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Transcriptional Responses of Human Respiratory Epithelial Cells to Nontypeable *Haemophilus influenzae* Infection Analyzed by High Density cDNA Microarrays

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Abstract Nontypeable *H. influenzae* (NTHi), a Gram-negative obligate human pathogen, causes pneumonia, chronic bronchitis, and otitis media, and the respiratory epithelium is the first line of defense that copes with the pathogen. In an effort to identify transcriptional responses of human respiratory epithelial cells to infection with NTHi, we examined its differential gene expression using high density cDNA microarrays. BEAS-2B human bronchial epithelial cells were exposed to NTHi for 3 h and 24 h, and the alteration of mRNA expression was analyzed using microarrays consisting of 8,170 human cDNA clones. The results indicated that approximately 2.6% of the genes present on the microarrays increased in expression over 2-fold and 3.8% of the genes decreased during the 24-h infection period. Upregulated genes included cytokines (granulocyte-macrophage colony stimulating factor 2, granulocyte chemotactic protein 2, IL-6, IL-10, IL-8), transcription factors (Kruppel-like factor 7, CCAAT/enhancer binding protein β, E2F-1, NF-κB), cell surface molecules (CD74, ICAM-1, ICAM-2, HLA class I), as well as those involved in signal transduction and cellular transport. Selected genes were further confirmed by reverse-transcription-PCR. These data expand our knowledge of host cellular responses during NTHi infection and should provide a molecular basis for the study of host-NTHi interaction.

Key words: Nontypeable *H. influenzae* infection, respiratory epithelial cells, high density cDNA microarray analysis

Bacterial infection is a very complex process in which both pathogenic microorganisms and host cells play crucial roles, and host cell response is the outcome of interaction between the two participants. To elucidate bacterial pathogenesis mechanisms, it is essential to understand the cellular and systemic response of the host as well as the virulence factors of the pathogen. Infection of a host by pathogenic bacteria causes significant changes in host cells, leading to activation of a wide range of genes and suppression of others at the same time. Recently, it has become possible, owing to the introduction of DNA microarray technology, to massively analyze changes in gene expression profiling [14, 25, 31]. In the field of bacterial pathogenesis, the microarray technique has been adopted by several groups to study host cellular responses to infection with *P. aeruginosa*, *B. pertussis*, *L. pneumophila*, *S. typhimurium*, and *B. abortus* [3, 7, 8, 13, 22].

Epithelial cells in the respiratory system are the first to come in contact with the external environment, and they not only serve as a physical barrier against potential microbial pathogen, but also actively participate in the inflammatory response to defend the airway. Human airway epithelial cells respond to external stimuli such as viruses, allergens, cigarette smoke, and air pollutants by secreting a number of cytokines, eicosanoids, and reactive oxygen species [2, 14, 19, 23, 24]. Airway epithelial cells also respond to the inflammatory cytokines interleukin-1β (IL-1β) and tumor necrosis factor-α (TNF-α) produced by various immune cells and structural cells [1, 28]. These cytokines are thought to activate the transcriptional factors NF-κB and AP-1 that govern the expression of various genes involved in the immune responses and inflammatory conditions [6, 28].

Haemophilus influenzae, a Gram-negative coccobacillus, is a strict human pathogen that frequently colonizes human respiratory mucosa and often causes a wide range of diseases, such as pneumonia, bronchitis, and meningitis [21]. Encapsulated *H. influenzae* strains are subgrouped into serotypes a-f on the basis of antigenically distinct capsular polysaccharides. Although serotype b strains account for most cases of illness due to *H. influenzae* infection, the introduction of efficient vaccines for this

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serotype has significantly decreased infection. Nontypeable *H. influenza* strains (NTHi), lacking capsular polysaccharides, are isolated from the asymptomatic carriers and are responsible for various diseases including infant otitis media, chronic bronchitis, sinusitis, and conjunctivitis [20, 26]. They colonize epithelial mucous membranes and the host-bacterium interaction is very tissue-specific. Outer membrane proteins, IgA protease, and lipooligosaccharide (LOS) are known virulence factors determining NTHi pathogenicity [10]. Although the pathogenesis mechanism of NTHi has been studied by many groups, little is known about the response of human respiratory epithelial cells to NTHi infection other than an increase in secretion of proinflammatory cytokines. In this study, in an attempt to characterize the cellular responses of the human respiratory epithelium to NTHi infection, high density cDNA microarrays were applied to the investigation of transcriptional responses of human respiratory epithelial cells upon exposure to NTHi.

MATERIALS AND METHODS

Bacteria Strains

The NTHi 2019 strain used for this study is a clinical isolate from a patient with chronic bronchitis [4], and was provided by M.A. Apicella of the University of Iowa, U.S.A. NTHi 2019 was grown on a Brain Heart Infusion media (BHI; Difco, Sparks, MD, U.S.A.) agar plate supplemented with 4% Fildes reagent (Difco) (BHIF) at 37°C under 5% CO_2 atmosphere. An overnight culture grown in BHIF media was inoculated into fresh media and grown to mid-logarithmic phase. For infections, bacterial cells that harvested were washed with phosphate-buffered saline (PBS) and adjusted to OD_{600} of 0.4. Viable cell counting of the bacterial suspension was performed at the time of each infection. To prepare killed NTHi cells, harvested bacteria washed with PBS were suspended in 0.3% formalin and kept at 4°C for 24 h. Cells were then washed twice with PBS and resuspended in PBS to OD_{600} of 0.4 for each infection.

Cell Culture and Bacterial Infection

The SV40-transformed human bronchial epithelial cell line BEAS-2B (ATCC CRL-9609) was used for experiments. The cells were routinely cultured in RPMI 1640 medium (Life Technologies, Rockville, MD, U.S.A.) supplemented with 10% fetal bovine serum (FBS) and antibiotics (100 μg/ml streptomycin, 100 U/ml penicillin) at 37°C in a humidified 5% CO_2 incubator. For experiments, cells were seeded in RPMI 1640 supplemented with 5% FBS at 2×10^6 cells per 100-mm dish, and cultured overnight. Next day, cells were washed twice with PBS, and fresh antibiotic/serum-free RPMI 1640 medium was added. Three hours later, bacteria were added at a ratio of 200 bacteria per human cell. Cells were then incubated at 37°C in a 5% CO_2 incubator until harvested. At harvesting, cells were carefully washed twice with ice-cold PBS to remove bacteria, scraped off from dishes, centrifuged, and used for RNA extraction.

Total RNA Isolation and Fluorescent cDNA Probe Preparation

Total cellular RNA was isolated using TRIzol® Reagent according to the manufacturer's instruction (Invitrogen, Grand Island, NY, U.S.A.), and treated with RNase-free DNase I (Promega) and extracted with phenol/chloroform (1:1, v/v) followed by ethanol precipitation. Purified RNA was quantified spectrophotometrically using GeneQuant Pro (Amersham Biosciences, Little Buckinghamshire, U.K.) and resolved on an ethidium bromide-containing agarose gel to check integrity and correct quantification.

Fluorescently labeled cDNA probes were prepared using Cyscribe First-Strand cDNA Labeling Kit (Amersham Biosciences) by incorporation of Cy3- and Cy5-conjugated dUTP during synthesis from 20 μg of total cellular RNAs extracted from bacteria-infected and uninfected control cells, respectively. The fluorescently labeled cDNAs were purified using a GFX column and checked for labeling efficiency before use. Equal amounts of the two probes were pooled and used for hybridization to microarrays.

cDNA Microarray Hybridization and Data Analysis

Labeled cDNAs were hybridized to TwinChip™ Human-8 K Microarrays (Digital Genomics, Seoul, Korea) containing 8,170 human cDNAs (6,990 known genes) at 42°C for 18 h. The arrays were washed with 1×SSC (150 mM NaCl, 15 mM sodium citrate) containing 0.2% SDS at 55°C for 10 min, and with 0.1×SSC containing 0.2% SDS for 10 min, followed by a rinse with 0.06×SSC at room temperature for 10 min. The arrays were then centrifuged, dried at room temperature, and scanned using ArrayWorx Biochip Reader (Applied Precision, Washington, U.S.A.). GeneSight 3.2 and Imagene 5.0 software (BioDiscovery, Los Angeles, U.S.A.) were used to analyze the data. The output was stored in an Excel spreadsheet. Three hybridizations were conducted using probes prepared with total RNAs from independent infections. The fluorescence intensity of each of the probes was normalized to the median value of all intensities measured in the corresponding array. Genes displaying a fluorescence intensity lower than 0.2 unit were considered as not being expressed and were excluded from analysis.

Reverse-Transcription-PCR Analysis

For a reverse-transcription (RT) reaction, 3 μg of total RNA was used as a template to synthesize cDNA. Total RNA was mixed with 4 μg of random hexamer (Amersham

Table 1. Oligonucleotides used for reverse-transcription-PCR of human genes.

Gene	Forward primer (5'-3')	Reverse primer (5'-3')	Size of PCR product (bp)	No. of PCR cycles
GAPDH	gagtcaacggatttggtcgt	gctgccatggaatttgccat	157	20
IL8	aaggaaccatctcactg	gattcttggataccacagag	369	25
IL6	agatggctgaaaaagatgga	ggtgcccatgctacatttgc	369	25
CD74	actctgctcctcgctggcca	tggcgggtacaccttcaggg	300	25
TAPBP	tccctttgggctagagtggc	gttgctggcatcagggacac	300	25
IFRD1	gggtcagctagtatgcaggc	atcatcacaagagaggaggc	300	25
ICAM1	cgactggacgagagggattg	cggctgctaccacagtgatg	280	30
ICAM2	gcaccacctgtaaccagcct	ggaacaggaagagggtgagg	306	30
SLC1A5	gcgtggccaagcacatcagc	caggctctgtgcttctcgac	384	25
ABCC6	ctgtccaggaacatgggagt	ctccttcttcatccaccacc	318	30
EFNA1	gcctggactgtgctgctcct	gaccatgcctgcacagcttg	349	25
DARS	ggctcagtccccacagctat	tctgtatcatacttttccttt	423	25

Biosciences), incubated at 65°C for 10 min and cooled on ice for 2 min. A RT reaction was carried out in 50 µl volume with 2 units of M-MLV RT (Invitrogen) at 42°C for 1 h, followed by heating at 95°C for 5 min to terminate the reaction. The RT reaction product was diluted to 50 µl with water, and 5 µl was used as template DNA for polymerase chain reaction (PCR). PCR was carried out in a 50 µl reaction mixture containing 5 µl cDNA template, 0.5 µM sense and antisense primers for the gene of interest, 1× Taq buffer, and 1 unit of Taq DNA polymerase (Takara Korea, Seoul, Korea). The sequences of gene-specific forward and reverse primers used for PCR are shown in Table 1. In order to optimize detection of small difference in mRNA level, PCR was performed for various cycles at 95°C for 30 sec, 55°C for 30 sec, and 72°C for 60 sec, followed by 72°C for 5 min. Negative control reactions were performed for each gene with total RNA without M-MLV RT to confirm the lack of genomic DNA contamination and with NTHi 2019 total RNA to exclude the possibility of detection of NTHi 2019 RNA. The resultant PCR products were resolved on an ethidium bromide-containing 2.5% agarose gel. The gel was scanned with Gel Document (BioRad, Hercules, CA, U.S.A.) on a UV-illuminator, and DNA bands quantitated using Quantity One software (BioRad).

RESULTS AND DISCUSSION

Expression Microarray Analysis of Human Respiratory Epithelial Cells Infected with NTHi

To investigate changes in respiratory epithelial mRNA expression in response to infection with NTHi, cultures of the human bronchial epithelial cell line BEAS-2B were infected with the NTHi strain 2019, and total RNA was extracted at 3 h and 24 h after infection. Control RNA was isolated from uninfected cells harvested at 3 h. Fluorescent-labeled cDNAs prepared from test and control RNA samples were mixed and used for hybridization with high density human cDNA microarrays. The fluorescence intensity

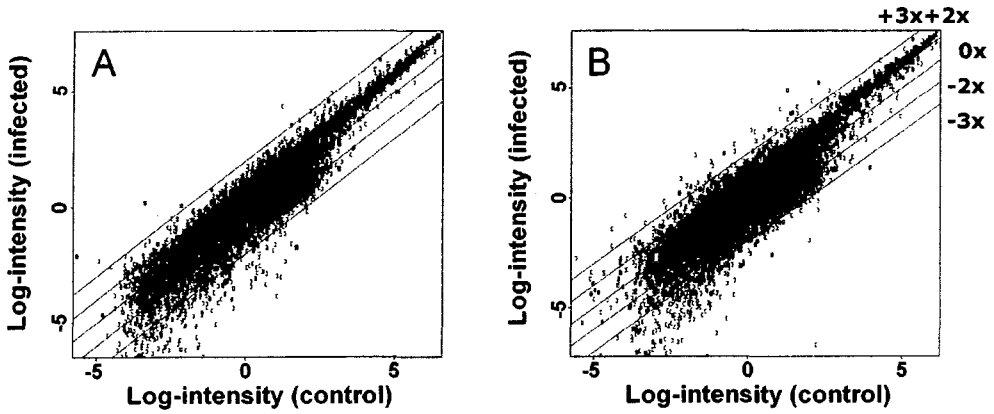

Fig. 1. Scatter plots of cDNA microarray analysis.
BEAS-2B human respiratory epithelial cells were infected with NTHi 2019 for 3 h (A) and 24 h (B), and total RNA extracted was used for hybridization of human high density cDNA microarrays. Lines in the graphs denote no change, ±2-fold, and ±3-fold changes, respectively, in gene expression.

of each spot was measured and normalized by the total spot intensity of the corresponding microarray, and data were expressed as relative changes in mRNA level between NTHi-infected samples and uninfected controls. Hybridization experiments were replicated three times using probes from independently infected cells, and the mean value of three experiments was calculated. Figure 1 shows the scatter plots of such experiments, which indicate that there were more genes displaying altered expression at 24 h than at 3 h after the bacterial infection, and that changes in intensity were also greater at 24 h after exposure.

Of the total 8,170 genes present on the microarrays, 210 genes in NTHi-infected samples displayed expression deviated from that in the untreated controls during a 24-h exposure to NTHi by at least 2-fold with a significance cutoff of $P<0.05$ (Student's *t*-test), indicating that approximately 2.6% of genes on the arrays positively responded to NTHi infection. In the meantime, 318 genes (3.8%) were found to have decreased more than 2-fold at $P<0.05$ at 24 h post-infection.

Differential Gene Expression in Respiratory Epithelial Cells in Response to NTHi

A list of selected genes upregulated during NTHi infection is shown in Table 2. Of the genes whose expression levels increased in abundance by greater than 2-fold, the most well represented category was that associated with immune responses and the inflammatory process. Serum amyloid A1, known to increase during inflammation, showed the highest increase among all the genes on the microarrays. Also up-regulated were genes encoding several cytokines and chemokines such as granulocyte chemotactic protein 2, granulocyte-macrophage colony stimulating factor 2

Table 2. List of genes upregulated in respiratory epithelial cells after exposure to nontypeable *H. influenzae* determined by cDNA microarray analysis.

Gene symbol	Accession no.	Protein or gene	Fold-increase[a]	
			3 h	24 h
Inflammation and cytokines/chemokines				
SAA1	AA829286	Serum amyloid A1	3.0±0.9	12.9±6.0
CXCL6	U81234	Granulocyte chemotactic protein 2	1.9±0.1	4.5±1.2
C3	K02765	Complement component 3	2.0±0.6	3.8±1.0
CSF2	NM_000758	GM-CSF	2.7±1.1	2.5±0.9
IL6	M54894	Interleukin 6	1.5±0.3	2.2±0.7
IL10	M57627	Interleukin 10	2.4±1.2	2.2±0.5
IL11	X58377	Interleukin 11	2.0±0.5	2.1±0.4
IL8	Y00787	Interleukin 8	2.4±0.9	2.0±0.7
Transcription/translation				
KLF7	AA486055	Kruppel-like factor 7	3.0±0.8	3.8±1.3
E2F1	NM_005225	E2F transcription factor 1	2.3±0.6	3.7±1.1
CEBPB	AA557306	CCAAT/enhancer binding protein beta	-	3.7±1.2
ZNF258	AI140947	Zinc finger protein 258	2.6±0.7	3.4±0.4
RPS21	AI026740	Ribosomal protein S28	4.5±2.0	3.5±1.1
EEF2	Z11692	Eukaryotic translation elongation factor 2	2.9±0.9	2.9±0.6
TCEA2	D50495	Transcription elongation factor A (SII), 2	2.6±0.8	2.7±0.6
CREBBP	U47741	CREB binding protein	2.3±0.6	2.3±0.6
Cell surface molecules/receptors				
CD74	NM_004355	CD74 antigen	0.7±1.2	7.4±1.7
IFITM3	X57352	Interferon induced transmembrane protein 3	4.0±1.6	4.4±1.2
PKD1	U24497	Polycystin-1	2.7±0.3	3.7±0.9
B2M	AI587122	Beta-2-microglobulin	2.6±1.1	3.6±1.4
ICAM2	NM_000873	Intercellular adhesion molecule 2	2.5±0.6	3.5±0.9
HLA-G	AI123699	HLA-G histocompatibility antigen, class I, G	2.2±0.7	3.1±0.8
FGFR1	X66945	Fibroblast growth factor receptor 1	2.0±0.7	3.1±0.8
MFGE8	AI340354	Milk fat globule-EGF factor 8 protein	2.4±0.9	2.9±0.5
HLA-B	U29057	Major histocompatibility complex IB	2.4±1.4	2.9±0.7
ICAM1	M24283	Intercellular adhesion molecule 1	2.5±0.6	2.7±0.6
IL13RA1	Y09328	Interleukin 13 receptor, alpha 1	2.3±0.6	2.3±0.6
IL3RA	AI765858	Interleukin 3 receptor alpha (low affinity)	2.3±0.5	2.3±0.7
IL10RB	Z17227	Interleukin 10 receptor, beta	2.4±0.5	2.3±0.4
EFNA1	M57730	Ephrin-A1	3.0±0.9	0.7±0.1

Table 2. Continued.

Gene symbol	Accession no.	Protein or gene	Fold-increase[a]	
			3 h	24 h
Signal transduction				
HAN11	AI632557	WD-repeat protein	2.5±0.5	2.7±0.9
IGFBP3	M31159	Insulin-like growth factor binding protein 3	3.8±1.4	2.4±0.8
TRAF5	U69108	TNF receptor-associated factor 5	2.2±0.5	2.2±0.5
DNA replication				
RNASEH2A	AA532741	Ribonuclease H2, large subunit	2.4±1.0	2.9±0.8
POLD1	M81735	DNA polymerase delta 1, catalytic subunit 125 kDa	3.36±1.2	2.45±0.6
Metabolic/synthetic				
PCK2	X92720	Phosphoenolpyruvate carboxykinase 2	1.1±1.1	8.1±3.2
PTS	AA428847	6-Pyruvoyltetrahydropterin synthase	3.7±1.3	4.5±1.3
CARS	AI753042	Cysteinyl-tRNA synthetase	1.2±0.2	4.5±0.5
PYCR1	M77836	Pyrroline-5-carboxylate reductase 1	2.3±1.1	4.3±0.8
NDUFC1	AA760866	NADH dehydrogenase (ubiquinone) 1	4.3±1.2	3.0±0.8
Cell cycle/proliferation				
MCM2	AI814040	MCM2 minichromosome maintenance deficient 2	2.5±0.8	2.6±0.8
Apoptosis/anti-apoptosis				
IER3	AI185199	Immediate early response 3	10.6±3.6	9.0±4.4
BIRC1	BE693227	Baculoviral IAP repeat-containing 1	2.8±0.8	3.3±1.3
LGALS1	AA035793	Galectin 1	4.2±1.9	2.8±1.0
Transport				
SLC1A5	AF102826	Neutral amino acid transporter	2.3±1.4	6.6±2.6
SLC1A4	L14595	Glutamate/neutral amino acid transporter	0.9±1.0	3.7±0.9
SLC21A3	U21943	Organic anion transporter	4.6±1.1	3.7±1.1
TAP1	L21204	Transporter 1, ATP-binding cassette, subfamily B	2.2±0.8	3.6±1.0
TETRAN	L11669	Tetracycline transporter-like protein	2.9±1.0	3.3±1.2
Others				
PSMB9	AI923532	Proteasome subunit, beta 9	2.9±1.4	5.8±1.2
SOD2	Y00472	Mitochondrial superoxide dismutase 2	2.9±0.6	5.2±1.2
BLVRB	AA280097	Biliverdin reductase B	2.9±0.9	4.7±1.3
KIFC3	AA703139	Kinesin family member C3	2.0±0.9	4.3±1.6
TAPBP	AF029750	TAP binding protein (tapasin)	3.1±1.1	4.3±1.2
PKP2	X97675	Plakophilin 2	4.5±1.1	3.6±1.0

[a]Fold-increase was determined by comparing with the intensity of uninfected control cells harvested at 3 h. The numbers are the mean±S.E. of values obtained from three independent hybridizations.

(GM-CSF), interleukin 6 (IL-6), interleukin 10 (IL-10), and interleukin 8 (IL-8). IL-8 and IL-6 activate and attract neutrophils to sites of inflammation, which play an important role in defense against pathogenic bacteria. It has been shown that IL-8 and IL-6 are the major chemokines secreted from respiratory epithelial cells upon NTHi infection [5, 18, 28]. This result confirmed that our approach to identifying host genes altered during pathogenic infection is reliable. As shown in this study, IL-8 and IL-6 induction upon NTHi exposure is far lower than those previously described for *H. influenzae* type b strain as well as other pathogenic bacteria [3, 17], explaining the difference in the pathogenicity of the two strains. *H. influenzae* type b is the most virulent *H. influenzae* strain, causing bacterial meningitis, pneumonia, and bacteremia [21], but infection with NTHi strains is rather limited to the respiratory tract and causes less severe diseases such as otitis media, sinusitis, and chronic bronchitis [10, 20, 26].

GM-CSF is a hematological growth factor and is required for the activation of a macrophage and subsequent host defense against bacterial invasion [30]. In contrast, IL-10 is an anti-inflammatory cytokine. It inhibits the release of proinflammatory mediators from macrophages, such as IL-1β, IL-6, and TNF-α, and is thus involved in the homeostatic control of innate immune reactions and cell-mediated immunity [9, 12, 16]. The secretion of GM-CSF and IL-10 by respiratory epithelial cells during bacterial infections has not been well described, but the results of this study revealed increased expression of the two cytokines, suggesting that they play roles in initiating and resolving host inflammatory responses against NTHi infection in the respiratory epithelium.

Other genes found to be induced upon NTHi infection are those whose products act to present endogenous antigens, e.g., the specialized proteasome component beta subunit, the ATP-binding cassette transporter, and the TAP-binding protein tapasin (TAPBP). Genes coding for surface molecules such as CD74 (MHC class II invariant chain) and MHC class I molecules were also upregulated. Other examples were genes associated with cell surface adhesion, e.g., intercellular adhesion molecules 1 (ICAM-1) and ICAM-2. These molecules play important roles in recruiting neutrophils and activated monocytes on the site of infection, and ICAM-1 has previously been reported as being activated during NTHi infection [11].

The bacterial infection also stimulated expression of a number of genes involved in cellular transport, e.g., neutral amino acid transporter, glutamate/neutral amino acid transporter, and organic anion transporter. The mRNA transcript levels of transcription and translation factors were seen to be elevated, reflecting a global activation of gene expression in response to pathogenic infection. Examples were Kruppel-like factor 7, E2F transcription factor 1, CCAAT/enhancer binding protein beta, eukaryotic translation elongation factor 2, and transcription elongation factor A. Nuclear factor-κB (NF-κB) was also found to be elevated by a 2.1-fold increase at 24 h (data not shown) confirming previous reports by Shuto *et al.* [27]. NF-κB is critical for both acquired and innate immune responses. It is activated by signals transduced by antigen receptors and Toll-like receptors and translocated into nuclei, leading to the induction of most of the genes responsive to bacterial infection.

Lipopolysaccharide (LPS) of Gram-negative bacteria is a major pathogenic factor provoking diverse host responses during bacteria infections. In this study, respiratory cell responses to NTHi LOS were examined by microarray analysis. BEAS-2B cells were co-cultured with NTHi 2019 LOS at a concentration of 2 μg/ml, and the total RNA isolated was used to prepare a cDNA probe for hybridization of human cDNA microarrays. The results revealed that overall transcriptional responses of the bronchial epithelial cells to NTHi 2019 LOS were largely similar to, but weaker in intensity than, those observed with live bacteria (data not shown), indicating that LOS stimulation evokes alteration of cellular gene expression, but bacterial factors other than LOS also are responsible for cellular responses to NTHi. As with the genes upregulated during infection, diverse groups of genes were found to be downregulated after incubation with NTHi. Few genes with roles in defense and immunity were present, however, indicating that there were substantially biased cellular responses towards immune activation (data not shown).

To verify the results obtained by microarray analysis, eleven genes were selected for RT-PCR analysis that were induced during exposure to NTHi. BEAS-2B cells were infected with NTHi 2019 and harvested for total RNA extraction at various time points up to 48 h. Total RNA was converted to cDNA, and the level of specific mRNA was assessed by PCR amplification. PCR was performed for various cycles to optimize detection of small differences in mRNA level. In Fig. 2, GAPDH was used as a control, which did not show significant changes in mRNA transcript level up to 48 h after the bacterial exposure. The mRNA levels of IL-6 and IL-8 reached a peak at 6 h and decreased thereafter, being in good agreement with the previous results [5, 18]. Ephrin A1 also exhibited an early transcriptional response. All the other genes showed increased expression over the time of observation with the highest expression at

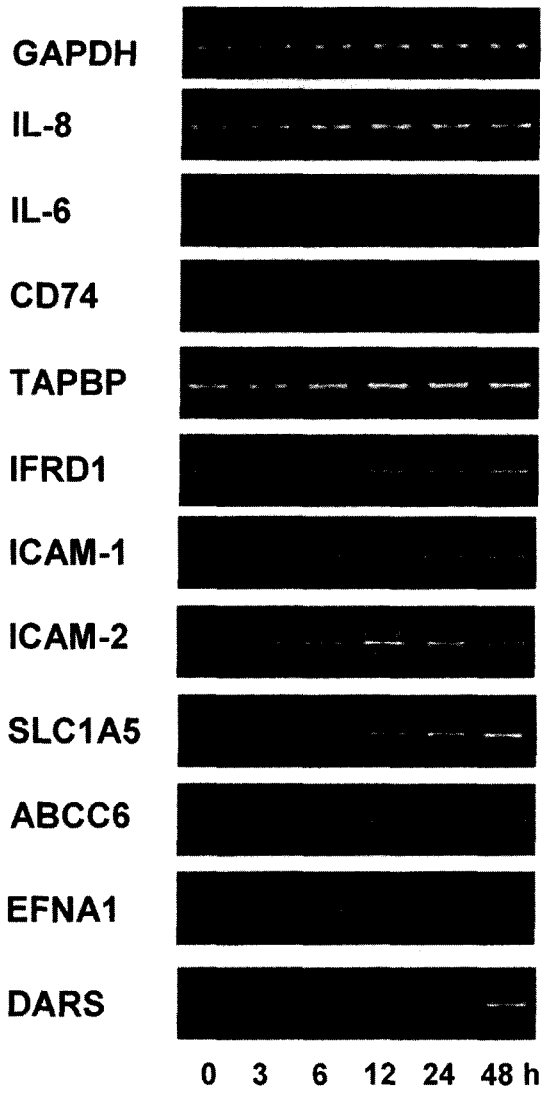

Fig. 2. Reverse-transcription-PCR analysis of selected gene transcription in human respiratory epithelial cell line BEAS-2B infected with NTHi.
BEAS-2B cells were infected with NTHi 2019 and harvested at indicated times for total RNA extraction. Cellular RNA was reverse transcribed using M-MLV RT, and PCR was performed with primers specific for each gene.

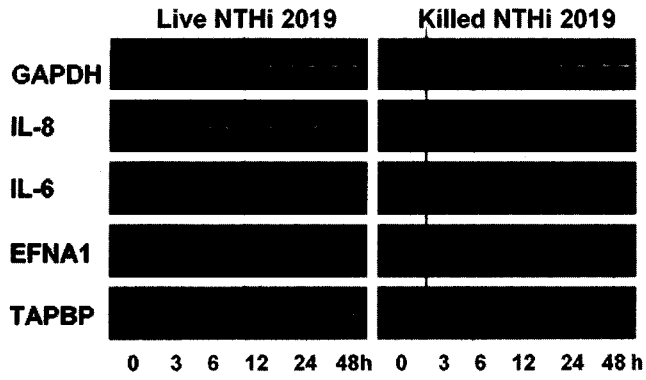

Fig. 3. Comparison of mRNA levels of selected genes determined by RT-PCR in BEAS-2B cells co-cultured with live and formalin-killed NTHi 2019.

24 or 48 h after the bacterial exposure. Expression patterns of these genes determined by RT-PCR confirmed the results obtained by microarray analysis.

To determine whether these epithelial responses to NTHi infection require live bacteria, we compared mRNA transcript levels of five genes upregulated in microarray analysis. BEAS-2B cells were co-cultured with live or killed NTHi, and the total RNA extracted was analyzed for gene expression by RT-PCR. As seen in Fig. 3, three genes of early transcriptional response (IL-8, IL-6, and Ephrin A1) were activated upon NTHi infection, but the responses were much weaker on exposure to killed bacteria. On the other hand, TAPBP exhibited a similar expression pattern. The difference in response to live and killed bacteria may reflect involvement of other bacterial signals from live bacteria in host response to NTHi infection. However, the possibility cannot be ruled out that the difference is primarily due to a higher number of live bacteria during the course of infection, and this requires further investigation. In this study, we used an established bronchial epithelial cell line for infection. Established cell lines cultured in experimental conditions may respond in a manner different from that of a host infected through natural infection routes, and respiratory epithelial cells surrounded by other types of cells are influenced by other factors. Therefore, it would be interesting to see whether the respiratory epithelial cells exhibit a similar response to NTHi *in vivo*, and this will be the subject of future studies.

Taken together, our data suggest that on contact with NTHi, the respiratory epithelium recruits and activates immune cells through the secretion of cytokines, such as IL-8, IL-6, GM-CSF, and granulocyte chemotactic protein 2, and increased expression of adhesion molecules like ICAMs. These effects should keep the pathogen at the respiratory portals of entry. In the meantime, the respiratory epithelium also activates the acquired immunity by actively processing and presenting invading antigens to immune cells. Further characterization of the specific ways in which NTHi alters the gene expression in the respiratory epithelium should provide important insights into the pathogenesis mechanism of NTHi.

In summary, we have shown in this study that microarray analysis can provide a useful tool in studying the molecular events of cellular responses to pathogenic bacteria infection, and the study results provide an overview of the interaction between human respiratory epithelial cells and NTHi at the transcriptional level.

Acknowledgments

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