Published in final edited form as:
J Allergy Clin Immunol. 2009 June ; 123(6): 1384–90.e2. doi:10.1016/j.jaci.2009.03.010.

GREATER *IN VITRO* SUSCEPTIBILTY TO RHINOVIRUS INFECTION OF BRONCHIAL THAN NASAL AIRWAY EPITHELIAL CELLS FROM HUMAN SUBJECTS

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Abstract

Background—Human rhinoviruses (HRVs) characteristically cause upper respiratory tract infection but they also infect the lower airways causing acute bronchitis and exacerbating asthma.

Objective—Our purpose was to study *ex-vivo* the differences in the response to HRV infection of nasal and bronchial epithelial cultures from the same healthy and asthmatic individuals, using conditions favoring development of fully differentiated pseudostratified mucociliary epithelium.

Methods—Cells from the inferior turbinates and bronchial tree of 5 healthy and 6 asthmatic individuals were cultured at an air-liquid interface. Cultures were infected with HRV-16 and after 48hrs the degree of infection was measured.

Results—Baseline median transepithelial resistance (R_{te}) was lower in human bronchial (HBE) than nasal (HNE) epithelial cell cultures (195Ω.cm^2 [95%CI=164–252] vs 366Ω.cm^2 [234–408] respectively, p<0.01). Virus replicated more easily in HBEs than HNEs based on virus shedding in apical wash ($LogTCID_{50}$/0.1ml=2.0 [1.0–2.5] vs. 0.5 [0.5–1.5], p<0.01), and on a 20–30 fold greater viral load and number of infected cells in HBEs than in HNEs. The increases in expression of RANTES and protein kinase PKR were greater in HBE than in HNE cultures, as well as the concentrations of interleukin (IL)-8, IL-1α, RANTES and IP-10 in basolateral medium. However,

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no significant differences between asthmatic and healthy subjects (including interferon beta1 expression) were found.

Conclusions—Differentiated nasal epithelial cells may have mechanisms of increased resistance to rhinovirus infection compared with bronchial epithelial cells. We could not confirm previous reports of increased susceptibility to HRV infection in epithelial cells from asthmatic subjects.

Keywords

Human Rhinovirus; nasal and bronchial airway epithelial cells; air-liquid interface

INTRODUCTION

Infection with human rhinovirus (HRV) characteristically causes common colds, but may also provoke acute bronchitis in healthy people and exacerbate airway disease in people with asthma, cystic fibrosis, or chronic obstructive pulmonary disease [1–4].

The primary site of HRV infection *in vivo* is the airway epithelial cell [5–7]. The involvement of the lower airways is likely due to direct infection of the bronchial mucosa because HRV RNA has been detected in tissues and secretions from the lower airways by application of polymerase chain reaction and *in situ* hybridization [7–9]. Infection of epithelial cells with HRV *in vitro* induces secretion of a variety of cytokines and chemokines, including interleukin (IL)-1, IL-6, IL-8, IL-11, IP-10, GM-CSF, RANTES [10, 11] and interferon β1 (IFN-β1) [12]. Because minimal or no discernible epithelial necrosis of sloughing are found in airway mucosal biopsies taken from people infected with HRV, or in cultures of airway epithelium infected *in vitro*, the symptoms of HRV infection are thought to reflect activation of host defense and inflammatory responses [7, 13].

In vivo, only a small proportion of cells are infected by HRV. Small and scattered foci of cells are infected in the upper [6, 14, 15] as well as lower airways [7]. In primary bronchial and adenoidal epithelial cells cultured on solid supports and infected *ex vivo*, again only a small subset of the cells are infected [7, 16].

Reasoning that the severity of the response to HRV infection may be related to the number of cells infected and the number of HRV virions produced, we examined the hypothesis that susceptibility to HRV infection may differ between nasal and. bronchial epithelial cells from the same individual and between cells from asthmatic and healthy subjects.

Asthmatic subjects develop similar upper respiratory tract symptoms, but more severe symptoms of lower respiratory tract symptoms than healthy subjects during acute respiratory viral infections [17]. A recent study reported that bronchial epithelial cells from asthmatic patients differ from those from healthy patients in supporting markedly greater viral replication and cell lysis, which was attributed to an impairment of virus-induced IFN-β1 production and induction of apoptosis [18]. This study, like virtually all *in vitro* studies of the effects of HRV, used undifferentiated human airway epithelium, in which cells are grown to form a single cell layer submerged in medium. We have previously shown that poorly differentiated tracheal and nasal airway epithelial cells produced more infectious virus particles per cell, a seven-fold higher proportion of cells were infected, a 30–130 greater replication of HRV was supported and a 16-fold greater production of RANTES was induced than when the same cells were grown at an air-liquid interface [19]. Air-liquid interface induces differentiation into a pseudostratified mucociliary epithelium closely resembling the *in vivo* appearance of native epithelium. Differentiation of airway epithelium therefore increases resistance to rhinovirus infection [19].

Because of the importance of the level of differentiation of airway epithelial cells as a determinant of their *ex-vivo* susceptibility to HRV infection, we undertook our study of differences in the response of nasal and bronchial epithelial cells from healthy and asthmatic subjects using only culture conditions favoring development of a normal-appearing mucociliary epithelium.

METHODS

Study subjects

Six subjects with allergic asthma and five healthy non-allergic subjects were studied (see Table I). Allergic status was determined by positive skin prick test (wheal >3mm with erythema and proper negative control reaction) to common allergens [20]. Asthmatic subjects had diagnosis of asthma, bronchial hyperresponsiveness (PC_{20} methacholine < 8mg/ml) 20, were allergic to at least one allergen, and had mild to moderate disease as defined by the NIH guidelines [21]. None had used inhaled corticosteroids for at least 4 weeks prior to enrollment. The healthy subjects had no previous history of lung disease, had normal spirometry and bronchial responsiveness to methacholine, and had no positive responses to skin prick tests. All subjects were non smokers. The study was approved by the University of California San Francisco's Committee on Human Research, and written informed consent was obtained from all subjects.

Human Airway Cell culture and Cell infection

Nasal scrapings were performed as we previously described [22]. Bronchial brushings were obtained by flexible Pentax bronchoscope (Model FB-18X; Pentax, Orangeburg, NY) using standard guidelines [23]. The nasal (HNE) and bronchial (HBE) epithelial cell cultures were processed in parallel, and they were cultured at an air-liquid interface, as described previously [19, 22]. See also the Methods section of the Online Repository at www jacionline.org.

Human rhinovirus-16 (HRV-16) passage 3 (grown from a stock sample from E. Dick and W. Busse, University of Wisconsin) with 1×10^6 tissue culture infectious dose of 50% (TCID50) per ml was added to the apical surface (multiplicity of infection=1.0), in triplicates. After 24h, viral suspension (or medium in control cells) was removed and the apical surface washed 3 times with PBS. Cells were then allowed to recover for 24 h, when the apical surface was washed with 200 to 300 μl of PBS, and this wash retained for titration of virus shedding. HRV was titrated in half-log dilutions of apical washes using confluent human fetal diploid lung cells (VRDL, Richmond, CA) and standard virology techniques [24]. Rte and Vte were measured, and basolateral medium was collected and stored at −80 °C. Filters with attached cells were cut in halves. One half was placed in RLT buffer, vortexed for 10 seconds and frozen at −80° C for future RNA isolation. The other half was fixed in buffered 3.5% formaldehyde for 10 min and stored in PBS at 4°C for future histology.

LINCOplex human cytokine kits (Linco Research, St. Charles, MI) were used to measure levels of cytokines and chemokines in basolateral medium samples.

Gene expression and Immunocytochemistry

Two-step real-time RT-PCR method and immunofluorescence to HRV-16 were performed as described previously [25] (see more details in the Online Repository at www jacionline.org).

Statistical analysis

Shapiro-Wilk test for normality showed that the data were not normally distributed even if log transformed. Therefore, we used non-parametric statistics to analyze the data. We compared baseline characteristics in Table 1 using Mann Whitney rank sum test for continuous variables and Fisher exact test for sex. We analyzed data on inflammatory mediators using generalized

estimating equations, which is a multivariate statistical test that allows simultaneous comparison of all characteristics of each culture (asthmatic or healthy group, nasal or bronchial site, and rhinovirus or sham infection) and also takes into account the subjects as sources for several replicate cultures. Differences were considered significant if the p value was < 0.05 using two-tailed tests. Data were analyzed using Stata 8 (College Station, TX).

RESULTS

Epithelial cell cultures

After 2 to 3 weeks of growth at an air-liquid interface (ALI), all nasal (HNE; n=70) and bronchial (HBE; n=84) epithelial cell cultures appeared fully confluent under the inverted microscope, developed properties resembling those of native epithelium, including an apical mucus layer and ciliated pseudostratified epithelium (see Figure 1), as well as permeability barrier, demonstrated by "dry" apical surface and development of transepithelial resistance (Rte), indicative of tight junction formation [22]. Cultures also developed vectorial transport of ions, demonstrated by the transepithelial potential difference (Vte) and equivalent short circuit current (Ieq).

Before HRV infection, Rte of nasal cell cultures was 366Ω.cm^2 (234–408) (median; 95%CI); Rte of the bronchial cell cultures was 195Ω.cm^2 (164–252) (p=0.0023). The values for Rte were closely similar for the cultures from the asthmatic and healthy subjects. Exposure to HRV-16 or sham led to similar and non-significant declines in Rte and transepithelial voltage at 48h in HNE and HBE cultures (see Results section of the Online Repository, which also contains the values for Vte and Ieq).

Virus replication in nasal and bronchial cell cultures

We first investigated susceptibility to HRV infection by quantifying HRV-16 viral load (RNA) in cell lysates using RT-PCR. There was a consistent and significant 20-to-30-fold greater HRV-16 replication in bronchial cell cultures (median = 12,279,579 relative gene copy number - RGCN; 95%CI = 9,051,454 to 20,010,944; n=46) than in nasal cell cultures (385,317 [258,999 to 1,287,758] n=39) in both groups (see Figure 2). The degree of infection was similar when comparing asthmatic versus healthy subjects.

The titer of infectious rhinovirus shed in the apical wash of infected cells 48h after infection was significantly greater (p<0.01) in apical washes from the HBE cell cultures than in washes from HNE cell cultures (see Table II).. HRV shedding tended to be greater in HBEs from asthmatic subjects than HBEs from healthy subjects, but the difference was not statistically significant.

By immunocytochemistry the number of HRV-16-infected cells was 5 to 30-fold higher in HBE than in HNE cultures. Essentially similar results were obtained in cultures from different subjects (duplicates of infected and controls). The average number of cells per field, viewed with confocal microscopy, was converted to the average number of cells per unit area of cell sheet. In bronchial cells exposed to virus, the number of positive cells was at least 20 times higher than control (see Figure 3). By contrast, nasal cells exposed to virus showed the number of positive cells to be insignificantly above background. In one experiment, there were 0 ± 0 positives per mm^2 (mean ±SE) in control nasal cells, 5 ± 3 per mm^2 in nasal cells exposed to virus, 0.6 ± 0.6 per mm^2 in unexposed bronchial cells, and 175 ± 32 per mm^2 in exposed bronchial cells. In a second experiment, background was higher, but the results were qualitatively the same: 20 ± 7/mm^2 in control nasal cells, 29 ± 13/mm^2 in exposed nasal, 9 ± 6/mm^2 in control bronchial, and 152 ± 58 per mm^2 in exposed bronchial cells.

Therefore, intracellular HRV-16 RNA load, apical shedding of infectious HRV-16 and immunocytochemistry count of cells infected with HRV-16 all showed that HBE cultures are about 20 times more susceptible to HRV-16 infection than HNE cultures.

Intercellular adhesion molecule-1 (ICAM-1) expression on upper and lower airway

To explore changes in expression of ICAM-1, the receptor for HRV-16, we analyzed mRNA of epithelial cell lysates. HRV-16 infection significantly increased expression of epithelial ICAM-1 mRNA by 1.2 to 1.4 fold in HBEs, but did not alter expression in HNEs (see Table III). There was no difference in ICAM-1 expression between uninfected HNEs and HBEs within group. However, comparison between groups showed that uninfected cells from healthy subjects expressed higher levels of ICAM-1 mRNA than cells from asthmatic subjects ($p=0.04$, GEE analysis).

Cytokine and chemokines mRNA expression after HRV infection

Consistent with our finding of low viral replication in HNE cultures infected with HRV-16, we found little change in mRNA for IL-6, IL-8, IL-1β, tumor necrosis factor α (TNFα), RANTES, or double-stranded RNA dependent protein kinase (PKR) after RV infection in these cells. In HBE cells, RV infection tended to increase expression of mRNA for ICAM-1, IL-8, RANTES, and PKR in both groups, but more markedly in asthmatic subjects (see Table III).

Cytokines and chemokines in basolateral medium

Largely consistent with the changes observed in mRNA expression, we found HRV-16 infection to induce significant production of IL-6, IL-1α, RANTES and IP-10 in samples from HBE cultures from both asthmatic and healthy subjects. HNE cultures did not have significant changes in production of these inflammatory mediators, except for RANTES in HNE cultures from healthy subjects (see Figure 4). There were again no differences in HBE cell production of these chemokines and cytokines between the asthmatic and healthy control groups.

Anti-viral defense mechanisms

Prompted in part by a recent report of a striking difference in HRV activation of anti-viral defense mechanisms in bronchial epithelial cells from asthmatic and healthy subjects [18, 26], we examined changes in mRNA in HBE cultures from 4 asthmatics and 2 healthy subjects of our study for products involved in the type-1 interferon pathways, for defensins, pro-apoptosis genes for neuronal and inducible nitric oxide synthase 2A and 1 (NOS2A, NOS-1) and signal transducer and activator of transcription 1A (STAT1A).

We found that HRV infection caused upregulation of apoptosis genes (Caspase 3 and 7) in HBE cultures from asthmatics and also from healthy subjects. Likewise, there were increases in interferon- β1 (IFN-β1: asthmatic group 74.2 fold induction; $p<0.001$; Healthy: 9.4, $p<0.001$) STAT1A (9.4 fold vs. 5.6 $p<0.001$) and NOS2A (11.6 fold $p=0.004$ vs. 4.6, $p=0.05$) expressions after HRV infection (see Table E1, see Online Repository). Infected bronchial cultures from asthmatics had significant higher mean levels of IFN-β1 and STAT1A than cultures from healthy subjects (see table IV). These data contradict previous findings that asthmatic epithelial cells may be deficient in production of interferons and in induction of apoptosis after HRV infection.

DISCUSSION

The two major findings of this study are that bronchial epithelial cells are more susceptible to infection with HRV-16 than are nasal epithelial cells from the same subjects, and that cultures of either airway site from asthmatic subjects did not have higher response to HRV infection

than cultures from healthy subjects. The first finding rejects our hypothesis that the predominance of upper airway symptoms with most rhinovirus infections is due to a greater susceptibility of nasal vs bronchial cells to HRV infection. Other factors must account for this, such as the site of deposition of infectious particles, whether transmitted by aerosol or by direct contact. Our second finding differs from those of previous investigators [18], who found bronchial epithelial cells from asthmatics to have greater susceptibility to HRV infection than bronchial epithelial cells from healthy volunteers. We believe the difference in our findings may be related to our practice of culturing cells at an air-liquid interface under conditions favoring development of a mucociliary epithelium resembling native epithelium in both structure (full confluence, formation of tight junctions and cilia) and function (electrical resistance, directional ion transport, mucus secretion) [19, 22]. Under these conditions, we found no difference in the susceptibility to RV infection of nasal and bronchial epithelial cells of asthmatic vs. healthy subjects, but did find that in comparison to nasal epithelial cells, bronchial epithelial cells support 20–40 fold greater HRV-16 replication and release of infectious progeny at 24 hours, whether measured as copies viral RNA load, as number of infected cells or as titer of infectious HRV shedding in the apical surface (Fig 1). This greater growth of virus was accompanied by greater production of pro-inflammatory cytokines and chemokines.

Our findings extend those of previous studies showing that human rhinovirus can directly infect epithelial cells of the lower as well as the upper airway [27]. A study using *in situ* hybridization showed HRV-16 to be present in epithelial cells of bronchial biopsies taken from volunteers after experimental infection with the virus [5, 6, 9]. And several *in vitro* studies have shown HRV-16 to replicate well in human bronchial cells cultured on solid support and submerged in medium, which prevents differentiation [7, 9, 10]. Under these culture conditions a study showed that non-differentiated squamous epithelial cells from the adenoids and bronchial epithelial cells obtained from different individuals were similarly susceptible to HRV infection [16]. Our study differed because we collected upper and lower airway respiratory epithelial cells from the same subjects [22], eliminating the effect of individual variability in susceptibility to HRV infection. In addition, we studied differentiated cell cultures [28, 29], which resemble the native pseudocolumnar ciliated epithelium, but are more resistant to infection than undifferentiated single-layer cell cultures [19, 30]. Undifferentiated cells resemble the basal cells of the pseudocolumnar epithelium. A recent study by Jakiela et al. has also demonstrated that basal cells from differentiated traqueobronchial epithelium are more susceptible to HRV than suprabasal cells, suggesting that differentiation is associated with resistance to HRV infection [31]. Taken together, these studies indicate that an intact differentiated epithelium is resistant to HRV infection and that damage to the superficial cells of the respiratory pseudocolumnar epithelium allows HRV to reach and infect the more susceptible basal epithelial cells.

The reason for the increased susceptibility of bronchial epithelial (HBE) cells to HRV compared with nasal (HNE) cells is unclear. Our HBE and HNE cell cultures differed modestly in their electrical properties. HNE tended to have slightly higher electrical resistance than HBE cultures (366 versus 195 $\Omega.cm^2$), but were morphologically similar (see Figure 1). A progressive proximal-to-distal airway decline in the electrical properties of the epithelium has been previously reported from comparison of the potential difference across the nasal, tracheal, and bronchial mucosa in vitro [32, 33]. This difference can not be explained by technique. To minimize the chances of differences in level of differentiation, HBEs and HNEs were collected and cultured in parallel under identical conditions regarding the density of cells plated, time to passage the cells (passage one), time to differentiation and duration of infection. The cells were infected with a single HRV-16 batch using the same MOI (1.0), and the samples of HNE and HBE cells from the same subject were processed together. Despite similar culture conditions, the resulting higher electrical resistance in HNEs than in HBEs raises the hypothesis that HNEs could form a tighter barrier or could express higher levels of ion channels than HBEs, which in turn could possibly increase protection against HRV infection. Another

hypothesis to explain the higher susceptibility of HBEs to HRV infection compared with HNEs is differences in innate immunity to RNA viruses. HNE and HBE cells could express different amounts of virus-RNA sensing molecules such as toll-like receptors and helicases, or of anti-viral molecules[12,35,36] such as human β-defensin (HBD)-2 and interferons. These hypotheses need to be further explored.

Cytotoxicity induced by HRV did not seem to be a confounder in our experiments. HRV did not affect cell viability significantly because epithelial electrical properties declined only modestly and to a similar extent in HNEs and HBEs after HRV infection. We also found few cells infected with HRV-16 by immunocytochemistry in either cell type, although the frequency of infected cells was higher in bronchial cultures than in nasal cultures. The good quality of RNA isolated from cells and non-significant alteration of expression of most genes related to apoptosis also indicate that cell viability was not affected to a large extent. This is in agreement with previous studies on confluent cultures using other rhinovirus strains [9, 11, 16, 34], although RV-49 can be cytotoxic [10]. It is also in agreement with studies of nasal and bronchial biopsies of patients with community-acquired or experimental colds, which have shown little evidence of epithelial cytotoxicity [6, 9]

We did not find statistically higher response to HRV-16 infection in cultures from asthmatic subject group compared with cultures from healthy group as described by Wark et al [18], and Contoli et al [26]. These researchers also studied subjects with mild asthma, but found that undifferentiated bronchial epithelial cells to have a markedly enhanced susceptibility to HRV infection that was associated with impaired production of IFN-β1, type III IFNs, and induction of apoptosis compared with cells from healthy subjects [18, 26]. A potential explanation for our discrepant results could be that culturing in conditions to induce differentiation for 3–4 weeks away from the original airway microenvironment could have generated cells with similar phenotypes between groups. However, if this were true, we would likely not have observed the marked differences between nasal and bronchial cells. In addition, Wark and Contoli et al. [18, 26] also grew their cells *in vitro* for few weeks before conducting their experiments. While we believe that the difference in our findings stems from our use of conditions that favor epithelial cell differentiation, we acknowledge that the infection of so few cells in our cultured cells may compromise our ability to detect differences in upregulation of the genes important in anti-viral defense. We can say, though, that our methods allowed productive infection of HBEs and detection of upregulation of other genes previously described as upregulated by viral infection (e.g., RANTES, IL-8, PKR) [37].

In summary, our data indicate that HBE cells are more susceptible to HRV infection than human HNE cells, in cultures from the same individual. Our data do not show a difference in the susceptibility to HRV infection of nasal or bronchial epithelial cells from asthmatic vs. non-asthmatic, healthy subjects. What remains to be determined, however, is the mechanism for the differences in viral infection between HBE and HNE cells. If our findings apply to native epithelium *in vivo*, further analysis of the mechanisms responsible for the greater resistance of nasal epithelial cells to viral infection may suggest approaches for prevention or treatment of acute viral bronchitis in healthy people and of virally-induced exacerbations of asthma, COPD, cystic fibrosis, and other airway diseases.

Acknowledgments

This work was supported by NIH grant P01AI050496. PCA and SF are currently supported by the Ernest S. Bazley Grant to Northwestern University.

We are grateful to Samantha Donnelly, Ph.D. and Jenny Shen, B.S. for their assistance in gene expression profiling.

List of Abbreviations

TCID50
: Tissue culture infective dose

HBE
: Human bronchial epithelial

HNE
: Human nasal epithelial

HRV
: Human rhinovirus

Rte
: transepithelial resistance

Vte
: transepithelial potential difference

Ieq
: equivalent short circuit current

ICAM-1
: intercellular adhesion molecule-1

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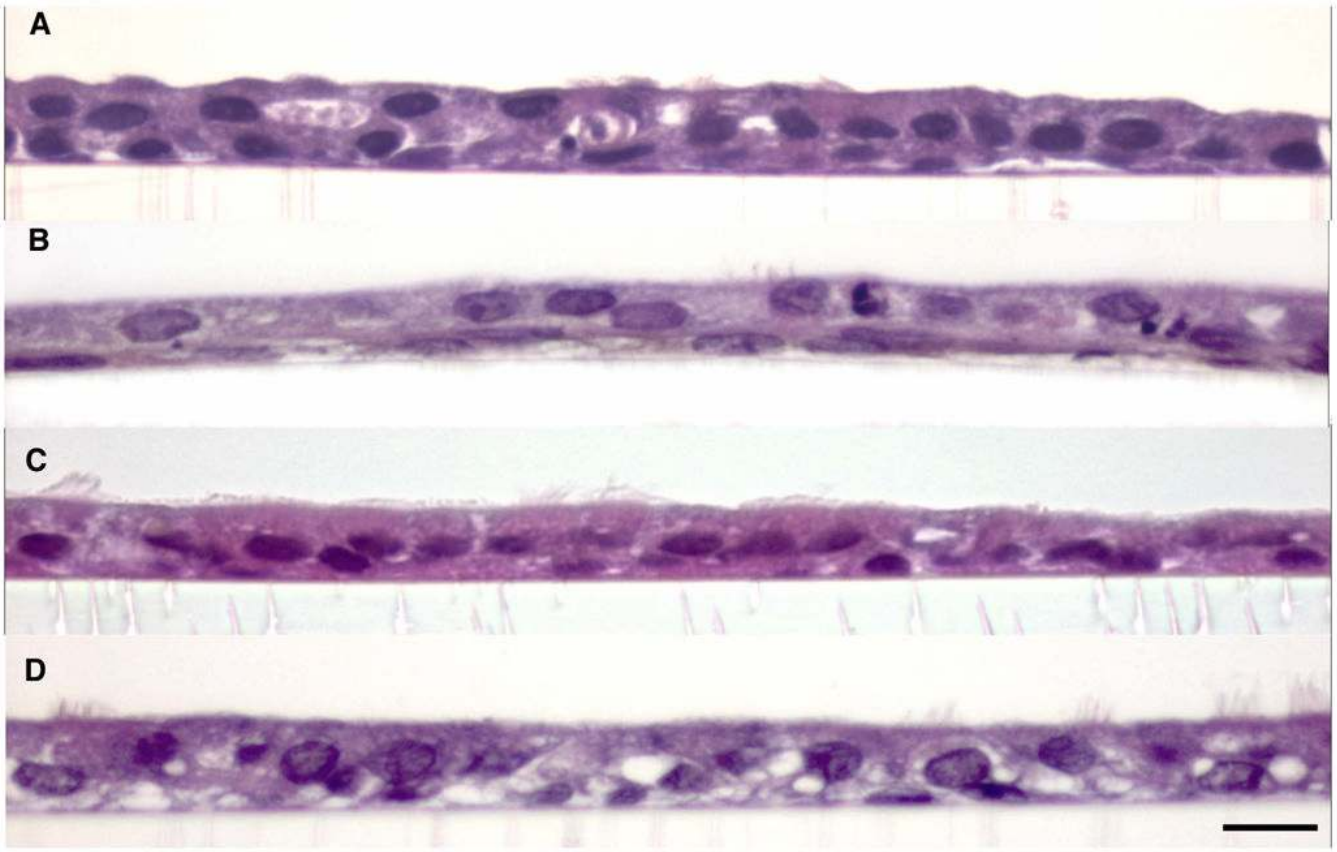


Figure 1.
Light microscopy of epithelial cultures. Cross-sections of passage 1 nasal (A,B) and bronchial (C.D) epithelial cell cultures from control (A,C) and asthmatic (B,D) subjects grown at an air-liquid interface for 21 days and stained with hematoxylin and eosin. Cultures obtained from all groups showed similar morphology forming multilayered sheets containing variable numbers of ciliated cells and scattered intraepithelial vacuoles. Scale bar = 25 μm.

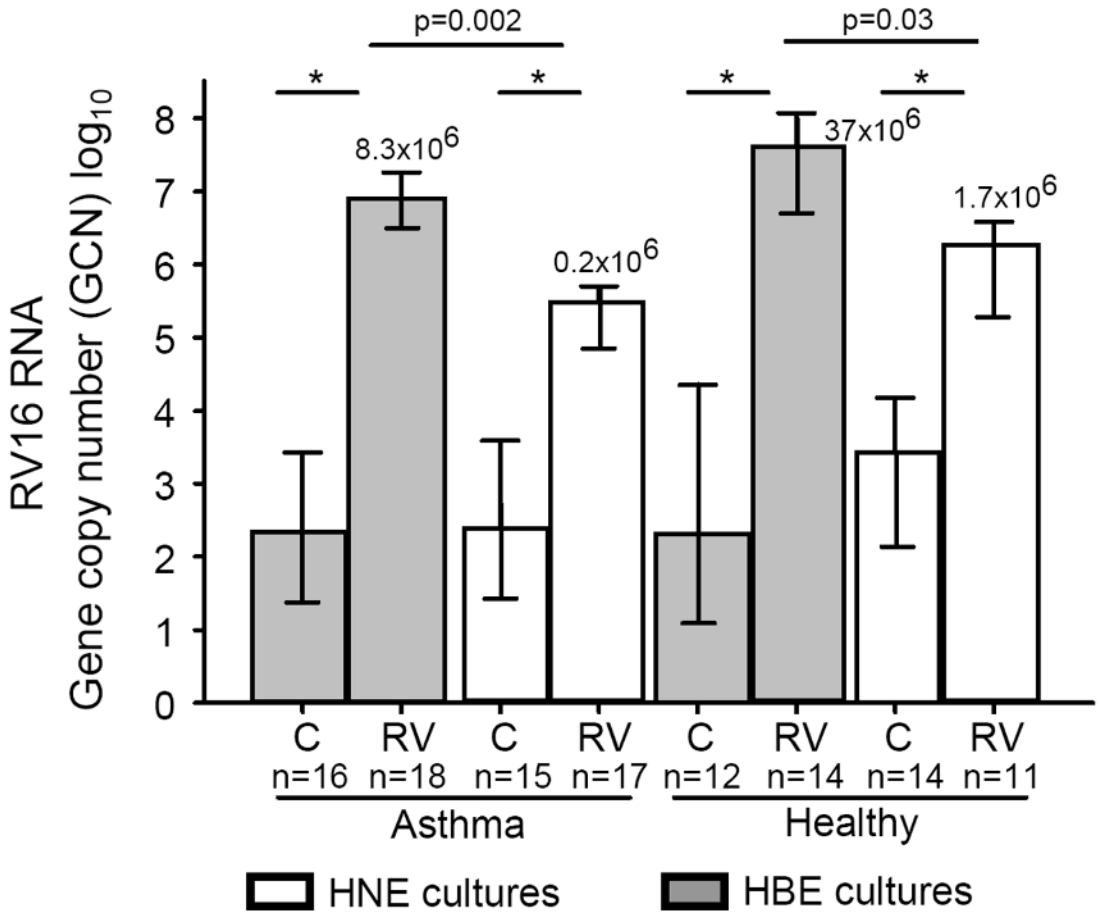
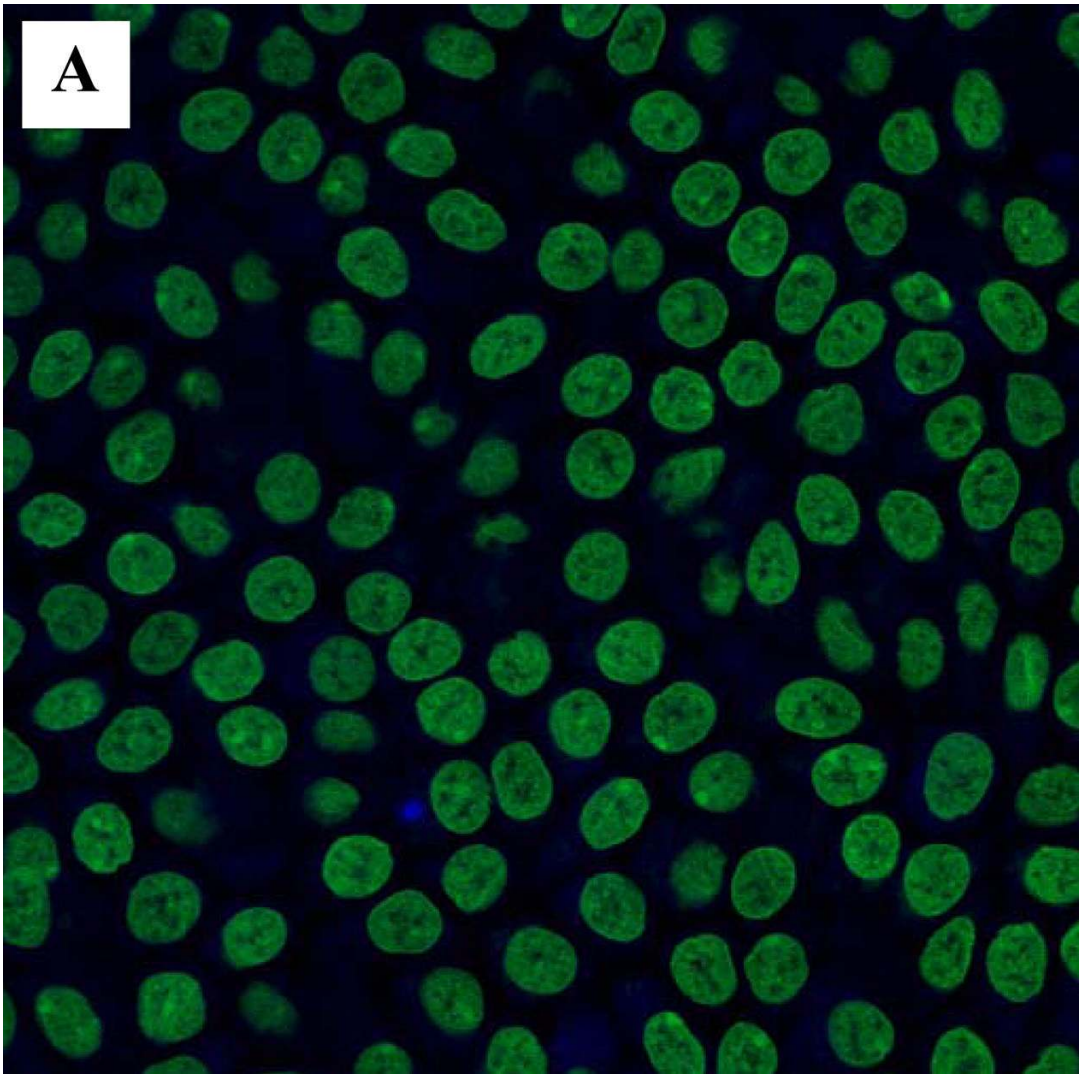


Figure 2.

HRV-16 replication on human nasal (HNE) and bronchial (HBE) epithelial cells measured by real-time PCR 48hs after viral inoculation. Intracellular HRV RNA load was higher in all infected compared to non-infected control cultures (*p<0.002, by GEE) from asthmatics (n=6) and healthy (n=5) subjects. HRV replicated to a greater extent in HBE than in HNE in both groups. There were no differences between groups for nasal cells or bronchial cells. Bars represent median and whiskers the interquartile range.



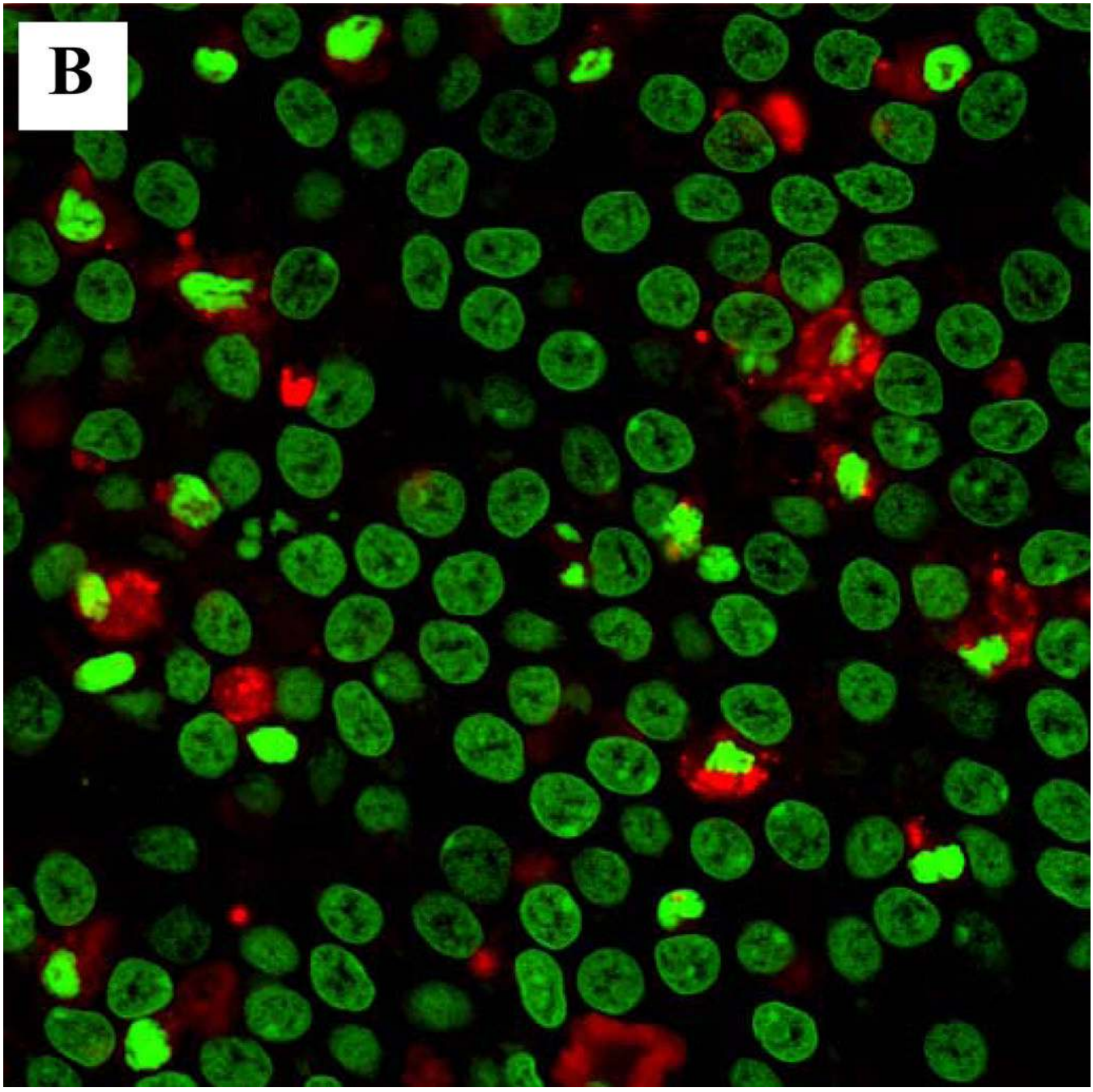


Figure 3.
Immunocytochemistry for rhinovirus RNA (HRV-16). HRV-16 virus capsid protein 2 was stained with Cyanine 3 (CY-3, red) and nucleic acid were stained with YO-PRO®-1 iodide (green). **A**: Micrograph of non-infected control HBE cells showing no HRV-16. B: Micrograph of infected HBE cells showing high number of HRV-16-infected cells, some with condensed chromatin/nuclei (bright green).

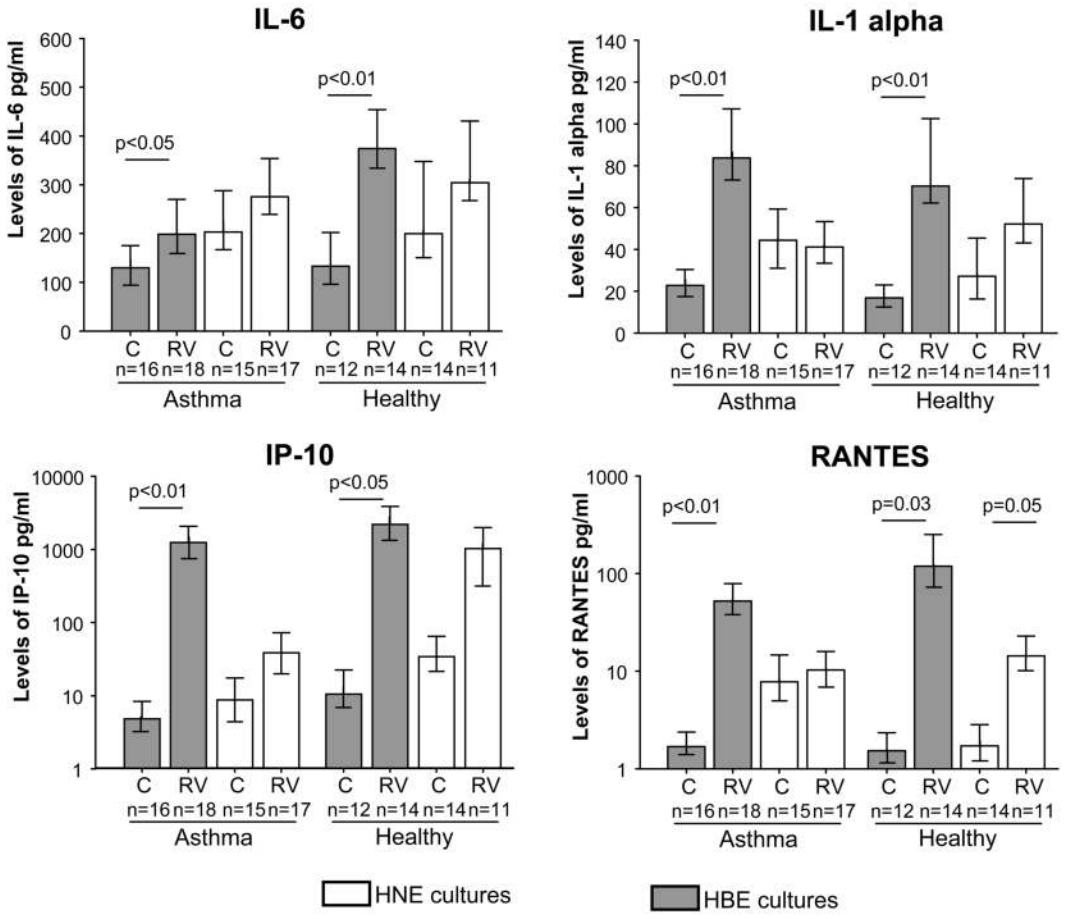


Figure 4.

Levels of interleukin (IL) -6, IL-1α, IP-10, RANTES in supernatants from HBE and HNE cultures collected 48h after HRV-16 (RV16) or sham (C, control) inoculation. Cultures from asthmatic (n=6) and healthy (n=5) subjects displayed similar results. HBE cultures, but not HNE cultures, produced greater amounts of mediators after HRV-16 infection. Bars represent median and whiskers the interquartile range. Data analysis by GEE.

Table I

Baseline characteristics of the subjects

	Asthma	Healthy	p value
	N=6	N=5	NA
SEX (% male)	67%	60%	0.40
Age (years)	33 (24–50)	37 (32–46)	0.43
FEV1% predicted	70 (59–84)	104 (90–124)	<0.01
PC_{20} (mg/ml)	0.509 (−0.03- 1.75)	>64	<0.01

Values are displayed as median and its 95% confidence interval. Allergy skin test (allergens to cat, house dust mites, local pollens and molds) showed that no healthy subject was allergic and all asthmatic subjects were allergic to at least 4 allergens. Groups were compared using Mann-Whitney rank test for continuous variables and Fisher exact test for proportions.

Table II

Viral shedding into apical side of nasal (HNE) and bronchial (HBE) epithelial cells infected with HRV-16 from asthmatics and healthy subjects.

	Asthmatic n=5	cultures	Healthy n=4	cultures	p value	All subjects n=9
HNE	1.0 (0.5–1.5)	n=20	0.5 (0.5–1.0)	n=13	0.17	0.5 (0.5–1.5)
HBE	2.5 (1.5–3.0)	n=20	1.5 (1.0–2.5)	n=16	0.07	2.0 (1.0–2.5)
p value	<0.001		0.03			<0.001

Virus titers were determined by $TCID_{50}$ assay in washes of the apical side of epithelial cells cultures 24 h after infection (See Methods). Titers are expressed as median (95% confidence interval) of $\log_{10}(TCID_{50}/0.1ml)$ values. Titers prior to infection and in sham-infected cultures were undetectable. p values were obtained by Generalized Estimating Equations.

Table III

mRNA expression of inflammatory markers in nasal (HNE) and bronchial (HBE) epithelial cells

	Asthmatic subjects n=6									
	HNE				HBE					Asthma vs. Healthy HNE
Cultures	**Infected n=21**	**Controls n=16**	**p***	**Ratio**	**Infected n=24**	**Controls n=21**	**p***	**Ratio**	**p†**	**p‡**
ICAM-1	11.6 (7.1–16.7)	12.3 (6.0–17.4)	ns	0.9	11.8 (10.1–15.0)	10.2 (8.2–12.0)	0.06	1.2	ns	ns
IL-6	1.2 (0.4–28.9)	8.4 (0.5–32.2)	ns	0.2	2.0 (0.4–15.4)	2.4 (0.3–12.5)	0.04	0.9	ns	ns
IL-8	20.8 (9.0–23.4)	21.3 (6.7–27.2)	ns	1.0	13.6 (8.8–23.0)	12.1 (8.0–14.0)	0.07	1.1	ns	ns
RANTES	0.25 (0.16–0.64)	0.10 (0.05–0.5)	ns	2.5	2.5 (1.8–5.3)	0.04 (0.02–0.12)	0.006	56.6	0.01	ns
PKR	9.4 (5.9–11.6)	6.1 (4.3–9.3)	<0.001	1.5	22.1 (18.2–37.3)	5.9 (3.8–7.5)	<0.001	3.7	0.001	0.06
IL-1β	1.5 (0.5–2.3)	2.6 (0.32–4.4)	ns	0.6	0.5 (0.32–0.85)	0.7 (0.17–1.1)	ns	0.6	0.01	ns
TNF-α	0.65 (0.4–1.8)	0.95 (0.51–1.5)	ns	0.7	0.19 (0.13–0.39)	0.17 (0.10–0.38)	ns	1.1	0.04	ns

	Healthy subjects n=5									
	HNE				HBE					Asthma vs. Healthy HBE
Cultures	Infected n=18	Controls n=15	p*	Ratio	Infected n=22	Controls n=17	p*	Ratio	p†	p‡
ICAM-1	17.4 (112.0–30.6)	23.3 (11.7–52.6)	ns	0.7	29.3 (18.6–81.1)	20.8 (9.9–31.3)	0.04	1.4	0.04	0.04
IL-6	2.92 (0.6–8.1)	4.6 (0.6–16.7)	ns	0.6	3.2 (1.2–6.4)	0.89 (0.51–27.4)	ns	3.5	ns	ns
IL-8	26.6 (10.7–54.7)	31.2 (14.0–59.7)	ns	0.9	45.2 (24.9–99.2)	31.9 (11.2–52.0)	0.01	1.4	0.01	0.02
RANTES	0.49 (0.2–0.8)	0.07 (0.015–0.11)	ns	6.8	2.0 (1.2–6.2)	0.04(0.003–0.8)	0.03	43.9	0.04	ns
PKR	27.4 (7.8–76.8)	10.7 (5.3–55.9)	0.006	2.5	38.5 (28.3–161.6)	11.0(5.5–38.7)	0.01	3.5	0.02	0.07
IL-1β	2.6 (1.2–5.4)	2.2 (0.85–13.8)	ns	1.2	2.0 (0.42–3.0)	0.63 (0.25–1.6)	0.07	3.2	ns	ns
TNF-α	0.74 (0.39–1.5)	0.63 (0.27–1.8)	ns	1.2	1.4 (0.21–1.8)	0.32 (0.10–1.18)	0.05	4.5	ns	0.03

Gene expression of inflammatory markers was determined by real-time RT-PCR (see methods). Messenger RNA levels were expressed as relative gene copy numbers (RGCN) and presented as median ($\times 10^6$) and its 95% confidence interval; p values were calculated between infected and control conditions for each type of epithelial cell; ns= p value non-significant *Definition of abbreviations*: IL-6 (interleukin 6), IL-8 (interleukin 8), IL-1β (interleukin 1beta), PKR (interferon-inducible double stranded RNA dependent protein kinase), TNF-α (tumor necrosis factor-alpha). p values were obtained by Generalized Estimating Equations.

* p (infected vs. control),

† p (infected HNE vs. infected HBE),

‡ p (infected cultures).