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# Respiratory Viruses Augment the Adhesion of Bacterial Pathogens to Respiratory Epithelium in a Viral Species- and Cell Type-Dependent Manner

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**Secondary bacterial infections often complicate respiratory viral infections, but the mechanisms whereby viruses predispose to bacterial disease are not completely understood. We determined the effects of infection with respiratory syncytial virus (RSV), human parainfluenza virus 3 (HPIV-3), and influenza virus on the abilities of nontypeable *Haemophilus influenzae* and *Streptococcus pneumoniae* to adhere to respiratory epithelial cells and how these viruses alter the expression of known receptors for these bacteria. All viruses enhanced bacterial adhesion to primary and immortalized cell lines. RSV and HPIV-3 infection increased the expression of several known receptors for pathogenic bacteria by primary bronchial epithelial cells and A549 cells but not by primary small airway epithelial cells. Influenza virus infection did not alter receptor expression. Paramyxoviruses augmented bacterial adherence to primary bronchial epithelial cells and immortalized cell lines by up-regulating eukaryotic cell receptors for these pathogens, whereas this mechanism was less significant in primary small airway epithelial cells and in influenza virus infections. Respiratory viruses promote bacterial adhesion to respiratory epithelial cells, a process that may increase bacterial colonization and contribute to disease. These studies highlight the distinct responses of different cell types to viral infection and the need to consider this variation when interpreting studies of the interactions between respiratory cells and viral pathogens.**

Respiratory infections are a leading cause of morbidity and mortality, but our understanding of their pathogenesis remains incomplete. Recent studies have drawn attention to the role that viral infections may play in predisposing persons to bacterial respiratory infections. There is considerable epidemiologic evidence that viral respiratory infections, particularly those caused by influenza virus and respiratory syncytial virus (RSV), increase the incidence and severity of severe secondary bacterial complications, such as pneumonia and sepsis (6, 18, 20, 33). Reducing the number of viral infections by immunization reduces the incidence of bacterial infections, supporting this concept (4, 35).

Viral infections might predispose to bacterial secondary infections by damaging respiratory epithelium, impairing mucociliary function, and triggering host inflammatory responses (26, 43). The most common bacterial respiratory pathogens, nontypeable *Haemophilus influenzae* (NTHi) and *Streptococcus pneumoniae*, are isolated more frequently and in larger numbers from the sputum of patients with viral infections than from those without these infections, suggesting a link exists between viral infection and bacterial colonization (36, 42). Jiang and colleagues noted that RSV infection of A549 respiratory epithelial cells significantly increased numbers of NTHi bacteria adhering to these cells, providing experimental support for this hypothesis (17). Influenza A virus and rhinovirus infection of tracheal epithelial cells similarly increased the number of adherent *S. pneumoniae*, indicating that the ability to augment bacterial adherence to host cells may be a general feature of respiratory viruses (16, 31).

NTHi adheres to a diverse group of host cell molecules on the respiratory epithelium. Phosphorylcholine residues present on NTHi lipoligosaccharide interact with PAF-r (37), P5 fimbriae specifically bind to host CEACAM1 (15) and ICAM-1 (2a), and sialic acid-containing oligosaccharides bind to respiratory epithelial mucin (32). Mucin also plays an important role in the adherence of NTHi. Pneumococcal cell wall phosphorylcholine also mediates bacterial adherence to lung epithelial cells via PAF-r (8). To clarify how viral infection modulates the adhesion of bacterial pathogens to respiratory epithelium, we determined how infection with the ubiquitous human respiratory viruses RSV, human parainfluenza virus 3 (HPIV-3), and influenza virus altered NTHi and pneumococcal adhesion to immortalized and primary respiratory cells and the expression of known epithelial receptors for these bacteria. Each of these viruses increased bacterial adhesion to respiratory epithelial cells. The mechanisms by which viruses promoted bacterial colonization, however, varied between respiratory pathogens and were cell type dependent. These studies also demonstrated that different respiratory epithelial cells

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have distinct responses to infection by the same virus. These differences must be considered when extrapolating results of in vitro assays to human disease.

## MATERIALS AND METHODS

**Epithelial cell culture.** A549 cells (American Type Culture Collection [ATCC], Manassas, VA) were grown in Hams F-12K medium (ATCC) with 10% heat-inactivated fetal bovine serum (Cambrex Corporation, East Rutherford, NJ). BEAS-2B human bronchial epithelial cells (ATCC) were propagated in flasks coated with 0.01 mg/ml fibronectin (Sigma, St. Louis, MO), 0.03 mg/ml collagen (Vitrogen 100; Collagen Corporation, Palo Alto, CA), and 0.01 mg/ml bovine serum albumin (BD Biosciences, San Jose, CA) in supplemented bronchial epithelial growth medium (Cambrex). Primary normal human small airway (SAE) and primary human bronchial epithelial (NHBE) cells (Cambrex) were maintained in supplemented serum-free medium (Cambrex) and used at the fourth passage.

**Virus and bacterial strains.** RSV (A2) Long strain stocks were prepared as described previously (19). Influenza virus strain A/Panama/2007/99 (H3N2) and HPIV-3 were obtained from the viral repository of St. Jude Children's Research Hospital. For NTHi, most assays were performed with NTHi strain 778, a clinical isolate obtained from both blood and respiratory tract cultures of an infant with pneumonia (29). This isolate expresses the Hap and high-molecular-weight 1 and 2 (HMW1/HMW2) adhesins, pili (Hif), P5 fimbriae, and phosphorylcholine (data not shown). Other NTHi strains tested included wild-type strains 781 (*hap*$^+$ *hmw*$^+$; lacking *hia* and *hif*), 786 (*hap*$^+$ *hia*$^+$; lacking *hmw* and *hif*), and 801 (*hap*$^+$ *hif*$^+$; lacking *hmw* and *hia*), Rd, and the encapsulated serotype B strain Eagan. NTHi was propagated in brain heart infusion broth (Difco, Sparks, MD) with 10 $\mu$g/ml hemin (Sigma) and 1 $\mu$g/ml of $\alpha$-NAD (Sigma) at 37°C in 5% $CO_2$. *S. pneumoniae* strain 357 (provided by E. Tuomanen, St. Jude Children's Research Hospital) is a serotype 19 nasopharyngeal isolate that expresses phosphorylcholine. It was propagated in C+Y medium at 30°C with 5% $CO_2$ (21). Bacteria were grown overnight in liquid broth, diluted 1:10 in fresh medium, and grown to log phase. Prior to assays, bacteria were washed three times in phosphate-buffered saline (PBS) and diluted in tissue culture medium to $1 \times 10^6$ CFU/ml. Bacterial numbers were determined by optical density at 600 nm and confirmed by plating dilutions on agar plates.

**Viral infection of epithelial cells.** Respiratory epithelial cells were grown to confluence and triplicate wells incubated with viruses at a multiplicity of infection (MOI) of one virus/cell for 1 h at 37°C in 5% $CO_2$ and washed, and medium was replaced. At 24-h intervals, cells were detached from plates using cell dissociation solution (CDS) (Sigma), and the percentages of cells expressing viral antigens were determined by fluorescence-activated cell sorting (FACS). Cells were resuspended in 1% bovine serum albumin in PBS and incubated for 1 h at 37°C with a 1/1,000 dilution of fluorescein isothiocyanate (FITC)-labeled goat anti-RSV (Chemicon International, Temicula, CA) or anti-HPIV-3 antibody (Ab) (Chemicon) or a 1/4,000 dilution of pooled mouse monoclonal antibodies (MAb) (immunoglobulin $G_1$ [$IgG_1$]) raised against the influenza virus nucleoprotein (41), followed by a 1/500 dilution of FITC-labeled goat antimouse secondary Ab (BD Biosciences), and analyzed on a FACSCalibur instrument using CellQuest software (BD Biosciences).

**Bacterial adhesion.** Epithelial cells, approximately $5 \times 10^5$ cells, were seeded in 24-well plates (Costar, Corning, Inc., Corning, NY) and triplicate wells inoculated with viruses as described above. At 24-h intervals after viral infection, cells were washed and treated with CDS, and numbers of cells per well were determined in parallel wells using a hemocytometer. Cell monolayers were incubated with bacteria for 1 h at 37°C, washed to remove loosely adherent bacteria, and detached from plates by incubation with 1× trypsin–EDTA (Mediatech, Inc., Herndon, VA), and serial dilutions were plated for quantitative cultures. For each assay, numbers of adherent bacteria were normalized to numbers of epithelial cells.

FACS analysis was used to determine bacterial binding to live and dead cells following viral infection. NTHi bacteria were labeled by incubation with FITC (1 mg/ml) (Sigma) in carbonate buffer containing 0.1 M NaCl, 0.09 M $Na_2CO_3$, and 0.015 M $NaHCO_3$, pH 9.2, for 30 min. Bacteria were then washed twice with PBS and resuspended in cell culture medium. FITC-labeled NTHi bacteria were then incubated with uninfected or RSV-infected A549 cells as described above, washed thrice with PBS, and detached using CDS. Cells ($10^6$) were resuspended in PBS containing 2 $\mu$g/ml of propidium iodide (PI) (Amersham Biosciences, Piscataway, NJ). Cells were analyzed on a FACSCalibur instrument using CellQuest software. Single-color controls containing only FITC-labeled bacteria or PI-stained cells were analyzed, and then the percentage of FITC-labeled NTHi bacteria binding to live (PI−) or dead (PI+) cells was quantified. A total of 10,000 events were acquired using forward and side scatter and gated to exclude cell debris.

**Cell surface receptor expression.** Epithelial cells were inoculated with viruses and, at 24-h intervals, detached with CDS, and $10^6$ cells resuspended in 1 ml of 1% bovine serum albumin in PBS. Cells were then incubated for 1 h at 25°C with a 1/1,000 dilution of mouse anti-human ICAM-1 (CD54/ICAM-1) ($IgG_1$; BD Biosciences), anti-human CEACAM1 ($IgG_1$; Calbiochem, La Jolla, CA, or Genovac GmbH, Freiberg, Germany), anti-human PAF-r MAb (Cayman Chemical, Ann Arbor, MI), or purified mouse $IgG_1$ Ab (an isotype control; BD Biosciences), followed by incubation with a 1/500 dilution of FITC-labeled goat antimouse Ab (BD Biosciences) for 30 min at 4°C prior to analysis. The mean fluorescence intensity of cells was compared to that of uninfected cells after subtracting background produced by the isotype control Ab.

**Mucin PCR.** A549 and SAE cells were infected with RSV as described above. At 24-h intervals, total RNA was extracted using Trizol (Invitrogen), according to the manufacturer's instructions, and *muc1* and *muc5AC* (which are expressed by respiratory epithelial cells) mRNA expression was evaluated by reverse transcription-PCR. RNA was treated with DNase (Promega), and 4 $\mu$g of total RNA was reverse transcribed using 200 U of Superscript II reverse transcriptase (Invitrogen) at 42°C for 55 min. cDNA (2 $\mu$l) was amplified by using *Taq* DNA polymerase (Promega) and the following amplification primers: *muc1* forward (5′-GCACTTCTCCCCAGTTGTCTACTG-3′) and reverse (5′-ATGGCCAGC GCAACCACCAGAACACAG-3′); *muc5AC* forward (5′-TACAACAACATCA TCACGAGTGCG-3′) and reverse (5′-TAGGGTGCTAGGAGCTGTCACAG-3′) (39). Amplification products were separated on 1.5% agarose gels and stained, and intensities of bands were normalized to that of $\beta$-actin amplified in parallel (38).

**Western blot analysis.** Epithelial cells were infected as described above. At 24-h intervals, $10^7$ cells were suspended in 1 ml of 300 mM NaCl, 50 mM Tris-HCl, pH 7.6, 0.5% Triton X-100, and then centrifuged at $10,000 \times g$ for 15 min at 4°C. The protein concentration of supernatants was determined by Bradford assay (Bio-Rad, Hercules, CA). Proteins (30 $\mu$g) were separated on a 7.5% sodium dodecyl sulfate-polyacrylamide gel, electroblotted onto Protran membranes (Schleicher & Schuell, Keene, NH) and incubated with mouse anti-human ICAM-1 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), mouse anti-human CEACAM1 MAb (Calbiochem), rabbit anti-human PAF-r Ab (Cayman Chemical) at a 1:1,000 dilution, or mouse anti-human $\beta$-actin Ab (Sigma) at a 1:5,000 dilution for 1 h at 37°C. Bound Ab was detected using peroxidase-conjugated goat anti-mouse or goat anti-rabbit Ab (1/4,000 dilution) and chemiluminescent substrate (ECL Western blotting analysis system; Amersham Biosciences, Piscataway, NJ). Differences in protein expression were quantified by integrated density analysis (Eagle Sight software; Stratagene) and normalized to $\beta$-actin expression on the same membrane.

**Inhibition of bacterial adhesion.** The ability of NTHi to adhere to RSV-infected A549 cells was assessed after incubation of A549 cells with increasing concentrations (5 to 25 $\mu$g/ml) of purified mouse anti-human CD54/ICAM-1 monoclonal antibody (BD Biosciences), purified mouse anti-human PAF-r monoclonal antibody (Cayman Chemical), or mouse anti-human LFA-3/CD58 antibody (BD-Biosciences) for 1 h at 37°C.

**Statistical analysis.** Data are expressed as means ± standard errors. Comparison between groups was performed using the Mann-Whitney U test, and other data were analyzed by Student's *t* test, with *P* values of $<0.05$ considered significant.

## RESULTS

**Viral infection of respiratory epithelial cells.** The proportion of epithelial cells expressing viral antigens was analyzed to determine the relative efficiency of viral infection. The percentage of cells expressing viral antigens increased with time after inoculation and was generally comparable among the different cell types and viruses (Table 1).

The viability of epithelial cells was determined using trypan blue exclusion. After 48 h of infection with all viruses, at least 93% of all cell types remained viable. After 72 h, 87 to 90% of cells infected with HPIV-3 or influenza virus and 80 to 85% of cells infected with RSV remained viable.

**Antecedent respiratory viral infection increased NTHi and *S. pneumoniae* adhesion to respiratory epithelial cells.** The proportion of the inoculum of NTHi strain 778 adhering to

TABLE 1. Percentages of cells expressing viral antigens following viral infection[a]

| Time (h) | % of cells expressing antigen after infection with: | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | RSV | | | | HPIV | | Influenza virus | |
| | A549 | BEAS-2B | SAEC | NHBE | A549 | NHBE | A549 | NHBE |
| 24 | 3 ± 0.7 | 4 ± 0.8 | 5 ± 0.8 | 10 ± 1.2 | 2 ± 0.8 | 7 ± 0.7 | 2 ± 0.3 | 6 ± 2.2 |
| 48 | 43 ± 6.3 | 45 ± 2.6 | 15 ± 1.7 | 52 ± 3.5 | 33 ± 9.8 | 35 ± 3.8 | 28 ± 3.6 | 32 ± 4.1 |
| 72 | 69 ± 12 | 68 ± 3.2 | 55 ± 3.4 | 71 ± 6.7 | 66 ± 8.5 | 65 ± 4.8 | 61 ± 8.1 | 59 ± 7.5 |

[a] A549, immortalized human type II alveolar cells; BEAS-2B, immortalized human bronchial epithelial cells; SAE, primary small airway epithelial cells; NHBE, primary human bronchial epithelial cells.

A549 cells was inoculum dependent: 15% at an MOI of 2 bacteria per cell, 11% at an MOI of 20, and 10% at an MOI of 200. For *S. pneumoniae* 357, the proportion of adherent bacteria was 6% at an MOI of 2, 3.5% at an MOI of 20, and 2.2% at an MOI of 200 (data not shown). An MOI of 2 was used for subsequent studies.

Adhesion of both NTHi and *S. pneumoniae* to respiratory epithelial cells was significantly increased following infection with RSV (Fig. 1). Adherence of NTHi strains 781, 786, 801, and Rd to A549 cells also increased by 4.7-, 3.5-, 3-, and 6-fold, respectively, after 72 h of RSV infection. In -contrast, adhesion of the encapsulated strain Eagan to RSV-infected A549 cells increased by only 1.4-fold (data not shown).

A549 cells were then infected with HPIV-3 and influenza virus to determine if the augmentation of bacterial adhesion observed with RSV was a general feature of respiratory viral infections. Infection with each of these viruses increased numbers of adherent NTHi and pneumococci (Fig. 2). NTHi and pneumococcal adherence to HPIV-3-infected A549 and NHBE cells increased progressively over 72 h. While NTHi

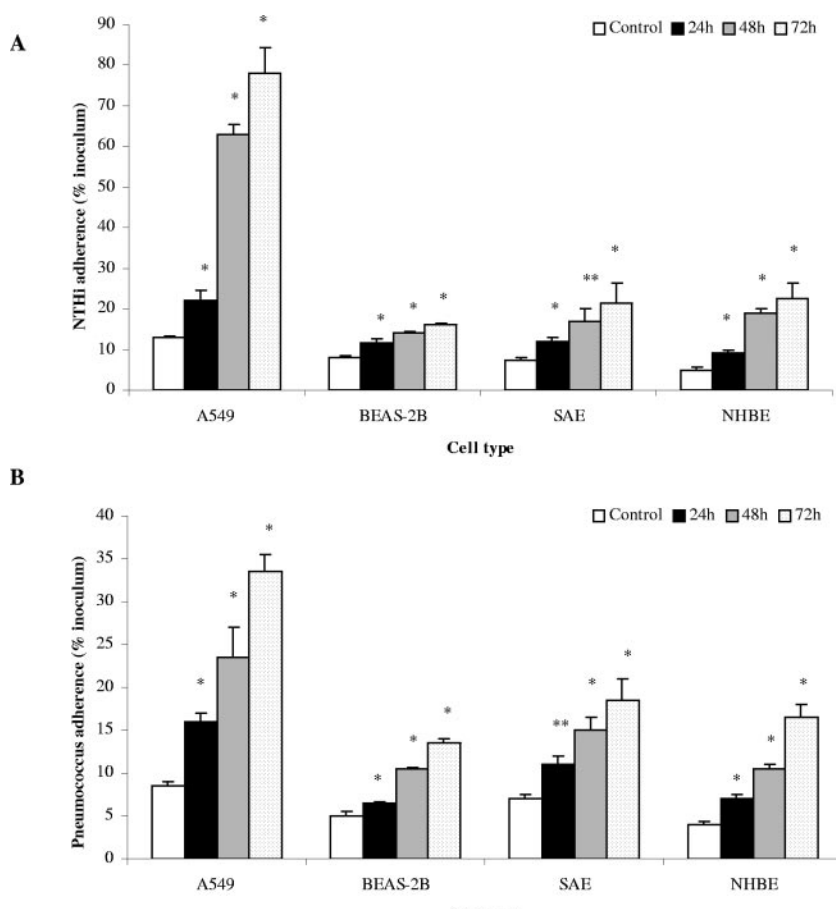


FIG. 1. Adhesion of NTHi and *S. pneumoniae* to respiratory epithelial cells is increased following infection with RSV. Respiratory epithelial cells were infected with RSV and at 24-h intervals incubated with NTHi-778 or *S. pneumoniae*. Adhesion is expressed as a percentage of inoculum. Antecedent infection of all cells with RSV resulted in a statistically significant increase in bacterial adhesion (*, $P < 0.005$; **, $P < 0.05$) compared to control cells not infected by RSV. (A) Adhesion of NTHi to respiratory epithelial cells. (B) Adhesion of pneumococci to respiratory epithelial cells. Data represent the mean ± standard error of the mean for six separate experiments.

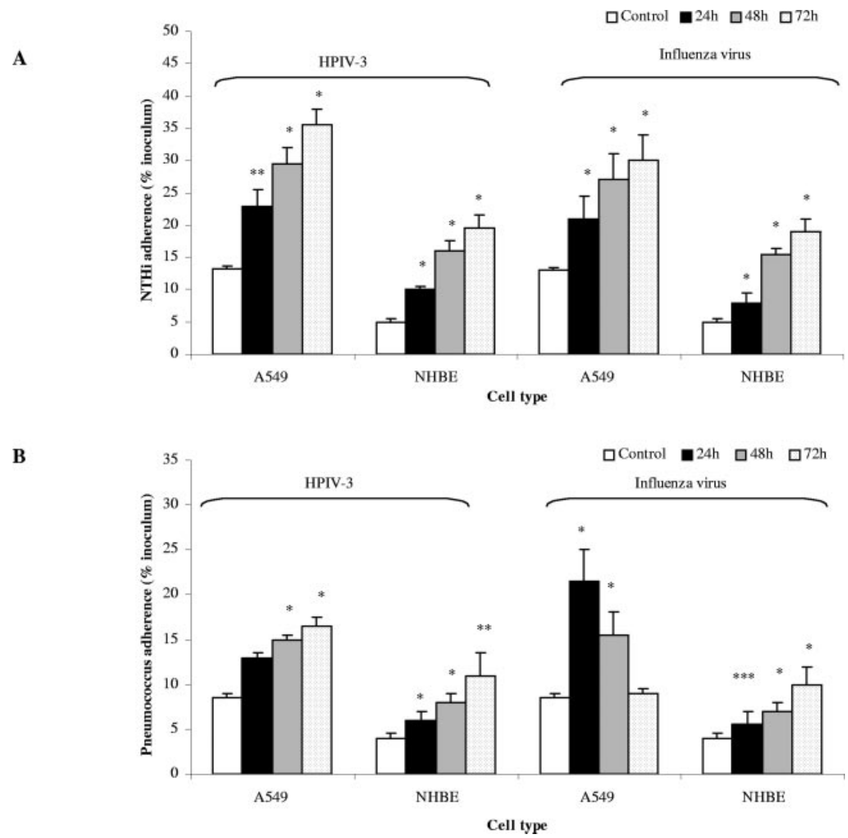


FIG. 2. Adhesion of NTHI and *S. pneumoniae* to respiratory epithelial cells is increased following infection with HPIV-3 and influenza virus. Respiratory epithelial cells were infected with HPIV-3 or influenza virus and at 24-h intervals incubated with NTHi-778 or *S. pneumoniae*. Adhesion is expressed as a percentage of inoculum. Bacterial adhesion was significantly increased to virally infected cells compared to adhesion to control cells not infected by viruses (*, $P < 0.005$; **, $P < 0.01$; ***, $P < 0.05$). (A) Adhesion of NTHi to respiratory epithelial cells. (B) Adhesion of pneumococci to respiratory epithelial cells. Data represents the means ± standard errors of the means of six separate experiments.

adherence to influenza virus-infected A549 and NHBE cells and pneumococcal adherence to influenza virus-infected NHBE cells similarly increased with time, pneumococcal adherence to influenza virus-infected A549 cells was maximal at 24 h after viral infection and fell to baseline levels of adherence at 72 h (Fig. 2).

FACS analysis demonstrated that in the absence of RSV infection, a mean of 95% of NTHi bacteria bound to the 95% of the cells that were viable. At 48 h after RSV infection, a mean of 89% of NTHi bacteria bound to the 91% of the cells that remained viable. Thus, NTHi bound live and dead cells approximately equally for both uninfected cells and cells infected with RSV (data not shown).

**RSV infection increased expression of eukaryotic receptors for respiratory bacteria in a cell type-dependent fashion.** We then determined if infection with RSV, HPIV-3, or influenza virus altered the expression of known epithelial receptors for these bacteria and augmented bacterial adhesion to epithelial cells. By FACS analysis, expression of ICAM-1 was only slightly greater than that of control uninfected A549 cells after 24 h of RSV infection but increased 30-fold ± 2-fold at 48 h ($P < 0.001$) and 50-fold ± 3-fold ($P = 0.008$) after 72 h (Fig. 3). CEACAM1 expression also did not differ from baseline expression at 24 h but increased 8-fold ± 1-fold after 48 h ($P = 0.006$) and 9-fold ± 1-fold ($P = 0.008$) at 72 h after RSV infection. PAF-r expression could not be quantified by FACS due to the low density of receptors on the cell surface. Western blot analysis of A549 cells lysates confirmed the increased expression of ICAM-1 and CEACAM1 and demonstrated increased PAF-r expression after infection with RSV (Fig. 3). ICAM-1 expression increased by up to 34-fold ± 3-fold and CEACAM1 expression by up to 12-fold ± 1-fold over the 72 h after RSV infection. PAF-r expression was unchanged at 24 h but increased by 4-fold ± 1-fold at 72 h.

RSV infection also increased expression of ICAM-1 and PAF-r by BEAS-2B cells (Fig. 3). Expression of ICAM-1 increased 1.2-fold ± 0.2-fold at 24 h, 1.3-fold ± 0.2-fold at 48 h, and 1.6-fold ± 0.2-fold ($P = 0.03$) at 72 h. PAF-r expression increased 1.1-fold ± 0.1-fold at 24 h, 1.5-fold ± 0.2-fold ($P = 0.03$) at 48 h, and 2-fold ± 0.3-fold ($P = 0.02$) at 72 h after RSV infection. Expression of CEACAM-1 by BEAS-2B cells, however, was not altered after RSV infection.

RSV infection also increased the expression of ICAM-1 by NHBE cells by 3.5-fold ± 0.8-fold at 48 h and 7-fold ± 1-fold ($P = 0.008$) at 72 h (Fig. 3) and CEACAM1 expression by 3-fold ± 0.5-fold ($P = 0.01$) at 48 h and 4-fold ± 0.3-fold ($P = 0.02$) at 72 h. Expression of PAF-r by NHBE cells did not increase. In contrast to other cells, RSV did not significantly alter the expression of ICAM-1, CEACAM1, and PAF-r by SAE cells (data not shown). Finally, RSV infection did not

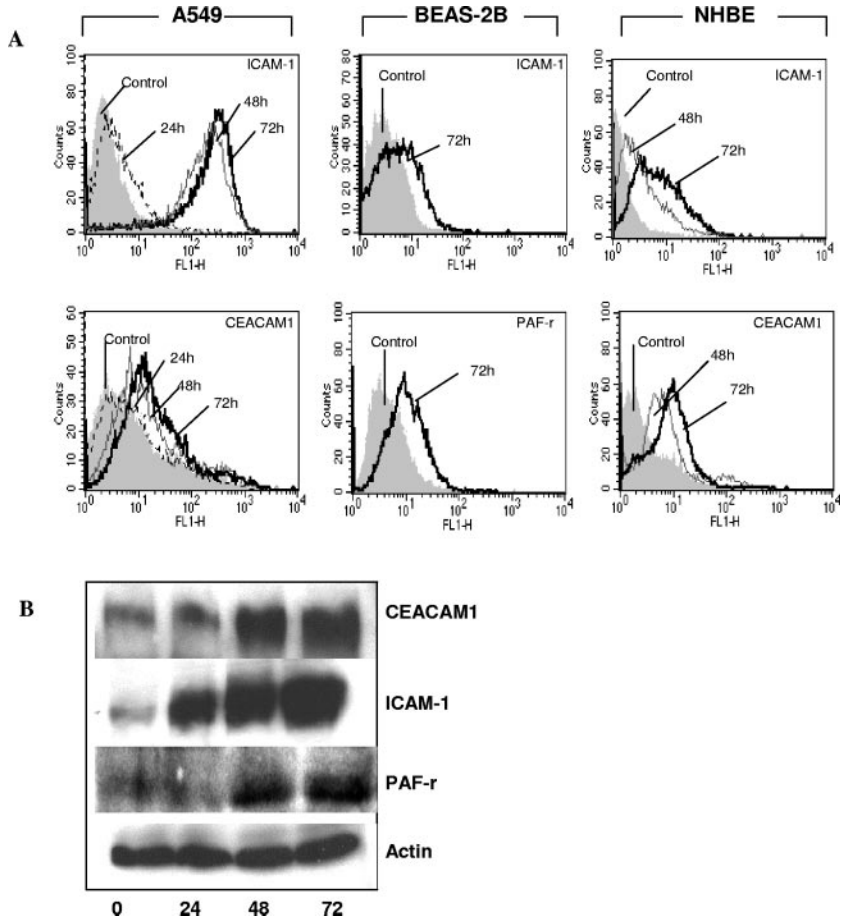


FIG. 3. ICAM-1, CEACAM1, and PAF-r expression by epithelial cells after RSV infection. (A) Cells were infected by RSV for 24, 48, or 72 h, and receptor expression was quantified by FACS. Histograms illustrate the fluorescence intensities of control cells (not infected with virus) compared to those of infected cells at 24, 48, and 72 h. RSV infection up-regulated A549 cell ICAM-1 and CEACAM-1 expression at 48 to 72 h, BEAS-2B ICAM-1 and PAF-r expression at 48 to 72 h, and NHBE cell ICAM-1 and CEACAM-1 expression at 48 and 72 h. (B) Up-regulation of receptors by RSV infection of A549 cells was confirmed by Western blot analysis. Data are representative of three independent experiments.

alter the expression of *muc1* or *muc5AC* by A549 or SAE cells (data not shown).

In summary, RSV increased the expression of ICAM-1, CEACAM1, and PAF-r by A549, BEAS-2B, and NHBE cells. The magnitude of this effect varied significantly between cell types.

**Blocking ICAM-1 and PAF-r after RSV infection prevented adhesion of NTHi and pneumococci to A549 cells.** Blocking assays were used to determine if the increased bacterial adherence of NTHi to RSV-infected cells was attributable to increased receptor expression. NTHi binding was inhibited in a dose-dependent manner when A549 cells were incubated with increasing concentrations of anti-ICAM-1, anti-PAFr, or both Ab but not by the control anti-LFA-3/CD58 Ab (Fig. 4). Anti-ICAM-1 Ab inhibited adhesion by 75% (from 0.75 ± 0.09 to 0.26 ± 0.06 bacteria/cell) after 24 h ($P = 0.005$), 65% (from 1.34 ± 0.13 to 0.47 ± 0.14 bacteria/cell) at 48 h ($P < 0.005$), and by 61% (from 1.56 ± 0.11 to 0.61 ± 0.11 bacteria/cell) after 72 h of RSV infection ($P < 0.001$). Anti-PAF-r Ab inhibited adhesion of NTHi by 48% (from 0.75 ± 0.09 to 0.39 ± 0.09 bacteria/cell) at 24 h ($P = 0.01$), 45% (from 1.34 ± 0.13 to 0.75 ± 0.14 bacteria/cell) at 48 h ($P = 0.002$), and by 30% (from 1.56 ± 0.16 to 1.09 ± 0.28 bacteria/cell) after 72 h of RSV infection ($P = 0.01$). The combination of anti-ICAM-1 and anti-PAF-r Ab inhibited NTHi adhesion by 85% (from 0.75 ± 0.09 to 0.11 ± 0.06 bacteria/cell) after 24 h ($P = 0.002$), 80% (from 1.34 ± 0.13 to 0.30 ± 0.08 bacteria/cell) at 48 h ($P = 0.004$), and by 79% (from 1.56 ± 0.16 to 0.33 ± 0.13 bacteria/cell) after 72 h of infection ($P = 0.003$). These data suggest that the increase in NTHi adhesion to RSV-infected cells was largely, but not completely, attributable to increased expression of specific receptors.

Pneumococcal adhesion to A549 cells after 72 h of RSV infection was reduced by 65% following preincubation of epithelial cells with anti-PAF-r antibody (25 μg/ml) but was not significantly reduced by anti-ICAM-1 antibody (data not shown). These data suggest that the increase in pneumococcus adhesion to RSV-infected A549 cells was due in part to up-regulation of PAF-r and are consistent with the observation that NTHi but not pneumococci bind ICAM-1 (2a).

**HPIV-3 infection also increased cell surface expression of eukaryotic receptors for respiratory bacteria, but influenza virus infection did not.** The expression of ICAM-1, CEACAM1, and PAF-r was measured after infection of A549 and NHBE

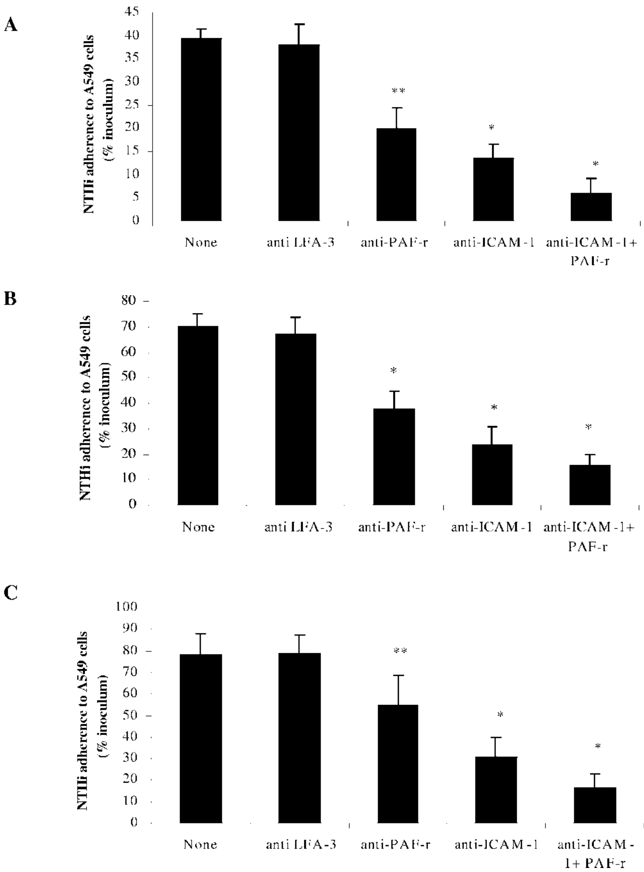


FIG. 4. Blocking ICAM-1 and PAF-r reduces NTHi adhesion to RSV-infected respiratory epithelial cells. A549 cells were infected with RSV for 24, 48, or 72 h and then incubated with anti-LFA-3/CD58, anti-ICAM-1, anti-PAF-r, or both anti-ICAM-1 and anti-PAFr Ab, and numbers of NTHi bacteria adhering to cells were determined. Shown is the inhibition observed after incubation of A549 cells with 25 μg/ml of Ab. (A) Adhesion of NTHi-778 at 24 h. (B) Adhesion after 48 h. (C) Adhesion after 72 h. Treatment with both anti-ICAM-1 and PAF-r antibodies but not control anti-LFA-3/CD58 Ab resulted in a significant inhibition of NTHi adhesion. (*, $P \leq 0.005$; **, $P = 0.01$). Data represent the means ± standard errors for three independent experiments.

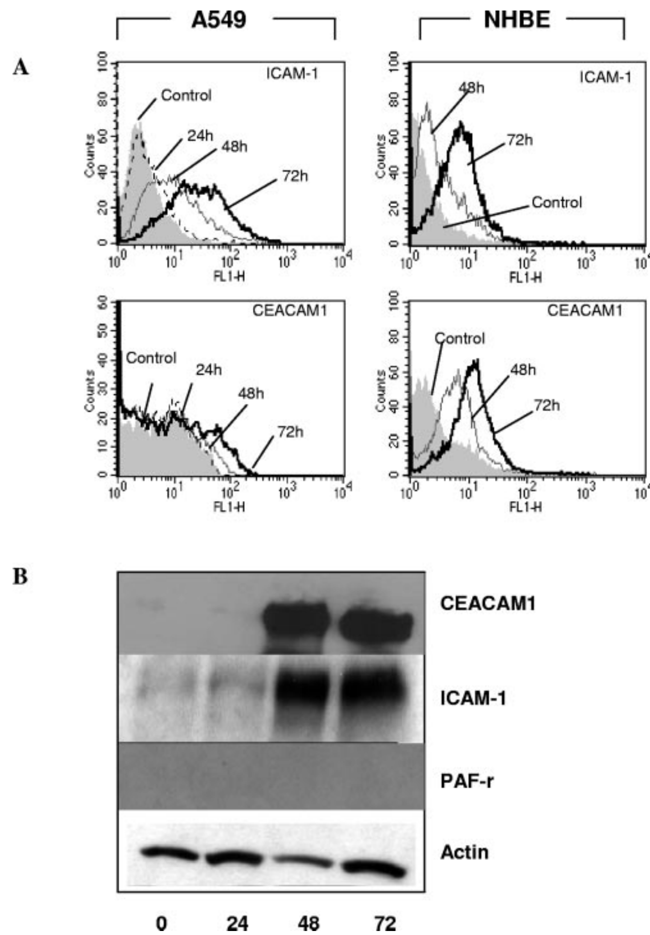


FIG. 5. HPIV-3 infection increases ICAM-1 and CEACAM1 expression by respiratory epithelial cells. (A) Cells were infected by HPIV-3 for 24, 48, or 72 h, and receptor expression was quantified by FACS. Histograms illustrate the fluorescence intensities of control cells (not infected with virus) compared to those of infected cells at 24, 48, and 72 h. HPIV-3 infection significantly increased expression of ICAM-1 and modestly increased expression of CEACAM1 by A549 cells and significantly increased ICAM-1 and CEACAM1 expression by NHBE cells at 48 to 72 h after infection. (B) The increased expression of receptors by A549 cells following HPIV-3 infection was confirmed by Western blot analysis. Data are representative of three independent experiments.

cells by HPIV-3 to determine if the ability to up-regulate receptor expression is shared by other paramyxoviruses. Expression of ICAM-1 by A549 cells after infection with HPIV-3 did not differ from that of uninfected control cells at 24 h but increased 4-fold ± 1-fold at 48 h ($P = 0.03$) and 9-fold ± 1-fold at 72 h ($P = 0.04$) (Fig. 5); expression of CEACAM1 on A549 cells was only minimally increased. HPIV-3 infection did not alter A549 cell expression of PAF-r. Western blot analysis of HPIV-3-infected A549 cell lysates confirmed the increased expression of ICAM-1, demonstrated an increase in CEACAM1 expression, and showed no difference in PAF-r expression (Fig. 5). Expression of ICAM-1 by HPIV-3-infected NHBE cells increased by 2.5-fold at 48 h ($P = 0.01$) and 4.8-fold ($P = 0.005$) at 72 h and CEACAM1 expression by 2.8-fold ($P = 0.02$) at 48 h and 3.8-fold ($P = 0.01$) at 72 h, and there was no change in the expression of PAF-r. In contrast to the case with other viruses, influenza virus infection did not alter receptor expression (data not shown).

## DISCUSSION

Several clinical observations suggest that viral infection might enhance bacterial colonization of the respiratory epithelium, allowing microorganisms to overcome physical barriers to infection and evade innate immune responses. Monso and colleagues found that patients with an acute exacerbation of chronic obstructive pulmonary disease (COPD) had greater bacterial loads than those with stable pulmonary disease (25). Viral respiratory infections were also associated with a greater likelihood of isolation of *H. influenzae* and *S. pneumoniae* from sputum cultures and a higher incidence of *H. influenzae* antibody seroconversion (36). In the current study, we show that RSV, HPIV-3, or influenza virus infection of respiratory epithelial cells increases adherence of NTHi and *S. pneumoniae* but that the mechanisms responsible for this phenomenon differ between the paramyxoviruses (RSV and HPIV-3) and influenza virus and vary according to cell type.

A549 and BEAS-2B are transformed cell lines derived from type II alveolar and normal bronchial epithelial cells, respectively. NHBE and SAE cells are primary epithelial cells obtained from bronchi and the distal bronchial tree and are likely to include a heterogeneous population of cells. Thus, none of these cells are completely representative of an individual cell type or respiratory epithelium as a whole. RSV and HPIV-3 up-regulated ICAM-1, CEACAM1, and PAF-r but not mucin on the surfaces of A549, BEAS-2B, and NHBE but not SAE cells, and much of the increased bacterial adhesion following RSV infection could be blocked by antibodies directed against these receptors. In contrast, influenza virus promoted bacterial adhesion without significantly altering expression of the receptors studied.

One explanation for these differences between cell types may be that these cells do not express the same baseline or stimulated number of surface receptors. A549 cells, for example, had low constitutive expression of ICAM-1 and PAF-r compared to SAE and BEAS-2B cells (data not shown). The expression of ICAM-1 was greater in RSV-infected A549 cells than in BEAS-2B cells, while the converse was true for expression of PAF-r. These data indicate that increased bacterial adhesion to SAE cells is mediated by receptors not tested in the present study. Although bacterial adhesion to each cell type is augmented by viral infection, alteration of receptor expression may be only one means by which bacterial adhesion is increased. Mechanisms independent of the expression of conventional receptors for bacteria, such as binding to viral proteins, could also be responsible for enhanced adhesion (12). Although these studies have focused on lower respiratory tract infections, RSV, HPIV-3, and influenza virus have also been implicated in other secondary bacterial infections, including otitis media and sinusitis. It is possible that the increased adhesion of bacteria to virus-infected cells and the regulation of eukaryotic receptors for bacteria by viruses may also be relevant to these infections.

Immunofluorescence microscopy demonstrated that bacteria binding to RSV-infected A549 cells adhere not only to those cells expressing viral antigens but also to uninfected epithelial cells (data not shown). These data suggest that the ability to augment bacterial adhesion may result from a factor secreted by infected cells that exerts a paracrine effect on adjacent epithelium. Cytokines or other inflammatory molecules are good candidates for such a mediator. Increased ICAM-1, PAF-r, and CEACAM1 expression by epithelial cells is mediated by interleukin 1 alpha (IL-1$\alpha$), tumor necrosis factor alpha, and interleukin 6, which have been reported to be secreted by RSV-infected epithelial cells (7–9, 21, 30). Furthermore, RSV infection of primary small airway, bronchial, and bronchiolar epithelial cells and A549 cells results in cell-specific inflammatory responses (28). Another explanation for variations in receptor expression is that the effects of inflammatory mediators, rather than their absolute concentrations of factors, may differ between cell types.

In this study, the greatest increase in both bacterial adhesion and receptor expression occurred following RSV infection. If inflammatory mediators are responsible for increased expression of ICAM-1, CEACAM1, and PAF-r, it is possible that the more-pronounced effect of RSV is related to differences in the character or magnitude of the inflammatory responses triggered by different viruses (2, 22, 27, 30). Recent studies suggest that the proinflammatory cytokine release triggered by influenza virus infection of bronchiolar epithelial cells is limited by the ability of the virus to induce host cell apoptosis (5). Finally, although differences in the degree of inflammation elicited by RSV, HPIV-3, and influenza virus infection described by other investigators appear to correspond to receptor expression, other mechanisms may also influence this process. HPIV-3, for example, induces ICAM-1 expression in a cytokine-independent manner (10).

The difference in the magnitude and kinetics of *S. pneumoniae* adhesion to virus-infected A549 cells implies that *S. pneumoniae* and influenza virus interact in a unique manner. Paramyxoviruses enhanced bacterial attachment progressively over 72 h. Increased adhesion of *S. pneumoniae* to influenza virus-infected cells, in contrast, peaked at 24 h and then gradually declined to a level comparable to that of adhesion to uninfected control cells. Previous studies have also demonstrated an increase in numbers of pneumococci adhering to A549 cells as early as 30 min after infection with influenza virus but did not study later time points (23). Consistent with our observation that influenza virus does not up-regulate PAF-r, mice treated with a PAF-r antagonist had no reduction in the severity of secondary pneumococcal pneumonia after influenza virus infection (24). Our data support an alternative mechanism of increased bacterial adherence, such as the model put forward by McCullers and colleagues, where influenza virus neuraminidase cleaves sialic acid on eukaryotic cells, allowing pneumococci to adhere to epithelial cells in greater numbers (23). Interestingly, HPIV-3 also possesses a hemagglutinin neuraminidase, and the role of this enzyme in promoting pneumococcal adherence cannot be excluded (1).

The ability of antecedent viral infections to trigger secondary bacterial infections is also likely to depend on host factors. It might be expected that infection by the most highly prevalent of these respiratory viruses, RSV, would lead to a disproportionately increased frequency of exacerbations of COPD. Although RSV has been implicated in 29 to 51% of exacerbations of COPD in some studies, this is not consistently the case (13, 34, 40). Differences in exposure to viruses, rates of influenza virus vaccination, and detection methods may account for some of the discrepancy between these studies. Repeated infections with RSV occur throughout an individual's life, usually producing partial immunity and more minor illness with subsequent infections (14). In contrast, antigenic drift of influenza viruses may result in the rapid loss of protective immunity, irrespective of age and previous infection.

Our data suggest that in addition to other effects, respiratory viral infection may predispose to bacterial secondary infections by promoting bacterial adhesion to the respiratory epithelium. Perhaps most importantly, these studies demonstrate marked differences in the responses of different respiratory epithelial cells to infection by the same virus and in the effects of infection by different viruses on the same cell type. These findings emphasize the importance of considering these experimental factors in future work and the need to translate important findings from in vitro studies to an appropriate in vivo model of infection (3, 11). Further examination of the interactions between respiratory bacterial and viral pathogens will clarify the mechanisms responsible for secondary bacterial infections, and strategies to circumvent these processes may help reduce these complications.

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