this cell line was previously used for the adherence studies with *H. influenzae* and defensins [11,12], as well as for unraveling regulatory mechanisms in the IL-6 and IL-8 responses [19,20].

In this study, we showed that in the presence of neutrophil defensins, *H. influenzae* synergistically increased IL-6 and IL-8 release by NCI-H292 epithelial cells. The increased IL-8 release was mainly due to increased stability of the IL-8 mRNA.

Materials and Methods

**Bacterial strains and culture**

*Haemophilus influenzae* strains A850048 and A950006 were cultured on chocolate agar plates (Oxoid, Haarlem, The Netherlands). These strains were used in previous studies [11,12,25] and were *in vitro* non-adherent and adherent, respectively, to NCI-H292 cells. The bacteria were resuspended in PBS at a density of OD$_{600\text{nm}}$ of 1 (~ $10^9$ CFU/ml). These
suspensions were used for the adherence assays. For the analysis of cytokine release, 10-fold dilutions in PBS were made.

Cell culture
NCI-H292 cells (ATCC CRL 1848) [2] were maintained in RPMI 1640 medium (In Vitrogen, Paisley, UK) supplemented with 10% fetal calf serum (FCS). The cells were maintained in the absence of antibiotics and grown in 25 or 75 cm² culture flasks (Falcon) at 37°C in a humidified atmosphere containing 5% CO₂. For the analysis of cytokine release, 3x10⁵ cells were plated and grown overnight in 500 μl in 24-wells plates. For the analysis of mRNA and that of transcriptional activity, 15x10⁵ cells were plated and grown overnight in 2.5 ml in 6-wells plates. For the adherence assays, 3x10⁵ cells were plated and grown overnight in 500 μl in 24-wells plates on round glass coverslips (12 mm diameter) (Menzelgläser, Braunschweitz, Germany). The culture medium was replaced by RPMI 1640 medium without FCS and incubated for another night prior to the addition of bacteria and/or defensins.

Isolation of neutrophil defensins
The neutrophil defensins were isolated as a mixture of HNP-1, HNP-2, and HNP-3 from an acetic acid extract obtained from human neutrophilic granules using gel filtration chromatography on Sephacryl S-200 HR (2.5x100 cm) (Pharmacia, Uppsala, Sweden) as described [27]. The purity of the defensins was assessed by SDS-PAGE and acid urea-PAGE and mass spectrometry [14,27].

Exposure of NCI-H292 cells to bacteria and defensins
Cells were exposed in serum-free RPMI medium to *H. influenzae* (10⁵ CFU/ml), and 20 μg/ml HNP1-3, unless mentioned otherwise. They were added in 1 ml final volume for both 24- and 6-wells plates. As a positive control, TNF-α (Sigma Chemical Co, St Louis, MO; 20 ng/ml in 24-well plates and 5 ng/ml in 6-wells plates) was used. Four hr after the initial exposure, 3 μg/ml chloramphenicol was added to all wells. This concentration of chloramphenicol caused bacterial growth inhibition, as determined by colony counts after 24 hr incubation and did not affect IL-6 and IL-8 release. In selected experiments, heat-inactivated bacteria (56°C for 30 min) and purified lipooligosaccharide (LOS) from *H. influenzae strain* 770235 (100 μg/ml) were used. To examine the effect of dexamethasone (dex) on IL-6 and IL-8 release, the cells were co-exposed to 1 μM dexamethasone (Sigma Chemical Co).

After the appropriate time of stimulation, the culture supernatants from the 24-wells plates were collected and stored at −20°C until analysis. The NCI-H292 cells from the 6-wells plates were lysed with TRIZol reagent (In Vitrogen) to isolate RNA according to the manufacturer’s protocol. Alternatively, after the exposure of the epithelial cells grown in 6-wells plates, nuclear extracts were prepared (as outlined below).

Determination of IL-6 and IL-8 proteins
The concentrations of IL-6 and IL-8 in culture supernatants were measured using ELISA kits from Sanquin (Amsterdam, The Netherlands) according to the manufacturer’s protocol.
RNA analysis

IL-6 and IL-8 mRNA were determined as described [19,20]. Briefly, RNA was dot spotted and hybridized with specific $^{32}$P-labeled probes for IL-6 or IL-8. The mRNA levels were quantified using a phosphorimager and variable loading was corrected by expressing mRNA levels relative to the housekeeping GAPDH mRNA. The stability of IL-6 and IL-8 mRNA was analyzed following co-incubation with actinomycin D (5 µg/ml) (Sigma Chemical Co) for 0, 40, and 80 min, after 8 and 24 hr of exposure to *H. influenzae* and defensins.

Isolation of nuclear extracts and electrophoretic mobility shift assay (EMSA)

Nuclear extracts were isolated as described [19,20]. Protein concentrations were measured using a protein assay kit (Bio-Rad, Hercules, CA). Two µg of the nuclear extracts were incubated with $^{32}$P-labeled oligonucleotides at 4°C for 1 hr as described [19,20] and separated on a 4% non-reducing poly-acrylamide gel at slowly increasing voltages (80-200V). The specificity of the bands was confirmed by supershifting using 1 µg of antibodies against C/EBP-β for C/EBP, p65 for NF-κB, and c-fos and c-jun for AP-1, (Santa Cruz biotechnology Inc., Santa Cruz, CA). The intensity of the bands were quantified using a phosphorimager. The following oligonucleotides were used in the EMSA: NF-κB, 5'-TTGCAAATCGTGGAATTTCCTCTGACATAA-3'; C/EBP, 5'-TTAAAGGACGTCACATTCACA TTGCACAATCTTAAATAA-3'; AP-1, 5'-TTAAGTGTGATGACTCAGGCACCA TTGCACAATCTTAAATAA-3'.

Bacterial adherence assay

The adherence assay was used as described [11,12,25] to determine whether adherence of the *H. influenzae* strains was affected by the presence of 1 µM dexamethasone. Briefly, the medium covering the cells grown on glass coverslips was replaced with RPMI 1640 medium without FCS. Bacteria and defensins were added in rapid succession at final concentrations of $10^6$ CFU/ml and 20 µg/ml, respectively. After 4 hr of incubation and subsequent washing, the cells were fixed in 4% paraformaldehyde and 1% glutaraldehyde and the number of adherent bacteria was counted using light microscopy [11,25].

Statistical analysis

The data were statistically analyzed by a student's t-test and differences were considered significant at p < 0.05.

Results

Synergistic effect of *H. influenzae* and defensins on IL-6 and IL-8 release by NCI-H292 cells

The effect of *H. influenzae* and neutrophil defensins on IL-6 and IL-8 release by NCI-H292 cells was determined. In initial experiments, cells were exposed to $10^6$ CFU/ml of the non-adherent *H. influenzae* strain A850048 or to 20 µg/ml
neutrophil defensins or to a combination of both. IL-6 release was not observed after 6 hr exposure to *H. influenzae* strain A850048 alone or defensins alone. However, exposure to the combination of *H. influenzae* strain A850048 and defensins resulted in a low release of IL-6 (table 1). The IL-8 release after 6 hr exposure was low for *H. influenzae* strain A850048 alone and defensins alone. The combination of *H. influenzae* strain A850048 and defensins resulted in a non-significant 2-fold increased IL-8 release (table 1). These results indicated that the IL-6 and IL-8 release may occur more rapidly in the presence of both *H. influenzae* and defensins. Exposure for 24 hr to either *H. influenzae* strain A850048 or defensins resulted in a limited release of IL-6 (table 1). The combination of *H. influenzae* and defensins synergistically increased the IL-6 release 3-fold compared to the IL-6 release by either *H. influenzae* or defensins (p < 0.04). Similarly, the IL-8 release by NCI-H292 cells was 3-fold enhanced upon exposure to the combination of *H. influenzae* A850048 and defensins compared to the release after exposure to *H. influenzae* alone and defensins alone (p < 0.001) (table 1). These results indicated that *H. influenzae* and neutrophil defensins synergistically enhanced IL-6 and IL-8 release by NCI-H292 cells. Exposure for 30 hr with *H. influenzae* strain A850048 alone resulted in increased IL-6 levels compared to that after 24 hr, diminishing the synergistic effect of defensins on the IL-6 release (table 1). For IL-8, the synergism was still observed after 30 hr of exposure to *H. influenzae* strain A850048 and defensins (p < 0.05) (table 1).

**Table 1.** Levels of IL-6 and IL-8 in cell culture supernatant of NCI-H292 cells after 6, 24, and 30 hr of exposure to *H. influenzae* (Hi) strain A850048 (10^6 CFU/ml), defensins (HNP1-3, 20 μg/ml), or a combination of both.

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*Data are expressed as the average ± SEM (pg/ml) for three independent experiments, each performed in triplicate.

* p < 0.05 compared to Hi A850048 alone, to defensins alone, and to the sum of both.
We next determined the IL-6 and IL-8 release following exposure to various CFU/ml of *H. influenzae* strain A850048 and various concentrations of defensins after 24 hr of exposure. At $10^5$ and $10^6$ CFU/ml of *H. influenzae*, defensins synergized in both IL-6 and IL-8 release (figure 1). A more than 5-fold increased IL-6 release was found for $10^5$ CFU/ml of *H. influenzae* and 10 or 20 μg/ml defensins ($p < 0.005$) compared to the exposure to either *H. influenzae* or defensins (figure 1A). For $10^6$ CFU/ml of *H. influenzae* and 2, 10 or 20 μg/ml neutrophil defensins, a more than 3-fold increase ($p < 0.02$) was observed compared to either *H. influenzae* or defensins (figure 1A). Similarly, IL-8 release increased more than 3-fold with $10^5$ and $10^6$ CFU/ml of bacteria and 10 or 20 μg/ml of neutrophil defensins ($p < 0.04$) (figure 1B). Higher concentration of *H. influenzae* ($10^7$ and $10^8$ CFU/ml) in combination with defensins did not result in a further increased IL-6 and IL-8 release (figure 1). These results indicated that the synergism of *H. influenzae* and defensins was most prominent at low amounts of bacteria.

![Graph A](image1)

![Graph B](image2)

**Figure 1.** The release of IL-6 (A) and IL-8 (B) by NCI-H292 cells after 24 hr exposure to various numbers of *H. influenzae* (Hi) strain A850048 and various concentrations of neutrophil defensins (HNP1-3). The results are expressed as the average and SEM of at least three independent experiments, each performed in triplicate. *, $p < 0.05$ compared to the IL-6 and IL-8 levels released after exposure to either *H. influenzae* or defensins as well as to their sum.
To determine whether this synergism was also observed for an adherent strain, the effects of exposure of the cells to *H. influenzae* strains A850048 and A950006 were compared. Exposure for 24 hr to both *H. influenzae* strains (10^6 CFU/ml) in

![Graph A](image)

![Graph B](image)

**Figure 2.** The release of IL-6 (A) and IL-8 (B) by NCI-H292 cells after 24 hr exposure to 10^6 CFU/ml *H. influenzae* strain A850048 or A950006 in the absence (-) and presence (+) of 20 μg/ml neutrophil defensins (HNP1-3). Viable or heat-inactivated (56°C for 30 min) *H. influenzae* strains were used. The results are expressed as the average and SEM of at least three independent experiments, each performed in triplicate. *, p < 0.05 compared to the IL-6 or IL-8 levels released after exposure to either *H. influenzae* or defensins as well as to their sum; **, p < 0.05 compared to the IL-6 or IL-8 levels released after exposure to either *H. influenzae* or defensins.
combination with defensins (20 µg/ml) resulted in a more than 3-fold increased IL-6 and IL-8 release compared to the IL-6 and IL-8 release upon exposure to either *H. influenzae* strain A850048, A950006 or defensins alone (p < 0.04) (figure 2). Exposure of heat-inactivated *H. influenzae* strain A850048 or strain A950006 to the NCI-H292 cells resulted in a slightly higher IL-6 release compared to the viable strains (figure 2A). This IL-6 release was 1.8-fold increased in the presence of defensins (not significantly). IL-8 release upon exposure to the combination of heat-inactivated *H. influenzae* strain A850048 and defensins was still significantly more than 2-fold increased (p < 0.05) compared to either heat-inactivated *H. influenzae* or defensins (figure 2B). This suggested that a heat-stable bacterial component is involved. Since lipooligosaccharide (LOS) from *H. influenzae* is a heat-stable component and LOS induces IL-6 and IL-8 release by epithelial cells [23], we studied whether purified LOS displayed a similar synergism with defensins on the IL-6 and IL-8 release as shown for defensins and *H. influenzae* bacteria. Exposure for 24 hr to 100 µg/ml purified LOS from *H. influenzae*, resulted in a release of 211 ± 33 pg/ml IL-6 and 322 ± 122 pg/ml IL-8 (n = 3). The combination of purified LOS and defensins (20 µg/ml) did not affect the release of these cytokines by the NCI-H292 cells compared to the release by LOS alone and defensins alone (IL-6: 99 ± 31 pg/ml and IL-8: 229 ± 77 pg/ml). Therefore, LOS did not mimic the synergistic effect of bacteria and defensins on the cytokine release by NCI-H292 cells.

Effect of *H. influenzae* and neutrophil defensins on IL-6 and IL-8 mRNA levels

To examine the mechanism by which *H. influenzae* and defensins synergized in the IL-6 and IL-8 release, we first determined IL-6 and IL-8 mRNA expression in time. There was no apparent increase in the IL-6 mRNA level after exposure to defensins (20 µg/ml). Upon exposure to *H. influenzae* alone (10⁶ CFU/ml), the level of IL-6 mRNA was slightly increased after 24 hr, but not yet after 8 hr (figure 3A). Exposure to *H. influenzae* (10⁶ CFU/ml) and defensins (20 µg/ml) increased IL-6 mRNA levels by 2.5-fold at 24 hr as compared to that with bacteria alone (non-significant). IL-8 mRNA levels were also increased after 24 hr exposure to *H. influenzae* (figure 3B), but only slightly further increased by *H. influenzae* and defensins (non-significant). Exposure to defensins did not increase IL-8 mRNA levels in NCI-H292 cells. These results indicated that the IL-6 and IL-8 mRNA levels were only increased after more than 8 hr exposure to *H. influenzae* and defensins.
Figure 3. IL-6 (A) and IL-8 (B) mRNA levels in NCI-H292 cells after exposure to *H. influenzae* strain A850048 (10^6 CFU/ml) and neutrophil defensins (HNP1-3, 20 μg/ml). The results are expressed as the fold increase compared to resting, non-exposed cells. The average and SEM of three independent experiments, each performed in duplicate or triplicate are shown.

Transcriptional activity of transcription factors NF-κB, C/EBP and AP-1
IL-6 gene transcription in TNF-α- and LPS-stimulated NCI-H292 cells is dependent on transcription factor CCAAT/enhancer binding protein (C/EBP) [19] and the production of IL-8 on nuclear factor κB (NF-κB) and activator protein-1 (AP-1) [20]. To determine whether the synergism between *H. influenzae* and defensins is related to
Figure 4A. The DNA-binding activities for NF-κB in nuclear extracts of NCI-H292 cells upon exposure to *H. influenzae* strain A850048 (10^6 CFU/ml) and neutrophil defensins (HNP1-3, 20μg/ml). The upper part of the figure shows a representative electrophoretic mobility shift assay (EMSA) gel and the lower part the quantification the gels from three independent experiments. The arrows indicate the specific bands. The supershift (ss) as determined using specific antibody against the p65 subunit of NF-κB for the samples after 24 hr exposure to the combination of *H. influenzae* and defensins is indicated on the right part of the gel.
Figure 4B. The DNA-binding activities for C/EBP in nuclear extracts of NCI-H292 cells upon exposure to *H. influenzae* strain A850048 (10^6 CFU/ml) and neutrophil defensins (HNP1-3, 20μg/ml). The upper part of the figure shows a representative electrophoretic mobility shift assay (EMSA) gel and the lower part the quantification the gels from three independent experiments. The arrow indicate the specific bands. The supershift (ss) as determined using specific antibody against the C/EBP-β subunit of C/EBP for the samples after 24 hr exposure to the combination of *H. influenzae* and defensins is indicated on the right part of the gel.
Figure 4C. The DNA-binding activities for AP-1 in nuclear extracts of NCI-H292 cells upon exposure to *H. influenzae* strain A850048 (10⁶ CFU/ml) and neutrophil defensins (HNP1-3, 20μg/ml). The upper part of the figure shows a representative electrophoretic mobility shift assay (EMSA) gel and the lower part the quantification the gels from three independent experiments. The arrow indicate the specific bands. The supershift (ss) as determined using specific antibody against respectively the fos and jun subunit of AP-1 for the samples after 24 hr exposure to the combination of *H. influenzae* and defensins is indicated on the right part of the gel.
an increased activation (i.e. increased nuclear localization and specific binding capacity) of the relevant transcription factors, we performed electrophoretic mobility shift assays (EMSA) with nuclear extracts. NCI-H292 cells were exposed for 1, 2, 4 and 24 hr to either *H. influenzae* strain A850048 (10^6 CFU/ml) or defensins (20 μg/ml) or a combination of both, after which nuclear extracts were obtained. The specific bands were identified by a specific antibody-induced shift, as shown in the right lanes of figure 4. For all conditions, no clear activation of the transcription factors was observed. Only after 24 hr of exposure to defensins or the combination *H. influenzae* and defensins, a slightly increased activation of C/EBP and AP-1 was observed. These results indicated that the synergism by *H. influenzae* and defensins in IL-6 and IL-8 release was not mainly due to an increased transcriptional activity.

![Graphs showing stability of IL-6 and IL-8 mRNA](image)

**Figure 5.** Stability of IL-6 (A,B) and IL-8 (C,D) mRNA after 8 hr (A,C) and 24 hr (B,D) of exposure to *H. influenzae* strain A850048 (10^6 CFU/ml) and neutrophil defensins (HNP1-3, 20 μg/ml). The results are expressed as the average and SEM of five different experiments, each performed in duplicate or triplicate. *, p < 0.05 compared to the rate of mRNA degradation upon exposure to either *H. influenzae* or defensins alone; **, p < 0.01 compared to the rate of mRNA degradation upon exposure to *H. influenzae* alone.
Stability of mRNA for IL-6 and IL-8

Previous studies have indicated that mRNA degradation plays an important role in regulating IL-6 and IL-8 mRNA expression [7]. To determine whether the synergism in IL-6 and IL-8 mRNA release was dependent on a prolonged half-life for IL-6 and IL-8 mRNA, mRNA half-lives were assessed after 8 and 24 hr of exposure to *H. influenzae* strain A850048 (10^6 CFU/ml) and defensins (20 μg/ml). For IL-6 mRNA at 8 and 24 hr of exposure and for IL-8 mRNA at 8 hr of exposure, no differences in mRNA stability were observed for the different stimuli (figure 5). IL-8 mRNA degradation in cells after 24 hr of exposure to *H. influenzae* and defensins, however, revealed mRNA stabilization (figure 5). This stabilization was significant at 40 min after the addition of actinomycin D as opposed to that in cells exposed to *H. influenzae* alone or defensins alone (p < 0.04) and significant at 80 min after the addition of actinomycin D compared to *H. influenzae* alone (p < 0.008). Defensins alone tended to increase the half-life of IL-8 mRNA, but this was not significant compared to the exposure to *H. influenzae* alone. These results indicated that the synergism between *H. influenzae* and defensins in IL-8 release was, at least in part, due to an increased stability of IL-8 mRNA.

Effect of dexamethasone on the cytokine release

Corticosteroids exert anti-inflammatory effects by modulating the expression of many inflammatory mediators such as that of IL-6 and IL-8 [5]. In this study, the effect of the corticosteroid dexamethasone on the synergistically increased release of IL-6 and IL-8 was investigated.

Table 2. Effect of dexamethasone (dex, 1 μM) on the adherence of *H. influenzae* to NCI-H292 cells in the presence and absence of neutrophil defensins (HNP1-3, 20 μg/ml)

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<tr>
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<tr>
<td>A950006</td>
<td>23</td>
</tr>
<tr>
<td>A950006 + HNP1-3</td>
<td>126</td>
</tr>
</tbody>
</table>

^a adherence (bacteria / epithelial cell) as determined by light microscopy of two different experiments performed in duplicate
IL-8 by NCI-H292 cells induced by *H. influenzae* strain A850048 and A950006 (10^6 CFU/ml) and defensins (20 μg/ml) was assessed. The adherence of both *H. influenzae* strains as well as the defensin-enhanced adherence of these strains were not affected by 1 μM dexamethasone (table 2). Dexamethasone (1 μM) markedly inhibited IL-6 release induced by *H. influenzae* strain A850048 as well as strain A950006 and by defensins alone (figure 6A). In contrast, dexamethasone did not significantly inhibit the IL-6 release after exposure with *H. influenzae* strain A850048.

**Figure 6.** Effect of dexamethasone on the IL-6 (A) and IL-8 (B) release by NCI-H292 cells after 24 hr of exposure to *H. influenzae* strain A850048 (10^6 CFU/ml) and neutrophil defensins (HNP1-3, 20 μg/ml). The results are expressed as the average and SEM of four independent experiments, each performed in triplicate. *, p < 0.05 compared to the control in the absence of dexamethasone.
and defensins. IL-6 release after exposure to *H. influenzae* strain A950006 was significantly inhibited (p < 0.04). IL-8 release upon exposure to *H. influenzae* strain A850048 as well as strain A950006 and defensins was significantly inhibited by dexamethasone. These results indicated that the synergistic increased IL-6 and IL-8 release was sensitive to dexamethasone.

**Discussion**

The results from the present study showed that the combination of *H. influenzae* and neutrophil defensins synergistically increased the production of the pro-inflammatory cytokines IL-6 and IL-8. The underlying mechanism involved at least stabilization of IL-8 mRNA. IL-6 and IL-8 release induced by *H. influenzae* and defensins was sensitive to downregulation by the corticosteroid dexamethasone.

The increased release of IL-6 and IL-8 was apparent already 6 hr after exposure to defensins together with a relatively low bacterial load of $10^6$ CFU/ml. At 24 hr of exposure, the synergistic effect was predominantly seen with low bacterial loads ($10^5$ and $10^6$ CFU/ml). These findings may indicate that in the respiratory tract of COPD patients, the inflammatory processes are affected by the combination of *H. influenzae* and defensins by lowering the threshold numbers of bacteria required to induce a rapid inflammatory response. Thereby, the local milieu can respond quickly to relatively low bacterial loads. It is of interest to note that in the presence of high numbers of bacteria, defensins did not further increase IL-6 and IL-8 release. It may be envisaged that this mechanisms limits extensive inflammation and subsequent tissue damage.

The synergistic increased IL-6 and IL-8 release with defensins was observed with viable and heat-inactivated *H. influenzae*, suggesting that the relevant bacterial component was heat-stable. Although LOS of *H. influenzae* is heat-stable and has been shown to induce IL-6 and IL-8 release by epithelial cells [23], purified LOS combined with defensins did not synergize in the release of IL-6 and IL-8. Purification of LOS may have affected its conformational state [24], which might explain the loss of synergism and thus does not exclude LOS as a relevant component in the synergized IL-6 and IL-8 release. Alternatively, another heat-stable bacterial component such as peptidoglycan may be involved in the synergism between defensins and bacteria in IL-6 and IL-8 release.
The induction of IL-6 and IL-8 protein by *H. influenzae* and defensins is slow as compared to previous studies with this cell line when exposed to TNF-α [19,20]. This is also reflected at the mRNA level, where no clear peaks were observed, but instead a rather gradual increase. The most pronounced effect was seen when *H. influenzae* and defensins were combined, resulting in further increased IL-6 and IL-8 mRNA levels after 24 hr of exposure. Also the transcriptional activation compared to the control treatment with culture medium only, as assessed by EMSA experiments, was hardly observed for *H. influenzae* and defensins as well as the combination of both. This is in line with the findings for mRNA expression. The most significant effect was on IL-8 mRNA degradation, which is an important mechanism to modulate mRNA expression. The combination of *H. influenzae* and defensins as well as defensins alone increased the stability of IL-8 mRNA after 24 hr of exposure. Previously, it was shown that neutrophil defensins did not affect the stability of IL-8 mRNA in A549 cells after 6 hr of exposure [27]. This is in line with our results, since 8 hr exposure to defensins did not affect the half life of IL-8 mRNA in NCI-H292 cells.

Glucocorticoids, such as dexamethasone, reduce the expression of the inflammatory cytokines by various means, such as by preventing translocation of transcription factors to the nucleus and/or by inducing IkBα, the inhibitor of NF-κB [5,8,21]. Previously, it was shown that dexamethasone also promoted IL-6 and IL-8 mRNA degradation [6]. Since exposure of NCI-H292 cells to *H. influenzae* and defensins resulted in a reduced IL-8 mRNA degradation and thus stability of the IL-8 mRNA, it may be envisaged that this increased stability will counteract the effect of dexamethasone. Pre-treatment of the NCI-H292 cells for 16 hr with dexamethasone resulted in a further reduced IL-6 and IL-8 release by NCI-H292 cells exposed to the combination of *H. influenzae* and defensins (data not shown). This is in line with the previously described observation that after pre-treatment, dexamethasone inhibited the neutrophil defensin-induced cytokine release by airway epithelial cells [28].

In summary, the results from this study show that the release of pro-inflammatory cytokines IL-6 and IL-8 is synergistically increased upon exposure to *H. influenzae* and neutrophil defensins. The synergized IL-6 and IL-8 release was most evident at low bacterial concentrations. The mechanism by which the IL-8 release was synergistically increased involved at least mRNA degradation. Since IL-8 is a chemoattractant for neutrophils [1], the enhanced cytokine release may result in the influx of neutrophils and consequently the release of neutrophil products, including
neutrophil defensins, subsequently resulting in the defensin-enhanced adherence and increased cytokine release. Therefore, the synergistic increased cytokine release by *H. influenzae* and defensins at low bacterial loads likely contributes to the chronic inflammation and infection as observed for the COPD patients.

Acknowledgments
The authors thank Sylvia Mannesse-Lazeroms (Department of Pulmonology, Leiden University Medical Center, Leiden, The Netherlands) for the isolation of HNP1-3. This work was supported by the Netherlands Asthma Foundation (grant numbers 95.36 and 99.27).

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