

Persisting *Haemophilus influenzae* strains induce lower levels of interleukin-6 and interleukin-8 in H292 lung epithelial cells than nonpersisting strains

P. Bresser^{*+,} L. van Alphen^{**,} F.J.M. Habets^{*+,} A.A.M. Hart^{++,} J. Dankert^{**,} H.M. Jansen^{*}, R. Lutter^{*+}

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ABSTRACT: Nonencapsulated *Haemophilus influenzae* strains isolated from patients with chronic bronchitis can be divided into those that persist in the lower respiratory tract and those that do not. We tested the hypothesis that persisting and nonpersisting strains differ in the extent to which they activate epithelial cells to produce two potent inflammatory mediators, interleukin (IL)-6 and IL-8.

A suspension of 10^7 and 10^8 colony forming units (cfu)·mL⁻¹ of *H. influenzae*, persisting and nonpersisting, induced a dose- and time-dependent production of IL-6 and IL-8 by the human pulmonary mucoepidermoid carcinoma-derived cell line H292, but levels of IL-6 were lower after exposure to persisting *H. influenzae* ($p < 0.05$). IL-8 production showed a similar trend ($p < 0.02$; analysis of variance). *H. influenzae* bacteria that adhered to H292 cells were equally distributed over persisting and nonpersisting isolates and induced IL-6 and IL-8 levels similar to their nonadhering counterparts. The difference between persisting and nonpersisting *H. influenzae* was not due to cytotoxic, antimetabolic or antiproliferative effects on H292 cells. Furthermore, pre-exposure of cells to persisting and nonpersisting isolates did not block subsequent IL-1 β -induced IL-6 production.

We conclude that persisting clinical isolates induce less interleukin-6 and interleukin-8 in H292 cells than nonpersisting isolates, probably because they excrete lower amounts of a stimulus of H292 cells. The stimulus is heat stable, hydrophilic and nonproteinous and probably not lipopolysaccharide alone. These findings support the suggestion that some strains of *Haemophilus influenzae* that persist in the airways of patients, may do so because they induce only a weak inflammatory response.

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*Dept of Pulmonology, +Clinical and Laboratory Immunology Unit, **Dept of Medical Microbiology, and ++Dept of Clinical Epidemiology and Biostatistics, Academic Medical Center, University of Amsterdam, PO Box 22700, 1100 DE Amsterdam, The Netherlands

Correspondence: R. Lutter
Dept of Pulmonology F4-208
Academic Medical Center
Meibergdreef 9
1105 AZ Amsterdam
The Netherlands

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Nonencapsulated *Haemophilus influenzae* are, either intermittently or continuously, isolated from sputum samples of a majority of patients with chronic obstructive pulmonary disease (COPD) [1–5]. Analysis of bacterial isolates from patients with COPD, by major outer membrane protein (MOMP) subtyping [6] and deoxyribonucleic acid (DNA) fingerprinting after restriction endonuclease digestion [7], revealed that some *H. influenzae* strains persisted for many months (referred to as persisting strains), whereas other strains were isolated only once (referred to as nonpersisting strains) [5]. The basis for this difference between strains is unknown, but is likely to entail both bacterial and host factors.

The interplay between *H. influenzae* and the airway mucosa in inflammatory airways disease is complex and involves several cell types. Bacterial staining of sections of airways from patients infected with *H. influenzae* showed accumulation of bacteria between and underneath epithelial cells [8, 9]. In addition, *in vitro* studies indicated that *H. influenzae* adheres to epithelial cells

via specific interactions [10, 11]. These findings have suggested a role for airway epithelial cells in the pathophysiology of *H. influenzae* colonization. Airway epithelial cells, both *in vitro* [12–14] and *in vivo* [15, 16], respond to adequate stimulation by the expression of inflammatory mediators, such as interleukin (IL)-6 and IL-8. In fact, it was shown recently that *H. influenzae*-derived lipopolysaccharide, at high concentrations, stimulated airway epithelial cells to release IL-6 and IL-8 [14]. IL-6 is a pleiotropic mediator implicated, among others, in immunoglobulin synthesis [17], and IL-8 mediates the recruitment of neutrophilic granulocytes [18–20]. Both immunoglobulins and neutrophilic granulocytes contribute to the killing of *H. influenzae* [21].

We hypothesized that persisting and nonpersisting *H. influenzae* strains differ in their ability to exert an effect on their local environment. In this hypothesis, we expected persisting strains to be less offensive than nonpersisting strains. Persisting and nonpersisting *H.*

influenzae strains isolated from chronic bronchitis patients were compared for the induction of IL-6 and IL-8 production by a human lung epithelial cell line. H292 cells were chosen as they originate from a continuous cell line derived from a pulmonary mucoepidermoid carcinoma. H292 cells show characteristics of differentiation as they express *muc-2*, indicative of its mucoid character [22], and tight junctions and desmosomes [23], which suggest a polarized phenotype. Furthermore, *H. influenzae* adheres to H292 cells and subsequently transmigrates across H292 monolayers [23].

We also assessed whether any difference in the induction of IL-6 and IL-8 could be related to adherence of *H. influenzae* to H292 cells [23], to cytotoxic properties displayed by *H. influenzae* [11], and to the release of a stimulus. In addition, the induction of IL-6 and IL-8 by *H. influenzae* isolated from patients having signs of lower respiratory tract infection and strains isolated from the throat of healthy carriers was compared.

Materials and methods

Epithelial cell culture

H292 cells (American Type Culture Collection CRL 1848, Rockville, MA, USA), were maintained in RPMI-1640 medium (Gibco BRL, Paisley, UK) with 0.5 mM glutamic acid (Merck, Darmstadt, Germany) and 10% heat-inactivated foetal calf serum (Gibco BRL), at 37°C in a humidified atmosphere of air with 5% CO₂. For experiments, confluent H292 cells were exposed in 2 mL of culture medium in 12-well plates (Costar, Badhoevedorp, The Netherlands).

Bacteria

Nonencapsulated *H. influenzae* (n=15) were isolated from sputum samples of patients with chronic bronchitis and characterized as described previously [5–7]. *H. influenzae* strains were considered persisting (n=9) when they were cultured for at least 6 months from various sputum samples from a patient. Nonpersisting *H. influenzae* strains (n=6) were cultured only on one occasion during a monthly follow-up for at least 6 months. Nonencapsulated carrier strains (n=9) were obtained from throat swabs of healthy individuals. All clinical isolates were used within two passages of the original isolation.

Incubation of epithelial cells with *H. influenzae*

Bacteria were grown overnight on chocolate agar plates at 37°C in a CO₂ incubator and were subsequently suspended in complete culture medium to a concentration of 10⁹ colony forming units (cfu)·mL⁻¹ as determined from the optical density of the suspension at 530 nm, and colony counting of serial dilutions as a reference. H292 cells were exposed to either 10⁶, 10⁷, 10⁸ or 10⁹ cfu·mL⁻¹, in culture medium supplemented with 25 mM Hepes. At various time intervals culture medium was collected and stored at -20°C until determination of IL-6

and IL-8. Chloramphenicol (CAM) was added to the medium at 3 µg·mL⁻¹. This concentration was bacteriostatic since bacterial growth was inhibited without killing them over a period of 24 h, as assessed by cfu countings with serial dilutions of bacterial suspensions. Interleukin-1β (IL-1β) (Genzyme, Cambridge, MA, USA) was used at 100 U·mL⁻¹ to study the effect of CAM on IL-6 and IL-8 production by the cells. To assess epithelial cell damage after 20 and 40 h of exposure to bacteria, the release of cytoplasmic lactate dehydrogenase (LDH) was measured as described previously [24]. The release of LDH is expressed as a percentage (mean±SEM of triplicate experiments) of total LDH as determined after cell lysis with 0.1% (v/v) Triton X-100 (Merck). In some experiments, formation of formazan from the tetrazolium salt 3-(4, 5 dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Sigma, St. Louis, MO, USA) was quantified as a measure of cell viability and cell proliferation [25]. The formazan levels were expressed as percentage of formazan formation by cells not exposed to bacteria (mean±SEM of triplicate experiments). To study the capacity of *H. influenzae* to inhibit IL-6 production by H292 cells, H292 cells were exposed for 24 h to bacteria, then washed three times with phosphate-buffered saline (PBS) to remove bacteria, incubated again for 4 h with culture medium without exogenous stimulus, and subsequently exposed to IL-1β (100 U·mL⁻¹) in fresh medium. Culture medium was collected 15 h later to determine IL-6.

Bacterial adherence to H292 cells

Adherence of *H. influenzae* to H292 cells was determined as described [23]. Bacteria were considered adherent when at least 50 bacteria·cell⁻¹ could be counted. Bacteria were considered nonadherent and intermediate when less than 5 bacteria or 5–50 bacteria·cell⁻¹ could be counted, respectively. The influence of CAM present in the cell culture medium on adherence was studied by comparing the adherence of the CAM-sensitive, adhering (nonpersisting) isolate A950009 with the adherence of the adhering but CAM-insensitive laboratory strain QC 2174 (maximal inhibitory concentration (MIC): 8 µg·mL⁻¹).

Characterization of the bacterial stimulus

Bacteria were grown in brain heart infusion broth (BHI) supplemented with hemin and nicotinamide adenine dinucleotide (10 mg·L⁻¹ each), with shaking (120 revolutions per minute (rpm)) at 37°C in a humidified atmosphere of air with 5% CO₂. Samples were taken at 6 h, after which the medium was separated from bacteria by centrifugation at 5,000 ×g for 15 min, and subsequently filtered through a filter with a 0.2 µm pore size (Schleicher and Schuell, Dassel, Germany). The culture filtrate was dialysed overnight against PBS. To denature protein, the filtrate was boiled for 5 min or exposed to 5% trichloroacetic acid (TCA; Merck) for 5 min at 0°C followed by centrifugation. The resulting acid supernatant was dialysed overnight against PBS before further analysis. Lipopolysaccharide was removed

by binding to polymyxin B immobilized to agarose (Boehringer Mannheim, Mannheim, Germany) for 18 h at room temperature followed by centrifugation to pellet the agarose beads. Lipids were removed by extraction with a mixture of chloroform and diethylether (1:1), followed by centrifugation to separate the aqueous phase from the lipid phase. Subsequently, the aqueous phase was exposed to a flow of nitrogen to remove traces of ether and chloroform.

Determination of pro-inflammatory mediators

IL-6 and IL-8 were determined by enzyme-linked immunosorbent assay (ELISA), essentially according to HELLE *et al.* [26] and to HACK *et al.* [27], respectively.

Statistical analysis

The induction of IL-6 and IL-8 production by each strain was determined in three to six independent experiments performed in duplicate. The IL-6 and IL-8 levels were expressed as the mean±SEM. The nonparametric Mann-Whitney U-test (MWU-test) and the Wilcoxon signed-ranks test (WSR-test) were used for comparison between different groups and different experimental conditions, as indicated in the text. The three groups of isolates were compared using the Kruskal-Wallis test. Bonferroni's correction for the risk of mass significance with multiple comparisons between the three groups was performed.

Analysis of variance (ANOVA) [28] was used to account for experimental differences between days in the induction of IL-6 and IL-8 production by persisting, nonpersisting and throat isolates. The day and the type of strain were used as fixed factors, and the individual strain as random factor. The IL-6 and IL-8 data were logarithmically transformed before analysis. Restricted maximum likelihood was used to fit the data. The p-values were calculated from the Wald test. In pairwise comparisons between types, a p-value adjusted for multiple comparisons was calculated according to the Hommel procedure [29]. Residual analyses were performed to check some assumptions (normality of between and within strain residuals and constancy of between and within SD) as well as to identify outliers. The statistical packages BMDP386 and BMDP-PC90 (BMDP Statistical Software, Inc., Los Angeles, CA, USA) were used for the calculations.

The Spearman rank correlation test was used to assess the correlation between the induction of IL-6 and IL-8 by *H. influenzae*.

A p-value of less than 0.05 was considered significant.

Results

Effect of chloramphenicol on IL-6 and IL-8 production in H292 cells

In the absence of bacteria, H292 cells spontaneously released 23±3 pg·mL⁻¹ of IL-6 (n=6; duplicate experiments) and 161±22 pg·mL⁻¹ of IL-8 (n=3; duplicate experiments) over 24 h. To be able to determine the dose-dependent induction of IL-6 and IL-8 by bacterial strains, the H292 culture medium was supplemented with 3 µg·mL⁻¹ chloramphenicol (CAM). In the absence and presence of CAM, H292 cells stimulated for 7 h with IL-1β (n=6) released 394±26 pg·mL⁻¹ and 396±35 pg·mL⁻¹ of IL-6, respectively, and 1609±165 pg·mL⁻¹

Table 1. – Interleukin (IL)-6 and IL-8 production by H292 cells induced by 10⁷ colony forming units (cfu)·mL⁻¹ of various *Haemophilus influenzae* isolates in the absence or presence of chloramphenicol (CAM) in the culture medium

	IL-6 pg·mL ⁻¹	IL-8 pg·mL ⁻¹
Without CAM	100 (30–1000)	2015 (860–7615)
With CAM	200 (40–450)	1758 (710–2640)

Values are presented as median, and range in parenthesis. All experiments were performed in duplicate. For IL-6 there were 12 isolates (four persisting, four nonpersisting and four throat isolates) (Wilcoxon Sign Rank (WSR)-test: p=0.25). For IL-8 there were four throat isolates (WSR-test: p=0.64).

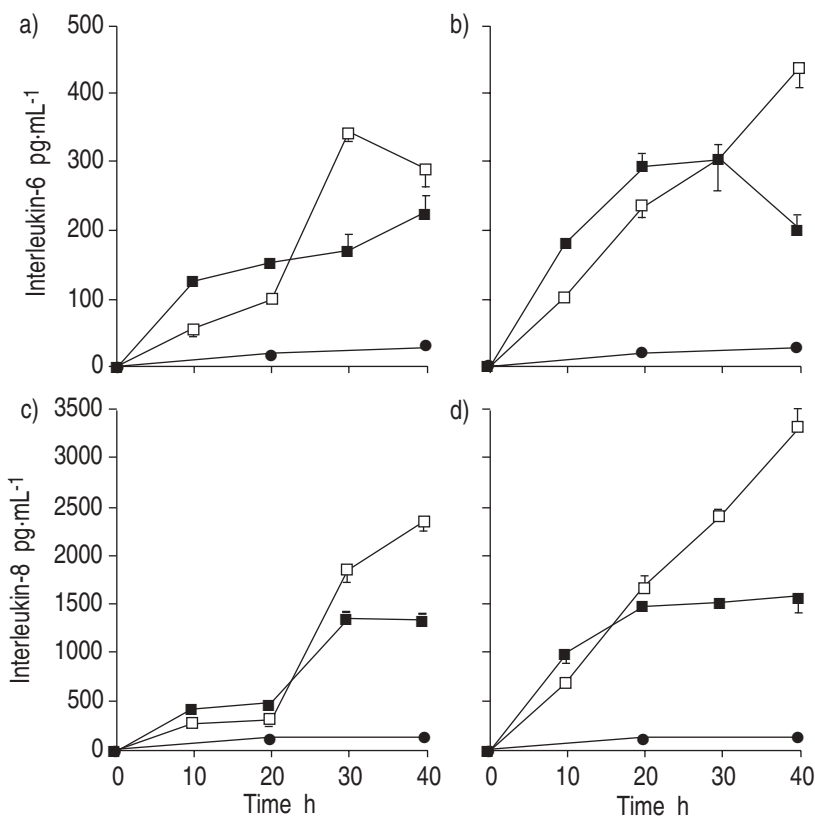


Fig. 1. – Time-dependent production of interleukin (IL)-6 by H292 cells exposed to *Haemophilus influenzae*: a) persisting isolate A950004; and b) nonpersisting isolate A950005; and production of IL-8 by: c) isolate A950004; and d) isolate A950005. Confluent H292 cells were exposed to 10⁷ colony forming units (cfu)·mL⁻¹ (□), 10⁸ cfu·mL⁻¹ (■) or no bacteria (●). Each point represents the mean±SD of triplicate experiments.

and 1703 ± 160 pg·mL⁻¹ of IL-8, respectively. Furthermore, the amounts of IL-6 and IL-8 produced after 24 h exposure of H292 cells to 10^7 cfu·mL⁻¹ in the presence of CAM were similar to those in the absence of CAM (table 1).

Dose-dependent induction of IL-6 and IL-8

H292 cells were exposed to 10^6 , 10^7 , 10^8 or 10^9 cfu·mL⁻¹ of 12 different *H. influenzae* strains for 24 h in the presence of CAM. Exposure of H292 cells to 10^6 cfu·mL⁻¹ did not, or only to a small extent, result in the production of IL-6 and IL-8. Exposure of cells to 10^9 cfu·mL⁻¹ resulted in the production of 100 pg·mL⁻¹ of IL-6 at the most. However, in the presence of 10^9 cfu·mL⁻¹ the culture medium acidified rapidly and the epithelial cells detached from the culture plates within 24 h. After exposure to 10^7 and 10^8 cfu·mL⁻¹ of *H. influenzae*, both IL-6 and IL-8 were produced and increased during at least 20 h. Figure 1 shows time courses of IL-6 and IL-8 production by H292 cells upon exposure to 10^7 and 10^8 cfu·mL⁻¹ of strains A950004 (persisting) and A950005 (nonpersisting), which are representative of the 12 isolates tested. It is of interest to note that levels of IL-6 and IL-8 induced by 10^8 cfu·mL⁻¹ of both strains during the first 20 h of exposure are usually higher than those induced by 10^7 cfu·mL⁻¹. Epithelial cells exposed to 10^7 or 10^8 cfu·mL⁻¹ during 20 h remained, like unexposed cells, apparently normal as assessed by light microscopy, and the pH of the culture medium remained between 7 and 8. After 40 h of exposure to 10^8 cfu·mL⁻¹, but not with 10^7 cfu·mL⁻¹, the culture medium acidified. In further experiments, IL-6 and IL-8 were determined in samples taken after 20–24 h of exposure to 10^7 or 10^8 cfu·mL⁻¹.

Comparison of IL-6 and IL-8 levels induced by persisting and nonpersisting *H. influenzae*

The induction of IL-6 and IL-8 by the various bronchitis isolates, *i.e.* nine persisting and six nonpersisting strains, was compared with nine throat isolates from healthy individuals. The data for IL-6 production are depicted in figure 2. Some strains evoked maximal IL-6 production at 10^7 cfu·mL⁻¹, others at 10^8 cfu·mL⁻¹ and some induced similar levels at 10^7 and 10^8 cfu·mL⁻¹, irrespective of whether persisting, nonpersisting or throat isolates were exposed to the H292 cells. Statistical analysis of the IL-6 levels induced by exposure to *H. influenzae* showed that persisting strains were relatively weak inducers of IL-6 production compared to nonpersisting strains, both evaluated for 10^7 cfu·mL⁻¹ (MWU-test: $p < 0.05$) and for the number of cfu·mL⁻¹ causing maximal production of IL-6 ($p < 0.05$). The levels of IL-6 induced by throat isolates at 10^7 cfu·mL⁻¹ and the maximal production did not differ significantly from those induced by persisting and nonpersisting isolates from bronchitis patients, although they tended to be higher than persisting isolates.

The data for IL-8 production are depicted in figure 3. The levels of IL-8 induced by 10^8 cfu·mL⁻¹ of all strains (median 2059

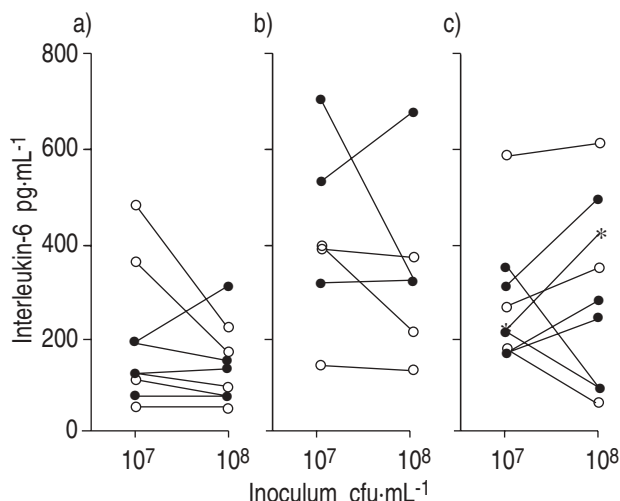


Fig. 2. – Production of interleukin (IL)-6 after 24 h of exposure to *Haemophilus influenzae* as a function of inoculum: a) nine persisting strains; b) six nonpersisting strains; and c) nine throat isolates. Means obtained for individual strains of 3–6 separate experiments in duplicate are shown. ●: adhering bacteria; ○: nonadhering bacteria; *: adherence not determined. cfu: colony forming units

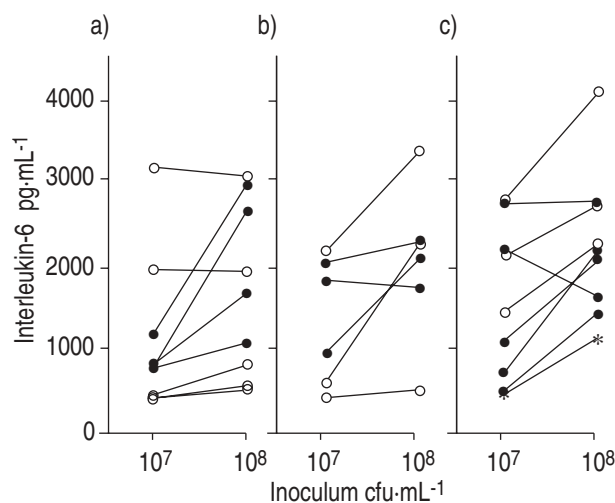


Fig. 3. – Production of interleukin (IL)-8 after 24 h of exposure to *Haemophilus influenzae* as a function of inoculum: a) nine persisting strains; b) six nonpersisting strains; and c) nine throat isolates. Means obtained for individual strains of 3–6 separate experiments in duplicate are shown. ●: adhering bacteria; ○: nonadhering bacteria; *: adherence not determined.

Table 2. – Maximal interleukin 6 (IL-6) production by H292 cells in the presence of 10^7 or 10^8 colony forming units (cfu)·mL⁻¹ of nine persisting, six nonpersisting and nine throat isolates of *Haemophilus influenzae*, adjusted for differences between days with analysis of variance after logarithmic transformation of the data

<i>H. influenzae</i>	IL-6 production*	SEM of difference with strain		p-value ⁺	
		Non-persisting	Throat	Unadjusted	Hommel adjusted
Persisting	4.89 ± 0.19	0.32	0.27	-	-
Nonpersisting	5.65 ± 0.26		0.33	0.017	0.017
Throat	5.73 ± 0.19			0.0015	0.0030

*: values presented as the mean \pm SEM of the natural logarithm of the values in pg·mL⁻¹; +: comparison with persisting strain.

pg·mL⁻¹, range 495–4,049 pg·mL⁻¹) were significantly higher compared to those induced by 10⁷ cfu·mL⁻¹ of all strains (median 1,011, range: 404–3,145 pg·mL⁻¹) (WSR-test: p<0.001). Analysis of the IL-8 levels induced by the three groups of isolates revealed only a slight tendency for persisting strains to induce lower levels than nonpersisting strains without reaching statistical significance.

The differences in the maximal induction of IL-6 between the three groups of strains was analysed by ANOVA and were found to be highly significant (table 2, p<0.005). Both throat isolates and nonpersisting bronchitis isolates induced significantly higher levels of IL-6 compared to the persisting bronchitis isolates. Essentially the same results were obtained in the analysis of persisting, nonpersisting and throat isolates for the induction of IL-6 with 10⁷ cfu·mL⁻¹. A similar analysis was also carried out for IL-8 (table 3). The maximal IL-8 production differed significantly between the three groups (p<0.02). After Hommel adjustment, the differences between nonpersisting and persisting bronchitis isolates and between throat isolates and persisting isolates were both statistically significant (table 3). The suboptimal levels of IL-8 induced by 10⁷ cfu·mL⁻¹ were similar for the three groups, but tended to be higher for nonpersisting than persisting isolates (Hommel adjusted: p=0.13).

The mean levels of IL-6 and IL-8 induced by 10⁷ cfu·mL⁻¹ of all 24 isolates, irrespective of their source or persistency, were significantly correlated (Spearman rho 0.62, p<0.002; data not shown). In contrast, no significant correlation was present if cells were exposed to 10⁸ cfu·mL⁻¹ of these isolates (rho 0.38, p=0.07; data not shown).

Effect of bacteria on viability and proliferation of H292 cells

H292 cells were incubated for 20 and 40 h with 10⁷ and 10⁸ cfu·mL⁻¹ of five *H. influenzae* strains, inducing different levels of IL-6 and IL-8. Table 4 summarizes the characteristics of two persisting, two nonpersisting and one throat isolate, with respect to the induction of IL-6 and IL-8. At 20 h, both for 10⁷ and 10⁸ cfu·mL⁻¹, the LDH release induced by either strain was less than 4% of total LDH. After 40 h of incubation with 10⁷ cfu·mL⁻¹, all strains induced 4.5±4.2% LDH release and for 10⁸ cfu·mL⁻¹ the release was 7.6±3.6%. At 20 h, formazan formation was 61.2±3.4% for 10⁷ cfu·mL⁻¹ and 50.3±4.6% for 10⁸ cfu·mL⁻¹ (WSR- test: p=0.06) of control values. At 40 h, formazan formation was 79.2±11.5% and 52.0±6.8% for 10⁷ and 10⁸ cfu·mL⁻¹, respectively, (WSR-test: p=0.13). The presence of CAM did not influence the formazan formation in H292 cells not exposed to bacteria. Differences between strains in LDH release and formazan formation were not observed, whilst induction of IL-6 and IL-8 differed between the strains (table 4).

Table 3. – Maximal interleukin 8 (IL-8) production by H292 cells in the presence of 10⁷ or 10⁸ colony forming units (cfu)·mL⁻¹ of nine persisting, six nonpersisting and nine throat isolates of *Haemophilus influenzae*, adjusted for differences between days with analysis of variance (ANOVA) after logarithmic transformation of the data

<i>H. influenzae</i>	IL-6 production*	SEM of difference with strain		p-value ⁺	
		Non-persisting	Throat	Unadjusted	Hommel adjusted
Persisting	7.01±0.20	0.32	0.27	-	-
Nonpersisting	7.89±0.26		0.33	0.0061	0.012
Throat	7.59±0.17			0.030	0.030

*: values presented as the mean±SEM of the natural logarithm of the values in pg·mL⁻¹; +: comparison with persisting strain.

Bacterial adherence and production of IL-6 and IL-8

The bacterial strains in the panel tested were either adherent or nonadherent. There was an equal distribution of adhering and nonadhering strains over persisting and nonpersisting isolates (figs. 2 and 3). The effect of CAM on the adherence was analysed for *H. influenzae* strain A950009. A twofold decrease in adherence was observed (52±16 bacteria·cell⁻¹ in the absence of CAM and 25±11 in the presence of CAM, respectively) after incubation with 10⁸ cfu·mL⁻¹. This reduction was not due to a direct effect of CAM on adherence, because the adherence of the CAM-resistant strain QC2174 was not influenced by CAM (22±9 compared to 20±8 bacteria·cell⁻¹, in the absence and presence of CAM, respectively). Subsequently, we evaluated IL-6 and IL-8 production induced by 10⁷ cfu·mL⁻¹ of 12 strains adhering to H292 cells and by 11 nonadhering strains. The levels of IL-6 and IL-8 production induced by adhering and nonadhering strains were similar (IL-6, adhering strains: median 201, range 76–701 pg·mL⁻¹; nonadhering strains: median 264, range 54–584 pg·mL⁻¹; MWU-test: p=0.98; IL-8, adhering strains: median 1010, range 497–2,716 pg·mL⁻¹; nonadhering strains: median 1,450, range 404–3,145 pg·mL⁻¹; MWU-test: p=0.83). Also, no difference was found in the analysis of maximal levels of IL-6 and IL-8 induced by adhering and nonadhering strains (data not shown).

Table 4. – Interleukin (IL)-6 and IL-8 production after 20 h and 40 h of exposure to *Haemophilus influenzae*

Strain	Inoculum cfu·mL ⁻¹	IL-6 pg·mL ⁻¹		IL-8 pg·mL ⁻¹	
		20 h	40 h	20 h	40 h
A950003*	10 ⁷	161±25	1583±62	474±32	1743±89
	10 ⁸	418±22	493±25	750±121	1852±134
A950004*	10 ⁷	100±8	288±33	335±16	2335±88
	10 ⁸	154±8	226±24	480±13	1349±56
A950005 ⁺	10 ⁷	236±19	437±27	1659±112	3302±174
	10 ⁸	294±17	200±22	1480±69	1575±155
A950006 ⁺	10 ⁷	339±13	753±11	2008±48	3187±88
	10 ⁸	429±13	393±14	1547±133	1835±78
A930065 [†]	10 ⁷	279±11	1175±68	911±36	996±52
	10 ⁸	540±15	489±46	1307±170	1620±112

Data are presented as mean±SEM values from six independent experiments. *: persisting strain; +: nonpersisting strain; †: throat strain.

Table 5. – Effect of bacterial pre-exposure of H292 cells on interleukin (IL)-1 β -induced IL-6 production

Strain	Inoculum cfu·mL ⁻¹	IL-6 production	
		Before IL-1 β [#] pg·mL ⁻¹	After IL-1 β [‡] pg·mL ⁻¹
A950004*	10 ⁷	198±13	1640, 1520
	10 ⁸	157±42	2580, 1650
A950005+	10 ⁷	351±33	1530, 1780
	10 ⁸	628±190	2980, 2420
No bacteria	-	23±2	1320, 1200

[#]: IL-6 production after 24 h exposure to bacteria, mean±SD of quadruplicate experiments; [‡]: IL-6 production after 15 h exposure to IL-1 β (100 U·mL⁻¹), duplicate experiments; *: persisting strain; +: nonpersisting strain.

Persisting H. influenzae isolates did not affect the capacity of H292 cells to produce IL-6

H292 cells were incubated for 24 h to 10⁷ and 10⁸ cfu·mL⁻¹ with either a persisting (A950004) or nonpersisting strain (A950005), after which IL-1 β was added to the cell culture medium. The results, summarized in table 5, show that IL-6 production by H292 cells was not hampered after pre-exposure to bacteria for 24 h, and that there was no difference between persisting and nonpersisting *H. influenzae*.

Initial characterization of bacterial component(s) that induced cytokine production by H292 cells

H292 cells were exposed for 20 h to the culture filtrates of the persisting strain A950004 and the nonpersisting strain A950005. Exposure of H292 cells for 20 h to the culture filtrate (1:10 diluted) of the persisting strain A950004 and the nonpersisting strain A950005 induced the production of 124±16 pg·mL⁻¹ (n=4, duplicate experiments), and 314±70 pg·mL⁻¹ (n=4, duplicate experiments), respectively, compared to the release of 23±3

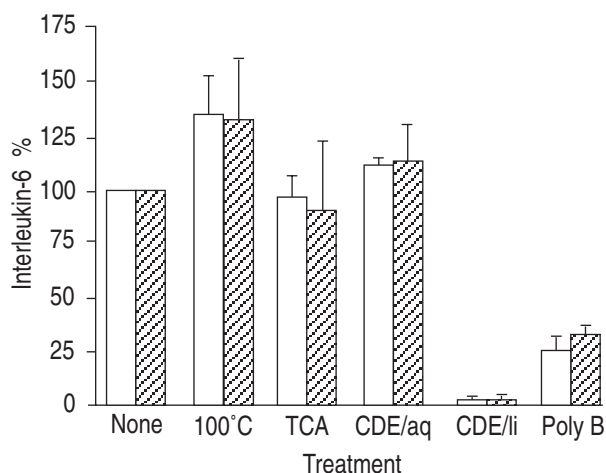


Fig. 4. – Production of interleukin (IL)-6 after 20 h of exposure to *Haemophilus influenzae* culture filtrates. Bacterial products released in brain heart infusion broth (BHI) were treated by boiling (100°C), 5% trichloroacetic acid (TCA), chloroformdiethylether (CDE) or polymyxin B (poly B), as described in Materials and methods. CDE/aq: aqueous phase; CDE/li: lipid phase. Values are mean±SEM of two independent duplicate experiments, expressed as the percentage of the IL-6 levels induced by untreated culture filtrates. Background IL-6 inductions was subtracted. ■: A950004; ▨: A950005.

pg·mL⁻¹ in the control experiments (n=6, duplicate experiments). The stimulating component(s) were not extracted with chloroform-ether, indicating that they were not associated with the lipid fraction. Boiling and TCA-precipitation also had no effect on the activity of the culture filtrate, practically ruling out a protein being responsible for the cytokine induction. However, the activity was partially reduced by incubation with immobilized polymyxin B (fig. 4).

Discussion

In the present study, we assessed whether persisting and nonpersisting *H. influenzae* isolates from patients with chronic bronchitis differ, particularly in the induction of IL-6 and IL-8 production by epithelial cells. All isolates induced IL-6 and IL-8 production in a time and dose-dependent fashion. Both a straightforward statistical analysis and a more extensive analysis showed that persisting isolates induced lower levels of IL-6 than nonpersisting isolates. For the IL-8 induction by persisting isolates in comparison to nonpersisting isolates there was a similar tendency, but not as clear cut as for IL-6. Levels of IL-6 and IL-8 induced by throat isolates from healthy carriers overlapped with those from chronic bronchitis isolates (figs. 2 and 3).

To be able to compare the various *H. influenzae* isolates for their ability to induce IL-6 and IL-8 production by H292 cells, we had to control the bacterial load during exposure to the H292 cells [14]. By using CAM at 3 μ g·mL⁻¹ we prevented bacterial replication without killing the bacteria. In addition, IL-1 β -induced IL-6 and IL-8 production by H292 cells was similar in the absence and presence of CAM, suggesting that IL-6 and IL-8 production by H292 cells was not modulated in the presence of CAM. Also, exposure of H292 cells to 10⁷ cfu·mL⁻¹ of *H. influenzae* in the absence or presence of CAM yielded similar amounts of IL-6 and of IL-8, indicating that, in general, the induction of IL-6 and IL-8 by *H. influenzae* was not affected by the presence of CAM, at least during a 24 h exposure. CAM did not directly affect the adherence of *H. influenzae* to H292 cells. The observed reduction in adherence with CAM-sensitive strains is probably the consequence of reduced growth of bacteria on the cells, since the adherence experiments involve a 6 h incubation [23]. Taken together, we have no indications that inclusion of CAM would bias comparison of the various isolates.

Using 10⁶ to 10⁹ cfu·mL⁻¹ of *H. influenzae* strains we found a pronounced IL-6 and IL-8 production only with 10⁷ and 10⁸ cfu·mL⁻¹. IL-6 and IL-8 levels increased during at least 20–24 h of exposure. Between 20–40 h of bacterial exposure to H292 cells, particularly with 10⁸ cfu·mL⁻¹, IL-6 and IL-8 levels were similar or reduced in comparison to those obtained after the initial 20 h of exposure (fig. 1, table 4). With 10⁷ cfu·mL⁻¹, IL-6 levels usually increased further after 20 h of exposure. This difference may be explained in part by limited cellular damage induced by 10⁸ cfu·mL⁻¹ as evidenced by the increased release of cellular LDH and a reduced formazan formation after 40 h of exposure to 10⁸ cfu·mL⁻¹ but not upon exposure to 10⁷ cfu·mL⁻¹. Since there was no cellular damage induced after 20 h of exposure to bacteria, and levels of IL-6 and IL-8 had

not reached plateau levels, we compared the various strains for the induction of IL-6 and IL-8 after 20–24 h of exposure to *H. influenzae*.

The numbers of cfu·mL⁻¹ we tested occur in clinical sputum samples (4×10⁴–4×10⁷ cfu·mL⁻¹; [30]). Since these values of cfu·mL⁻¹ induced IL-6 and IL-8 production *in vitro*, this may indicate that *H. influenzae* may induce IL-6 and IL-8 *in vivo*, thus contributing to the eradication of *H. influenzae* [21]. Nevertheless, extrapolation of the present findings to the interactions of *H. influenzae* with airway epithelial cells *in vivo* should be performed with caution.

The induction of IL-6 and IL-8 by isolates correlated significantly for 10⁷ cfu·mL⁻¹ suggesting that the same stimulus induces IL-6 and IL-8 production by H292 cells. The reason for the more obvious differences between strains for the induction of IL-6 than for IL-8 are unknown, but may be due to differences in the regulation of the IL-8 response by H292 cells. The initial characterization of the stimulus indicated that it is released by the bacteria and that it is probably not a lipid or protein. A polysaccharide can be excluded since these *H. influenzae* strains are unencapsulated. LPS or LPS-bound component(s) [31] are responsible for a major part of the interleukin induction, since approximately 70% of the activity was removed by polymyxin B. The remaining activity may be caused by a second stimulus, which is also neither a protein nor a lipid. Therefore, we propose that persisting and nonpersisting isolates differ in their ability to induce IL-6 and IL-8 because they differ in the excretion of a (complex) stimulus.

Future studies should be directed to further characterization of the bacterial stimulus that induces IL-6 and IL-8 production by H292 cells. This would allow quantitative comparison of persisting and nonpersisting isolates and also assessment of IL-6 and IL-8 induction by this stimulus in cultured primary airway epithelium.

In conclusion, *Haemophilus influenzae* isolates that differ clinically, *i.e.* persisting *versus* nonpersisting, differ in the induction of interleukin-6 and interleukin-8 by H292 cells. We suggest that persisting isolates generate lower amounts of a stimulus that activates epithelial cells to produce interleukin-6 and interleukin-8, which may, at least in part, contribute to their persistence. It is likely that *H. influenzae* isolates in the lower respiratory tract originate from the upper airways, especially the nasopharynx, the natural habitat for *H. influenzae*. If anything, our results for throat isolates from healthy carriers may point to the possibility that persisting *H. influenzae* strains are selected from throat strains that induce low levels of interleukin-6 and interleukin-8.

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