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Nontypeable *Haemophilus influenzae* Adheres to Intercellular Adhesion Molecule 1 (ICAM-1) on Respiratory Epithelial Cells and Upregulates ICAM-1 Expression

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Nontypeable *Haemophilus influenzae* (NTHI) is an important respiratory pathogen. NTHI initiates infection by adhering to the airway epithelium. Here, we report that NTHI interacts with intracellular adhesion molecule 1 (ICAM-1) expressed by respiratory epithelial cells. A fourfold-higher number of NTHI bacteria adhered to Chinese hamster ovary (CHO) cells transfected with human ICAM-1 (CHO-ICAM-1) than to control CHO cells ($P \leq 0.005$). Blocking cell surface ICAM-1 with specific antibody reduced the adhesion of NTHI to A549 respiratory epithelial cells by 37% ($P = 0.001$) and to CHO-ICAM-1 cells by 69% ($P = 0.005$). Preincubating the bacteria with recombinant ICAM-1 reduced adhesion by 69% ($P = 0.003$). The adherence to CHO-ICAM-1 cells of NTHI strains deficient in the adhesins P5, P2, HMW1/2, and Hap or expressing a truncated lipooligosaccharide was compared to that of parental strains. Only strain 1128f$^-$, which lacks the outer membrane protein (OMP) P5-homologous adhesin (P5 fimbriae), adhered less well than its parental strain. The numbers of NTHI cells adhering to CHO-ICAM-1 cells were reduced by 67% ($P = 0.009$) following preincubation with anti-P5 antisera. Furthermore, recombinant ICAM bound to an OMP preparation from strain 1128f$^+$, which expresses P5, but not to that from its P5-deficient mutant, confirming a specific interaction between ICAM-1 and P5 fimbriae. Incubation of respiratory epithelial cells with NTHI increased ICAM-1 expression fourfold ($P = 0.001$). Adhesion of NTHI to the respiratory epithelium, therefore, upregulates the expression of its own receptor. Blocking interactions between NTHI P5 fimbriae and ICAM-1 may reduce respiratory colonization by NTHI and limit the frequency and severity of NTHI infection.

Nontypeable *Haemophilus influenzae* (NTHI) is a gram-negative nonencapsulated coccobacillus. It is the most frequent cause of bacterial exacerbations of chronic obstructive pulmonary disease; a major cause of acute otitis media, sinusitis, and bronchitis; and an occasional cause of serious invasive infections, such as bacteremia and meningitis. Adhesion of NTHI to the respiratory epithelium promotes colonization and subsequent epithelial and endothelial invasion by the bacteria. NTHI binds specifically to a variety of receptors on the host cell membrane, including carcinoembryonic antigen cell adhesion molecule 1 (CEACAM1) and CEACAM3, platelet-activating factor receptor, fibronectin, laminin, and respiratory epithelial mucins (10, 14, 18, 31, 42). The importance of NTHI interaction with each of these receptors during human colonization and infection, however, is not yet well understood.

Intercellular adhesion molecule 1 (ICAM-1; CD54) is a member of the immunoglobulin (Ig)-like superfamily that contains five Ig domains, a transmembrane domain, and a short cytoplasmic tail (30). ICAM-1 makes possible reversible adhesion and signal transduction between cells, processes critical to normal leukocyte recruitment and to T-cell development, by interacting with specific receptors. As is the case with other eukaryotic receptor molecules, the expression of ICAM-1 by a variety of human cells has been subverted by pathogens to facilitate their own adhesion and uptake. ICAM-1 serves as a receptor for the majority (90%) of human rhinoviruses; is required for cell entry by coxsackieviruses; and, in concert with CD36, it mediates the adherence of *Plasmodium falciparum*-infected erythrocytes to endothelial cells (21, 33, 41). Here, we demonstrate that NTHI also adheres to human respiratory epithelium by binding specifically to ICAM-1 and that the NTHI ligand for ICAM-1 is P5 fimbria, a major outer membrane protein that also binds respiratory mucin and CEACAM1 (14, 28).

MATERIALS AND METHODS

Bacterial culture. Most assays were performed with NTHI strain 778. This clinical isolate expresses Hap, high-molecular-weight 1 and 2 adhesins (HMW1/HMW2), pili (Hif), P5 fimbriae, and phosphorylcholine (data not shown) (1, 26, 36, 38). NTHI strains 781 (*hap*$^+$ *hmw*$^+$ *hia*/*hif* negative) and Rd, which also express P5 fimbriae (data not shown), were also tested. Wild-type NTHI strain 1128f$^+$ and its isogenic P5 fimbrial mutant, 1128f$^-$, have been previously described (34). NTHI strain 12 (*hmw1*/*hmw2* positive; *hia*/*hif* negative) and its isogenic mutant lacking *hmw1*/*hmw2* (37), N187 (*hmw1*/*hmw2* and *hap* positive), N187 Hap$^-$ (an isogenic mutant of N187 lacking *hap*), N187 Hap$^-$HMW$^-$ (an isogenic mutant of N187 lacking both *hap* and *hmw1*/*hmw2*) (13), Rd, and Rd lacking outer membrane protein P2 were provided by J. St Geme III, Washington University School of Medicine. NTHI strains 2019 (wild type) and 2019 *pgmB*::*erm*, which expresses a truncated LOS lacking branch oligosaccharides (42), were provided by M. Apicella, University of Iowa. NTHI was grown in brain heart infusion broth (Difco, Sparks, MD) supplemented with 10 μg/ml hemin (Sigma, St. Louis, MO) and 1 μg/ml of α-NAD (Sigma) (sBHI) or on sBHI or chocolate II agar (Edge Biologics, Memphis, TN) at 37°C in 5% CO_2.

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S. pneumoniae strain 357 is an encapsulated strain isolated from the nasopharynx of a child and was provided by E. Tuomanen, St. Jude Children's Research Hospital. It was grown on tryptic soy agar (Difco) with 3% defibrinated sheep's blood (Enova Medical Technologies, St. Paul, MN), or in C+Y medium at 30°C and 5% CO_2 (19).

Cell culture. A549 respiratory epithelial cells (CCL-185; American Type Culture Collection [ATCC], Manassas, VA) and Chinese hamster ovary (CHO) cells (CRL-9618; ATCC) were cultivated in Hams F-12K medium (ATCC) supplemented with 10% heat-inactivated fetal bovine serum (FBS). CHO cells stably transfected with human ICAM-1 (CHO-ICAM-1) (CRL-2093; ATCC) were maintained in RPMI 1640 (ATCC) supplemented with 10% FBS.

Bacterial-adhesion assays. Bacteria were grown overnight in broth, diluted 1:10 in fresh medium, and grown to log phase for adhesion assays. Prior to the assays, the bacteria were washed three times in phosphate-buffered saline (PBS) and resuspended in tissue culture medium. Bacterial concentrations were determined by optical density at 600 nm, and the numbers of bacteria were retrospectively confirmed by plating serial dilutions of the starting inocula. A549 or CHO cells were grown to confluence in 24-well plates (Costar; Corning Inc., Corning, NY). The cell monolayers were washed twice with PBS, and then triplicate wells were incubated with bacteria at a multiplicity of infection (MOI) of two bacteria per eukaryotic cell for 1 h at 37°C in 5% CO_2. The cells were then washed three times with PBS to remove loosely adherent bacteria, detached from the plates by incubation for 10 min at 37°C with 1× trypsin-EDTA (Mediatech Inc., Herndon, VA), and washed twice with PBS, and serial dilutions were plated for quantification of adherent bacteria. For each assay, the numbers of bacteria were normalized to the numbers of epithelial cells.

Confocal microscopy. NTHI 778 and *Streptococcus pneumoniae* (5×10^7 CFU) were incubated with fluorescein isothiocyanate (FITC) solution (1 mg/ml FITC in carbonate buffer containing 0.1 M NaCl, 0.09 M Na_2CO_3, and 0.015 M $NaHCO_3$, pH 9.2 [Sigma]) for 30 min, washed twice with PBS, and resuspended in cell culture medium. CHO and CHO-ICAM-1 cells were grown to confluence in LAB-TEK glass chamber slides (Nalge Nunc International, Naperville, IL). The cells were inoculated with FITC-labeled bacteria at an MOI of 100 as described above. A larger inoculum of bacteria was used in these studies than in adhesion assays to more readily visualize adherent bacteria. The cells were then washed, fixed with 4% paraformaldehyde (Sigma) for 10 min at room temperature, and rinsed. After being blocked with 4% normal goat serum (Sigma), 0.5% bovine serum albumin, and 1% Triton X-100 in PBS, the cells were rinsed with PBS and incubated with a 1/250 dilution of anti-ICAM-1 antibody (Ab) (BD Biosciences) in blocking buffer for 1 h at 37°C and then incubated with a 1/500 dilution of Alexa 594-conjugated goat anti-mouse secondary antibody (Ab) (Molecular Probes, Eugene, OR) in blocking buffer for 1 h at 37°C. After thorough rinsing with PBS, the cells were mounted with the DNA-specific dye TO-PRO-3 (Molecular Probes). The cells were examined with a Leica TCS NT SP confocal laser scanning microscope equipped with argon (488-nm) and helium-neon (633-nm) lasers.

Inhibition of ICAM-1-mediated adhesion of NTHI to epithelial cells. The ability of NTHI to adhere to A549 and CHO-ICAM-1 cells was compared to its ability to bind to cells on which cell surface ICAM-1 was blocked by preincubation with purified mouse anti-human CD54/ICAM-1 monoclonal antibodies (MAbs): clone LB2, IgG2b (BD Biosciences, San Jose, CA), and clone R1/1, IgG1, and clone 8.4A6, IgG1 (Alexis Biochemicals, San Diego, CA). Epithelial cell monolayers were incubated with 0 to 20 μg/ml of anti-ICAM-1 or control mouse IgG2b or IgG1 Ab (BD Biosciences) in cell culture medium for 1 h at 37°C in 5% CO_2. The cells were then rinsed with PBS and inoculated with NTHI, and the numbers of adherent bacteria were quantified as previously described.

The ability of NTHI to adhere to respiratory epithelial cells was also determined after the bacterial surface ligands that bind to the ICAM-1 receptor were blocked using recombinant ICAM-1 (rICAM-1; R&D Systems, Minneapolis, MN). Bacteria were incubated with 0 to 4 μg/ml of rICAM-1 for 2 h at 37°C in RPMI 1640 without FBS (to prevent bacterial replication) and used in adhesion assays as previously described.

Inhibition of P5-mediated NTHI adhesion to ICAM-1 cells. The specificity of NTHI P5 fimbrial binding to ICAM-1 on respiratory epithelial cells was tested by comparing binding to CHO-ICAM-1 cells by untreated bacteria to that of bacteria in which fimbrial binding was blocked by preincubation with anti-P5 antiserum. Bacteria were incubated with a 1:200 to 1:1,000 dilution of rabbit polyclonal anti-P5 antiserum (23) or control preimmune rabbit serum for 1 h at 37°C in RPMI 1640 without FBS, and adhesion assays were performed as described above.

P5 binding by rICAM-1. Whole outer membrane proteins (OMPs) of NTHI strains $112f^+$ ($P5^+$) and $1128f^-$ ($P5^-$) and peptides corresponding to the four predicted surface-exposed loops of P5 were prepared as described previously (24, 34). Proteins, peptides, and rICAM-1 (10 μg; R&D Systems) were spotted on a nitrocellulose membrane using a 96-well vacuum apparatus (Bio-Rad, Hercules, CA). The membrane was blocked with 5% nonfat dry milk in PBS for 1 h, washed, and incubated with 5 μg/ml of rICAM-1 in 5% nonfat dry milk in PBS for 1 h, followed by mouse anti-human ICAM-1 monoclonal Ab (BD Biosciences) at a 1:1,000 dilution for 1 h. Bound Ab was detected using peroxidase-conjugated goat anti-mouse secondary Ab (ECL Western Blotting Analysis System; Amersham Biosciences, Piscataway, NJ).

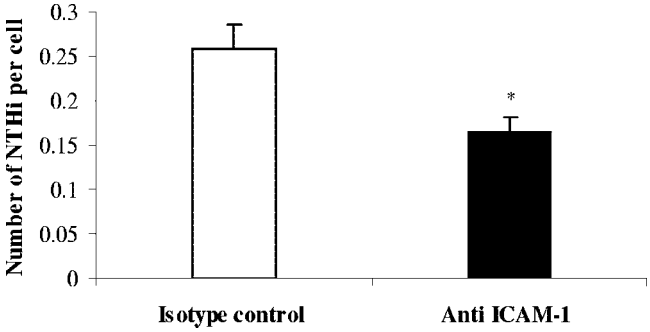


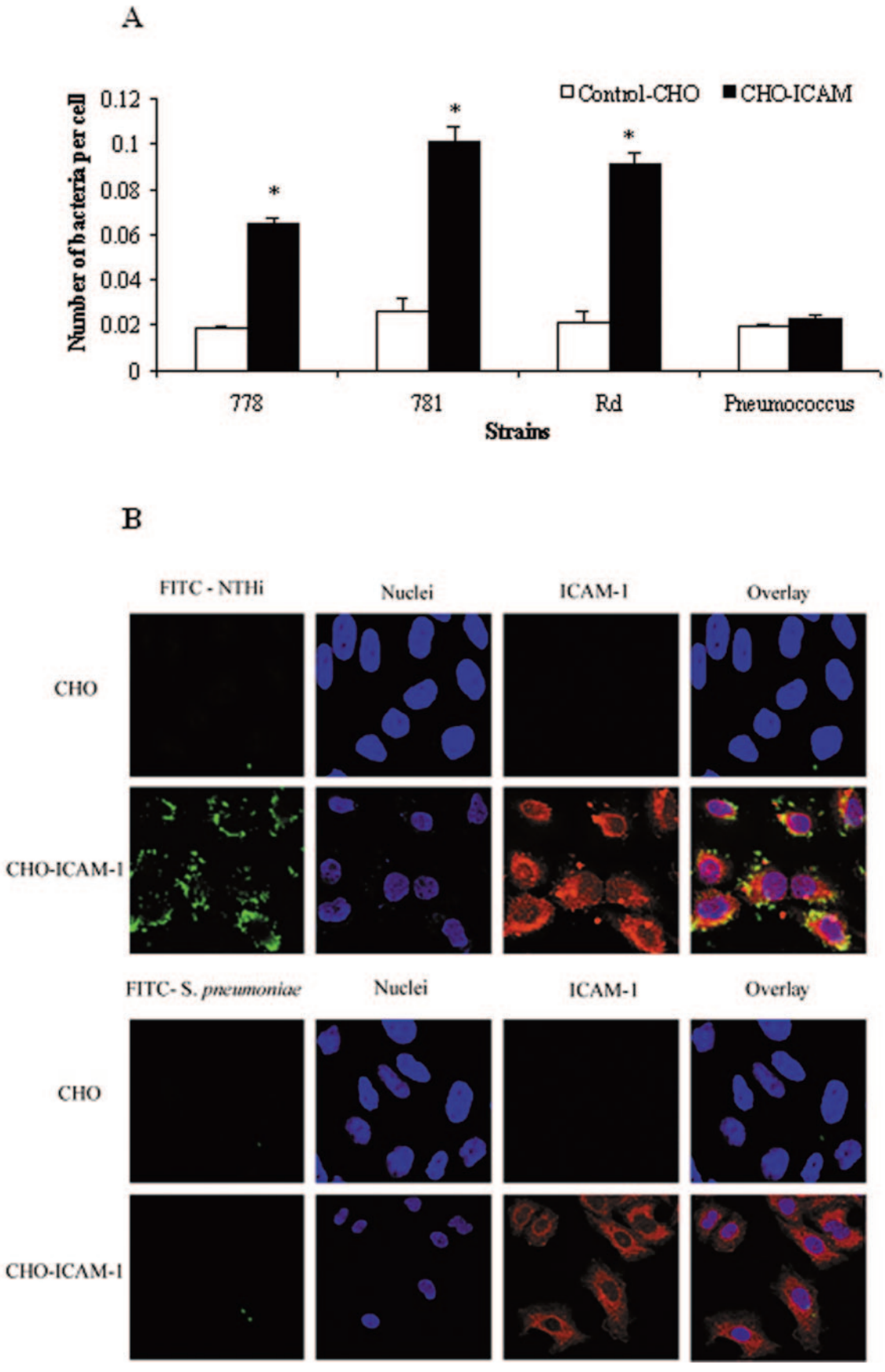
FIG. 1. Adhesion of NTHI to A549 respiratory epithelial cells is blocked by anti-ICAM-1 antibodies. A549 cells were incubated with anti-ICAM-1 Ab or an isotype control (15 μg/ml) for 1 h, infected with NTHI for 1 h, and washed to remove loosely adherent bacteria. Adherent bacteria were quantified by plating serial dilutions of A549 cell lysates on agar. Preincubation of A549 cells with anti-ICAM-1 Ab (black bar) significantly reduced the adhesion of NTHI compared to preincubation with an isotype control Ab (white bar) (*, $P = 0.001$). Adhesion is expressed as the number of bacteria/cell. The data represent the mean ± standard error of the mean of four separate experiments.

Flow-cytometric analysis of ICAM-1 expression. A549 cells were propagated in 12-well plates and infected with NTHI at an MOI of 1, 10, or 100 as previously described. After 6 h of incubation, the cells were washed and detached from the plates by incubation with 500 μl/well cell dissociation solution and washed with PBS, and 10^6 cells were resuspended in 1 ml of 1% bovine serum albumin-PBS. The cells were then incubated with a 1/1,000 dilution of mouse anti-human CD54–ICAM-1 (IgG1; BD Biosciences) or purified mouse IgG1 Ab (BD Biosciences) (an isotype control) for 1 h at 25°C. The cells were washed once with PBS and then incubated with a 1/500 dilution of FITC-labeled goat anti-mouse Ab (BD Biosciences) for 30 min at 4°C in the dark and washed, and 10^4 cells were analyzed by flow cytometry. The mean fluorescence intensity of the cells was compared to that of uninfected cells after background staining produced by isotype control Abs was subtracted.

Statistical analysis. Data are expressed as the mean ± standard error. 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

NTHI binding to respiratory epithelial cells is reduced by monoclonal anti-ICAM-1 antibody. Previous work by ourselves and other investigators determined that NTHI is able to adhere to epithelial cells using a variety of cell surface ligands, including respiratory mucin, CEACAM1 and -3, platelet-activating factor receptor, fibronectin, and laminin (10, 14, 18, 31, 42). In preliminary studies, efforts to completely prevent NTHI from adhering to the respiratory epithelium by blocking interactions with these receptors were not successful (data not shown), suggesting that another, as-yet-unidentified NTHI receptor might exist on these cells. Several important human pathogens adhere to and invade host cells using the immunoglobulin-like molecule ICAM-1. We therefore hypothesized that ICAM-1 might also be a ligand for NTHI. Blocking



ICAM-1 by preincubating A549 cells with anti-ICAM-1 antibody reduced the numbers of adherent bacteria by 37% ($P = 0.001$), suggesting that this was the case. The adhesion of NTHI to A549 cells was reduced from 0.25 ± 0.02 bacterium per cell (12.5% of the inoculum) when incubated with isotype control antibody to 0.16 ± 0.01 bacterium per cell (8% of the inoculum) ($P = 0.001$) when incubated with anti-ICAM-1 antibody (Fig. 1).

NTHI binds to CHO cells transfected with ICAM-1. The ability of anti-ICAM-1 antibody to block NTHI adhesion to respiratory epithelial cells suggested that ICAM-1 itself might serve as a ligand for the bacteria. Interpretation of these assays, however, is complicated by the presence of multiple receptors for NTHI on A549 cells. Additionally, the expression of some of these receptors may be up- or downregulated, depending on experimental conditions (7, 22, 44, 45). The adhesion of NTHI to CHO cells stably transfected with human ICAM-1 was studied to clarify this interaction.

Adhesion of NTHI 778 was tested at MOIs of 2, 20, and 200. The number of bacteria adhering to CHO-ICAM-1 increased with the increase in the inoculum. At an MOI of 2, 0.07 ± 0.011 bacterium per cell (3.9% of the inoculum) adhered to CHO-ICAM-1 cells compared to 4.2 ± 0.04 bacteria per cell (21% of the inoculum) at an MOI of 20 and 58 ± 1.2 bacteria per cell (29% of the inoculum) at an MOI of 200. In contrast, the numbers of NTHI cells binding to nontransfected CHO cells did not differ significantly with an increase in the inoculum (data not shown). An MOI of 2 was used for subsequent studies.

Adhesion of *H. influenzae* strains 778, 781, and Rd to nontransfected CHO cells was low: 0.018 ± 0.008 bacterium adhered per cell (0.9% of the inoculum) for 778, 0.025 ± 0.006 bacterium adhered per cell (1.2% of the inoculum) for 781, and 0.021 ± 0.005 bacterium adhered per cell (1.06% of the inoculum) for Rd. The adherence of NTHI strains 778, 781, and Rd to CHO-ICAM-1 cells was increased by 4-, 5-, and 4.5-fold, respectively, compared to untransfected CHO cells. The adherence of 778 increased to 0.064 ± 0.002 bacterium per cell (3.2% of the inoculum) ($P < 0.001$), that of 781 increased to 0.101 ± 0.006 bacterium per cell (5% of the inoculum) ($P = 0.001$), and that of Rd increased to 0.091 ± 0.006 bacterium per cell (4.5% of the inoculum) ($P = 0.005$) (Fig. 2A). In contrast, the numbers of *S. pneumoniae* cells adhering to CHO cells and to CHO-ICAM-1 cells did not differ significantly; 0.019 ± 0.001 bacterium per cell (0.95% of the inoculum) bound to CHO cells, while 0.022 ± 0.001 bacterium per cell (1.1% of the inoculum) bound to CHO-ICAM-1 cells. These differences were confirmed by confocal microscopy of eukaryotic cells incubated with FITC-labeled NTHI 778 and pneumococci (Fig. 2B). These data suggest that NTHI cells, but not pneumococci, bind to human ICAM-1.

Anti-ICAM-1 antibodies and rICAM-1 reduce the adhesion of NTHI to CHO- ICAM-1 cells. CHO-ICAM-1 monolayers were preincubated with anti-ICAM-1 antibodies to determine the specificity of NTHI binding to ICAM-1. Adhesion of NTHI to CHO-ICAM-1 cells was inhibited in a dose-dependent manner with increasing concentrations of antibody. Adhesion of NTHI 778 was reduced from 0.064 ± 0.002 bacterium per cell (3.25% of the inoculum) following preincubation with 0 μg/ml anti-ICAM-1 MAb (clone LB2) to 0.020 ± 0.001 bacterium per cell (1% of the inoculum) following preincubation with 20 μg/ml of anti-ICAM-1 MAb ($P = 0.005$) (Fig. 3A). Adhesion of strains 781 and Rd also decreased by 64% and 70%, respectively, following incubation with 20 μg/ml anti-ICAM-1 antibody (data not shown). In contrast, NTHI adhesion was not affected by preincubation with an irrelevant isotype control antibody.

ICAM-1 is composed of five tandemly linked extracellular domains, designated D1 to D5. The binding site of the physiological ligand of ICAM-1, LFA-1, is located in D1 (39). D2 also plays an important role in the conformation of D1. Since the anti-ICAM-1 MAb used in the above-mentioned experiments, clone LB2, has been reported to block the interaction of LFA-1 with ICAM-1 (20), this suggested that NTHI also binds to D1 or D2. Two additional antibodies, R1/1 and 8.4A6, that block the interaction of LFA-1 with ICAM-1 by binding to D1 and D2, respectively, were tested to investigate if preincubation of CHO-ICAM-1 cells with these antibodies would interfere with NTHI adherence. The numbers of adherent NTHI cells decreased from 0.064 ± 0.002 bacterium per cell (3.25% of the inoculum) to 0.055 ± 0.004 (2.75% of the inoculum) ($P = 0.03$) and 0.045 ± 0.009 (2.25% of the inoculum) ($P = 0.01$) bacterium per cell after incubation with 10 μg/ml of the anti-ICAM-1 antibodies clones R1/1 and 8.4A6, respectively (Fig. 3B). R1/1 and 8.4A6 did not inhibit the adhesion of NTHI as well as LB2, even at high concentrations. These data suggest that NTHI may bind at a site adjacent to the D1 and D2 junction or overlapping these domains.

The adhesion of NTHI to CHO-ICAM-1 cells was also reduced in a dose-dependent fashion by preincubation of bacteria with rICAM-1. The adhesion of NTHI was reduced from 0.075 ± 0.005 bacterium per cell (3.75% of the inoculum) following preincubation with 0 μg/ml rICAM-1 to 0.023 ± 0.004 bacterium per cell (1.15% of the inoculum) following preincubation with 4 μg/ml rICAM-1 (69% reduction; $P = 0.003$) (Fig. 3C). The adhesion of strains 781 and Rd decreased by 68% and 73%, respectively, following incubation with 4

FIG. 2. Adhesion of NTHI and *S. pneumoniae* to CHO and CHO-ICAM-1 cells. CHO and CHO-ICAM-1 cells were incubated with *H. influenzae* strain 778, 781, or Rd or *S. pneumoniae* for 1 h and washed to remove loosely adherent bacteria. Adherent bacteria were quantified by plating serial dilutions of cell lysates on agar plates. Adhesion is expressed as numbers of bacteria/cell. (A) More NTHI cells adhered to CHO-ICAM-1 cells (black bars) than to control CHO cells (white bars) (*, $P \leq 0.005$), whereas there was no significant difference in the adhesion of *S. pneumoniae* to CHO or CHO-ICAM-1 cells. The data represent the mean ± standard error of the mean of four separate experiments. (B) CHO-ICAM-1 cells were incubated with FITC-labeled NTHI or *S. pneumoniae* for 1 h, stained with anti-ICAM-1 antibody, and then washed and incubated with Alexa 594-conjugated anti-IgG secondary antibody and the double-stranded DNA specific fluorochrome To-PRO3. (Top) FITC-labeled NTHI (green) binds primarily to cells expressing ICAM-1 (red). Cell nuclei are counterstained blue. (Bottom) Few FITC-labeled *S. pneumoniae* cells (green) adhere to CHO-ICAM-1 cells.

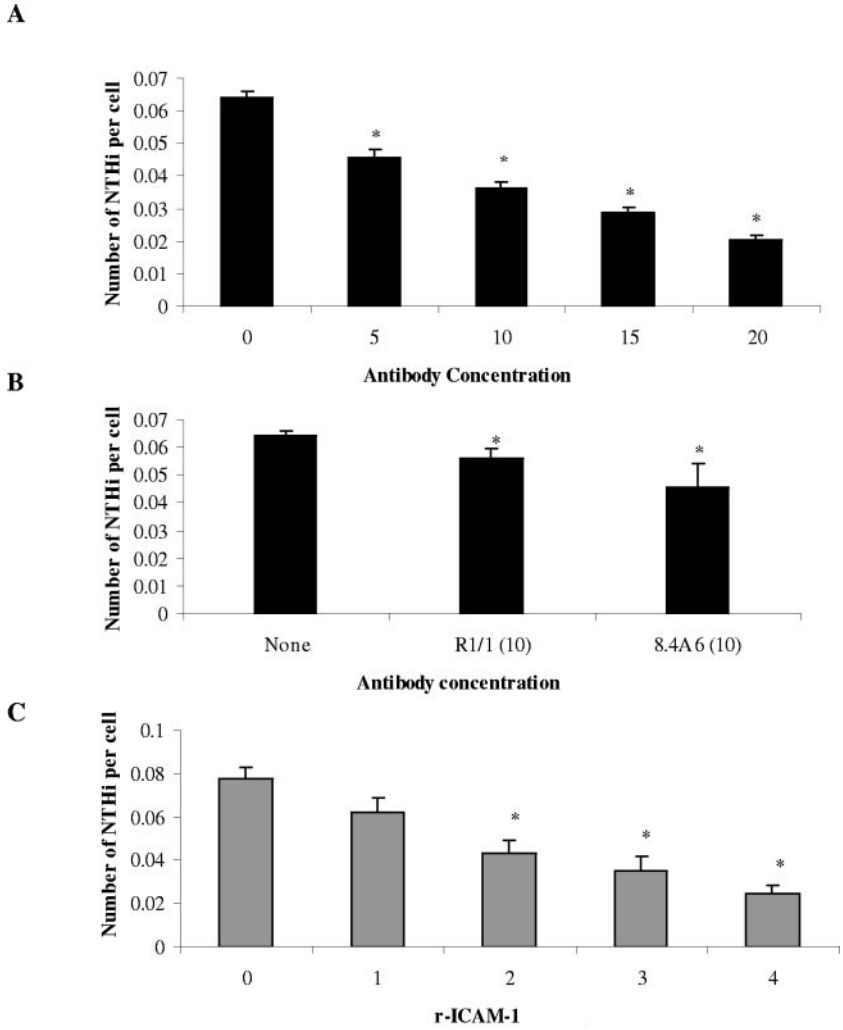


FIG. 3. Blocking with anti-ICAM-1 antibodies and rICAM-1 reduces NTHI adhesion to CHO-ICAM-1 cells. CHO-ICAM-1 cells were incubated with anti-ICAM-1 MAb at the indicated concentrations (in μg/ml). The cells were then infected with NTHI, and the numbers of bacteria adhering to the cells were compared to the numbers of bacteria binding to untreated cells. (A) Treatment with anti-ICAM-1 MAb (LB2) resulted in a dose-dependent inhibition of NTHI adhesion. The data represent the mean ± standard error of five independent experiments (*, $P = 0.005$). Preincubation of CHO-ICAM-1 cells with an isotype control antibody did not alter NTHI adherence (data not shown). (B) Adhesion of NTHI was also determined after incubating CHO-ICAM-1 cells with MAbs R1/1 and 8.4A6, which bind ICAM-1 D1 and D2, respectively. Both R1/1 and 8.4A6 at concentrations of 10 μg/ml reduced the numbers of adherent NTHI cells (*, $P = 0.03$ for R1/1 and $P = 0.01$ for 8.4A6). In contrast, preincubation of CHO-ICAM-1 cells with isotype control antibodies did not alter NTHI adherence (data not shown). The data represent the mean ± standard error of four independent experiments. (C) NTHI was preincubated with rICAM-1 at the indicated concentrations and then used to infect CHO-ICAM-1 cells. Preincubation of bacteria with rICAM-1 resulted in a dose-dependent inhibition of NTHI adhesion to ICAM-1 cells (*, $P = 0.003$). The data represent the mean ± standard error of five independent experiments.

μg/ml rICAM-1 (data not shown). Together, these data suggest that NTHI specifically binds human ICAM-1.

P5 fimbriae mediate binding to ICAM-1. NTHI expresses five well-characterized adhesins: LOS, HMW1/2, Hap, P2, and P5. We compared the adherence of mutant NTHI strains lacking each of these structures and their parental strains to CHO-ICAM-1 cells to determine if any of these structures might confer the ability to bind ICAM-1. Of the NTHI strains tested, only strain 1128f$^-$, which lacks P5 fimbriae, adhered less well to CHO-ICAM-1 cells than its parent strain. Only 0.015 ± 0.007 (0.75% of the inoculum) P5-deficient bacterium adhered per CHO-ICAM-1 cell compared to 0.043 ± 0.007 (2.15% of the inoculum) wild-type bacterium (a reduction of 65%; $P = 0.003$) (Fig. 4).

NTHI 778 was preincubated with anti-P5 polyclonal rabbit antiserum (or control preimmune rabbit serum) to determine if blocking P5 on the bacterial surface interfered with binding to ICAM-1. The adhesion of NTHI was reduced in a dose-dependent manner with increasing concentrations of anti-P5 antisera but not with control preimmune serum. Binding of NTHI was reduced by 67%, from 0.071 ± 0.008 bacterium per cell (3.6% of the inoculum) to 0.025 ± 0.0009 bacterium per cell (1.25% of the inoculum) ($P = 0.009$), following preincubation with a 1:200 dilution of anti-P5 antiserum (Fig. 5).

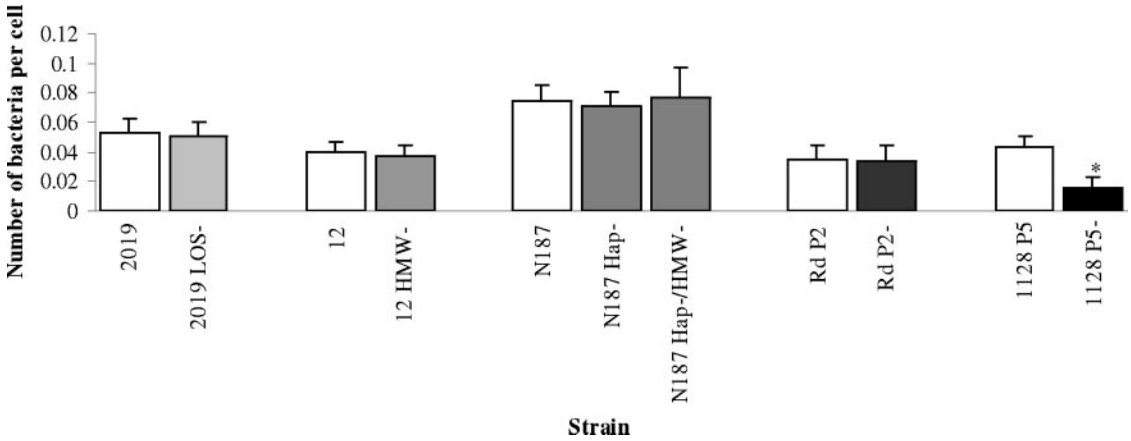


FIG. 4. An isogenic mutant strain of NTHI lacking P5 adheres poorly to CHO-ICAM-1 cells compared to its P5-sufficient parental strain. CHO-ICAM-1 cells were incubated with NTHI strains and their isogenic mutants lacking HMW1 and HMW2 (strain 12, HMW$^-$); HMW1, HMW2, and Hap proteins (strain N187, HMW$^-$, and N187, Hap$^-$); P2 fimbriae (strain Rd, P2$^-$); and P5 fimbriae (strain 1128f$^-$) and expressing LOS (strain 2019) or truncated LOS (strain 2019, *pgmB*::*erm*), and adherent bacteria were quantified. Only the P5 fimbria-deficient strain 1128f$^-$ had significant reduction in adhesion to CHO-ICAM-1 cells compared to its parental strain (*, $P = 0.003$). The data represent the mean ± standard error of five independent experiments.

NTHI was incubated with medium alone or with a 1:200 dilution of control preimmune serum or anti-P5 antiserum for 1 h to determine if nonspecific agglutination might give the appearance of reduced binding to ICAM-1 cells. Serial dilutions of bacteria were then plated on agar plates. There was no reduction in the number of bacteria treated with control preimmune serum or anti-P5 antiserum compared to medium alone, suggesting that decreased adhesion is more likely to be attributable to blocking of ICAM-1–P5 fimbrial interactions (data not shown).

The ability of rICAM-1 to bind directly to outer-membrane preparations of NTHI and to peptides corresponding to predicted surface-exposed regions of P5 fimbriae of NTHI strain 1128 (24) was also determined. rICAM-1 bound to OMPs from NTHI 1128f$^+$ (P5 sufficient) but not to 1128f$^-$ (P5 deficient). ICAM-1 also bound specifically to P5 peptide 4, suggesting that this region may be involved in the interaction with ICAM-1 (Fig. 6).

NTHI stimulates the expression of ICAM-1 on A549 cells. Incubation of respiratory epithelial cells with NTHI was previously reported to upregulate the expression of ICAM-1 and thus has the potential to increase inflammatory responses to infection (11). To confirm this observation, the expression level of ICAM-1 was measured following incubation with NTHI-778 using fluorescence-activated cell sorter analysis. Incubation of NTHI with A549 cells increased the expression of ICAM-1 in an inoculum-dependent manner, with an MOI of 10 resulting in a threefold increase ($P = 0.01$) in ICAM-1 expression and an MOI of 100 resulting in a fourfold increase ($P = 0.001$) (Fig. 7). Thus, adhesion of NTHI to the respiratory epithelium upregulates the expression of its own receptor, ICAM-1, in an inoculum-dependent fashion.

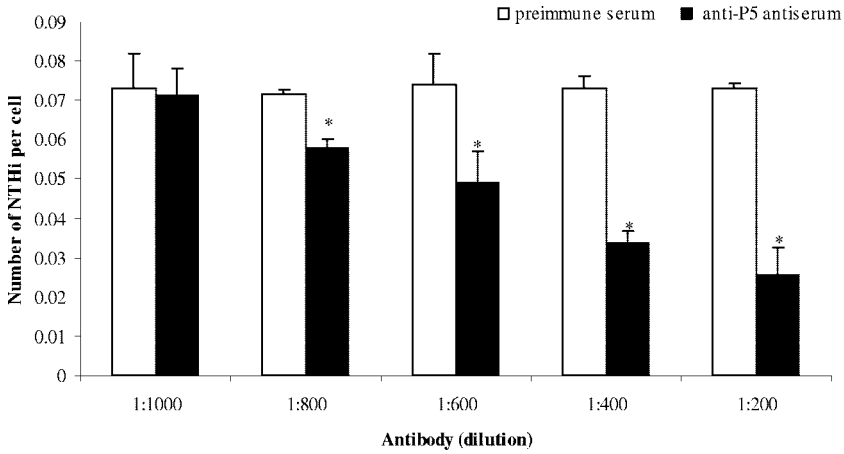


FIG. 5. Incubation with anti-P5 antibodies inhibits adhesion of NTHI 778 to ICAM-1 cells. NTHI 778 was preincubated with increasing concentrations of rabbit anti-P5 antiserum (black bars) or control preimmune serum (white bars) at the indicated concentrations and then incubated with CHO-ICAM-1 cells. Preincubation of bacteria with anti-P5 Ab resulted in a dose-dependent inhibition of NTHI adhesion to CHO-ICAM-1 cells. The data represent the mean ± standard error of four independent experiments (*, $P = 0.009$).

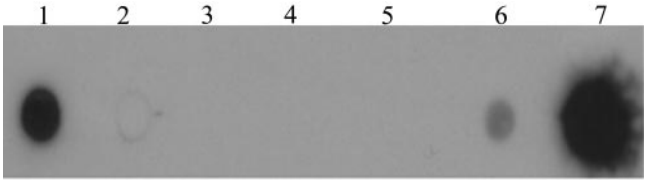


FIG. 6. rICAM binds to outer membrane protein P5. Whole OMPs of NTHI 1128f$^+$ (lane 1) and 1128f$^-$ (lane 2); peptides 1 to 4 (lanes 3 to 6), corresponding to the four predicted surface-exposed regions of P5; and rICAM-1 (positive control) (lane 7) were spotted on a nitrocellulose membrane and incubated with rICAM-1. Anti-ICAM-1 Ab was used to detect bound ICAM-1. rICAM-1 binds to OMP preparations from P5-sufficient strain 1128f$^+$, but not P5-deficient strain 1128f$^-$, and to a peptide corresponding to loop 4 of P5 fimbriae.

DISCUSSION

The adherence of NTHI to respiratory epithelial cells allows the bacteria to overcome many of the body's physical barriers to infection. Most infections caused by NTHI are preceded by bacterial colonization of the respiratory epithelium, and the likelihood of symptomatic disease appears to increase with an increase in the extent of colonization (27, 32). The success of NTHI as a human pathogen may be, in part, attributable to its ability to adhere to a diverse group of host cell molecules. Pili and the major outer membrane P2 protein bind sialic acid-containing moieties on epithelial cell surfaces (18, 28). In the latter instance, sialic acid-containing oligosaccharides of respiratory epithelial mucin appear to be most important for bacterial adhesion. The phosphorylcholine residue of NTHI LOS interacts with platelet-activating factor receptor to enhance respiratory epithelial cell adherence and invasion (42). NTHI Hap promotes bacterial adhesion to epithelial cells and microcolony formation by binding eukaryotic fibronectin, laminin, and collagen IV (10), and finally, P5 fimbriae specifically bind to host CEACAM1 (14). In the current study, we show that NTHI also adheres to respiratory epithelial cells by directly binding ICAM-1, and P5 fimbriae mediate this interaction.

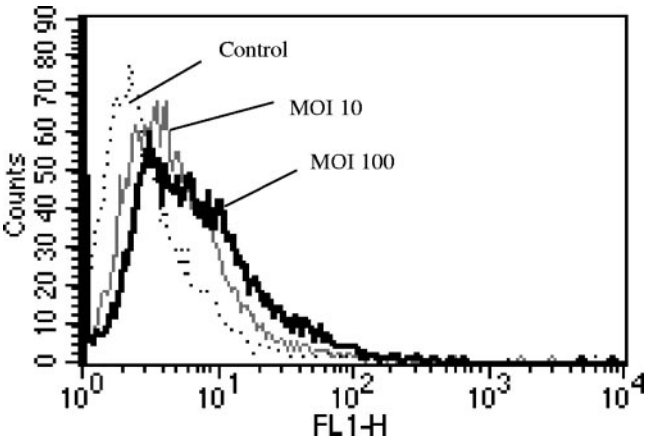


FIG. 7. NTHI infection increases ICAM-1 expression on A549 cells. A549 cells were infected by NTHI 778 at an MOI of 1, 10, or 100 for 6 h, and ICAM-1 expression was quantified by fluorescence-activated cell sorting. The histograms illustrate the fluorescence intensity (ICAM-1 expression; horizontal axis) of uninfected control cells (dotted line) compared to that of cells infected with increasing numbers of NTHI. The expression level of A549 cell ICAM-1 increased in an inoculum-dependent manner by threefold at an MOI of 10 (gray line) ($P = 0.01$) and by fourfold at an MOI of 100 (black line) ($P = 0.001$). The data are representative of three independent experiments.

ICAM-1 is a member of the immunoglobulin superfamily, a type I transmembrane glycoprotein with an extracellular region comprised of five tandemly repeated immunoglobulin constant-region-like domains (40). Its major physiologic function is to promote cell adhesion during inflammation and immune responses. Physiological ligands of ICAM-1 include the alpha-beta integrin receptors, leukocyte-associated function antigen 1 (LFA-1; CD11a/CD18), and macrophage 1 antigen (Mac-1; C3; CD11b/CD18), CD43, fibrinogen, and hyaluronan (15, 35). Expression of ICAM-1 by CD8$^+$ T cells facilitates interactions between the T-cell receptor and major histocompatibility class I and II peptides (43). The expression of ICAM-1 on epithelial and endothelial cells directs the trafficking of leukocytes from the circulation to sites of inflammation by stabilizing the initial, weaker interactions of leukocytes with other selectin molecules (4).

ICAM-1 also contributes to innate immune responses to bacterial pathogens by promoting the uptake of bacterial pathogens by macrophages and increases neutrophil recruitment in the lung (16, 25). Frick and coworkers and Humlicek et al. found that adherence of NTHI to respiratory epithelial cells rapidly induced ICAM-1 expression, a process they hypothesized would facilitate the recruitment of neutrophils to sites of NTHI infection (11, 16). Although advantageous to the human host, this process is likely to adversely affect NTHI and other bacterial pathogens by increasing the numbers of phagocytes at sites of colonization. The ability of NTHI to bind to ICAM-1 may, to some degree, compensate for the disadvantages of increased ICAM-1 expression by increasing the numbers of adherent bacteria. This strategy would be particularly useful if NTHI binding to ICAM-1 blocked subsequent leukocyte adhesion to ICAM-1.

In contrast to other integrin receptors, ICAM-1 does not possess an Arg-Gly-Asp (RGD) binding motif. The binding site on ICAM-1 for Mac-1 is located on the third NH_2-terminal immunoglobulin-like domain (8), whereas the binding sites for LFA-1, human picornaviruses, *Plasmodium*-infected erythrocytes, and fibronectin are located on overlapping but nonidentical segments of the most amino-terminal D1 and D2 domains, which are most distant from the host cell surface (2, 49). Our data suggest that NTHI shares this binding site. The LB2, R1/1, and 8.4A6 MAbs all block the interaction of LFA-1 with ICAM-1, although each binds to a distinct ICAM-1 epitope (3).

The P5 fimbrin is an important virulence factor in a chinchilla model of otitis media (34). P5 has been previously reported to bind to both respiratory mucin and CEACAM1 (14, 28). Our data indicate that it also binds ICAM-1, and therefore, like another important NTHI adhesin, Hap, P5 is able to interact with a very diverse group of eukaryotic cell surface molecules (10). Antibodies directed against P5 are protective in rat and chinchilla models of middle-ear infection; however, during chronic infections with NTHI, the protein is antigenically variable, potentially permitting bacteria to evade immune recognition and killing (9, 12, 23). Determining whether the antigenic variability of P5 proteins is significant in normal

human infections will require further study. The ability of P5 and other important NTHI adhesins to bind to a number of eukaryotic receptors, however, is likely to contribute to the organism's ability to colonize and infect humans.

ICAM-1 is expressed constitutively on a wide variety of cells, generally at a low basal level. Its expression is transcriptionally regulated through one of four pathways: NF-κB, gamma interferon–Janus kinase/signal transduction-activating transcription (JAK/STAT), mitogen-activated protein (MAP) kinase/activator protein 1 (AP1), and, indirectly, protein kinase C (PKC) (29). NTHI infection of tracheal epithelial cells induces the secretion of a number of proinflammatory cytokines, including tumor necrosis factor alpha, monocyte chemoattractant protein 1 (MCP-1), interleukin 6, and interleukin 8 (5). Additionally, NTHI and tumor necrosis factor alpha synergistically induce NF-κB activation through NF-κB-inducing kinase–IκB kinase β/γ-dependent phosphorylation and ubiquitination of IκBα and by the MEKK1-dependent activation of the p-38 MAP kinase kinase (MKK3/6) MAPK pathway (48). It therefore seems probable that NF-κB pathway activation is responsible for the NTHI-stimulated upregulation of ICAM-1 expression on respiratory epithelial cells (6, 17, 46, 47). Further studies, however, are needed to confirm this hypothesis and to identify what other positive regulatory pathways, if any, may be initiated by NTHI.

The ability of NTHI to upregulate ICAM-1 expression on respiratory cells is reduced by inhibiting protein synthesis with chloramphenicol or by killing bacteria with gentamicin. Sterile filtered NTHI culture supernatants also failed to increase ICAM-1 expression (11). These data imply that the factor responsible for the induction of ICAM-1 expression is a constitutive, cell surface-expressed molecule. In other studies, incubation of epithelial cells with purified *H. influenzae* lipooligosaccharide did not increase ICAM-1 expression, and both wild-type and mutant NTHI strains lacking HMW1, HMW2, or Hap had equivalent capacities to induce ICAM-1 expression on A549 cells. It is therefore tempting to hypothesize that NTHI both adheres to epithelial cells and stimulates ICAM-1 expression by interactions with the same receptor, P5; however, further studies will be required to address this issue.

In summary, we report that NTHI binds to respiratory epithelial cells by binding of P5 fimbriae to ICAM-1. The NTHI ICAM-1 binding site appears to be identical or closely related to the binding sites of LFA-1, human rhinoviruses, and coxsackieviruses. Our studies confirm that adherence of NTHI to epithelial cells increases ICAM-1 expression by respiratory epithelial cells. Thus, NTHI is able to positively regulate the expression of its own cell surface receptor. Blocking P5–ICAM-1 interactions has the potential to modify bacterial adherence and inflammatory responses to infection and to reduce the risk or severity of disease.

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