Effects of Rhinovirus Infection on the Adherence of *Streptococcus pneumoniae* to Cultured Human Airway Epithelial Cells

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To examine the effects of rhinovirus (RV) infection on the adherence of *Streptococcus pneumoniae* to human tracheal epithelial cells, cells were infected with RV-14, and *S. pneumoniae* were added to the culture medium. The number of *S. pneumoniae* adhering to epithelial cells increased after RV infection. Y-24180, a specific inhibitor of the platelet-activating factor receptor (PAF-R); PAF; and the pyrrolidine derivative of dithiocarbamate, an inhibitor of transcription factor nuclear factor–κB (NF-κB), decreased the number of *S. pneumoniae* adhering to cells after RV-14 infection. RV-14 infection increased PAF-R expression and the activation of NF-κB and promoter-specific transcription factor 1. These findings suggest that RV-14 infection stimulates *S. pneumoniae* adhesion to airway epithelial cells via increases in PAF-Rs that are partly mediated through activation of transcription factors. Increased adherence of *S. pneumoniae* may be one of the reasons that pneumonia develops after RV infection.

Respiratory bacterial infection causes the exacerbation of respiratory symptoms and pneumonia after respiratory viral infection and is a common problem for clinicians [1, 2]. Examination of the bacterial flora after respiratory viral infection has revealed infection with various microorganisms, including *Streptococcus pneumoniae* [3, 4]. Infection with *S. pneumoniae* after respiratory virus infection is associated with greater severity of illness and more frequent hospitalization [1]. Bacterial flora examination has revealed the presence of *S. pneumoniae* in secretions in the upper respiratory tract of patients infected with various viruses, including rhinovirus (RV) [5]. Because infection with viruses such as influenza virus and adenovirus damages ciliated and nonciliated airway epithelial cells [6], it has been suggested that impairment of mucociliary transport by viral infection allows the colonization of microorganisms in airways [7]. However, cell cytotoxicity does not appear to play a major role in the pathogenesis of RV infection [8].

The adherence of *S. pneumoniae* to epithelial cells is an important step in the development of respiratory tract infection with this bacterium [9]. *S. pneumoniae* adheres to the airway epithelial cells and vascular endothelial cells via binding to a receptor for the G protein–coupled platelet-activating factor (PAF), *N*-acetylgalactosamine β-1–4-galactose, or *N*-acetylgalactosamine β-1–3-galactose [9–14]. Activation of human epithelial and endothelial cells by interleukin (IL)–1α and tumor necrosis factor (TNF)–α produced in inflamed sites induces the adherence of this bacteria to PAF receptor (PAF-R) [13, 14]. Because respiratory viral infection induces the production of a variety of inflammatory cytokines by the alveolar and bronchial epithelial cells [15, 16], we hypothesized that RV infection may stimulate airway epithelial cells to induce the production of PAF-R, thereby increasing susceptibility to *S. pneumoniae* infection. However, the effects of RV infection on the development of *S. pneumoniae* infection have not been examined [17].

We recently demonstrated that *S. pneumoniae* ad-
heres to cultured human tracheal epithelial cells via binding to PAF-R [18]. In the present study, we investigated whether RV infection increases the adherence of S. pneumoniae to human tracheal epithelial cells.

MATERIALS AND METHODS

Culture media. Reagents for cell culture media were obtained from a variety of sources. Eagle’s MEM, Dulbecco’s modified Eagle’s medium (DMEM), Ham’s F-12 medium, fetal calf serum (FCS), and ultralow-IgG FCS were obtained from Gibco-BRL Life Technologies; fluorescein isothiocyanate (FITC), trypsin, EDTA, dithiothreitol, Sigma type XIV protease, human placental collagen, penicillin, streptomycin, gentamicin, and amphotericin B were from Sigma Chemical; Ultroser G serum substitute (USG) was from BioSepra; and pyrrolidine derivative of dithiocarbamate (PDTC) was from Calbiochem-Novabiochem.

Culture of human embryonic fibroblast cells and human epithelial HEp-2 cells. Human embryonic fibroblast cells were cultured in a Roux-type bottle (Iwaki Glass) sealed with a rubber plug in MEM containing 10% FCS supplemented with 10^5 U/L penicillin, 100 mg/L streptomycin, 50 mg/L gentamicin, and 2.5 mg/L amphotericin B. We used primary cultures of human tracheal epithelial cells for experiments done on days 6–14 of culture.

Virus stocks. RV-14 was prepared in our laboratory from isolates obtained from patients with common colds [19]. RV-14 was identified with a microneutralization test, using the antibody for RV-14, as described elsewhere [22]. Stocks of RV-14 were generated by infecting human embryonic fibroblast cells cultured in glass tubes in 1 mL of MEM supplemented with 2% ultralow-IgG FCS, 5 × 10^5 U/L penicillin, and 50 mg/L streptomycin at 33°C. The cells were incubated for several days in glass tubes in 1 mL of MEM supplemented with 2% ultralow-IgG FCS until the cytopathic effects were obvious, after which the cultures were frozen at −80°C, thawed, and sonicated. The virus-containing fluid thus obtained was frozen in aliquots at −80°C. The content of virus stock solutions was determined using the human embryonic fibroblast cell assay described below.

Respiratory syncytial virus (RSV) was also prepared in our laboratory from isolates from patients with common colds [19]. RSV was identified by indirect immunofluorescence, using guinea pig antisera, as described elsewhere [19]. Stocks of RSV were generated by infecting HEp-2 cells cultured in glass tubes in 1 mL of MEM supplemented with 2% ultralow-IgG FCS, 5 × 10^5 U/L penicillin, 50 mg/L streptomycin, and 1.7% glucose at 33°C.

Detection and titration of viruses. RVs were detected by exposing confluent human embryonic fibroblast cells in glass tubes to serial 10-fold dilutions of virus-containing medium in MEM supplemented with 2% ultralow-IgG FCS and 1.7% glucose. Glass tubes were then incubated at 33°C for 7 days, and the cytopathic effects of viruses on human embryonic fibroblast cells were observed using an inverted microscope (Mit; Olympus), as reported elsewhere [19]. RSV was also detected by exposing confluent HEp-2 cells in glass tubes to serial 10-fold dilutions of virus-containing medium in MEM supplemented with 2% ultralow-IgG FCS and 1.7% glucose [19]. The amount of specimen required to infect 50% of human embryonic fibroblast cells or HEp-2 cells (TCID50) was determined.

Viral infection of human tracheal epithelial cells. Infection of human tracheal epithelial cells with RV-14 and RSV was performed using methods described elsewhere [21]. RV-14 (10^4 TCID50 units/mL) or RSV (10^4 TCID50 units/mL) was added to the epithelial cells. After 1 h of incubation at 33°C, the virus solution was removed, and the epithelial cells were rinsed 1 time with 1 mL of PBS. The cells were then fed with fresh DMEM–Ham’s F-12 medium containing 2% USG supplemented with antibiotics, placed in an incubator, and cultured at 33°C [21]. We measured the time course of virus release.
from the epithelial cells as described elsewhere [21]. The supernatants were stored at \(-80^\circ\text{C}\) for the determination of virus content. Virus content in the supernatant is expressed as TCID\(_{50}\) units per milliliter [21].

**Bacterial stocks.** *S. pneumoniae* R6 (unencapsulated) was isolated from expectorated sputum obtained from patients with acute bronchopneumonia. The isolated bacteria were seeded on blood-agar plates consisting of Mueller-Hinton broth (Difco) with 5% sheep blood and 0.5% agar [23]. Colonies were gathered, resuspended in PBS to a concentration of 10\(^8\) cfu/mL, and then sonically dispersed into a single cell.

**Labeling of bacteria.** Bacteria (10\(^8\) cfu/mL) were mixed for 1 h at 4\(^\circ\)C with FITC (1 mg/mL) dissolved in a buffer containing 0.05 mol/L Na\(_2\)CO\(_3\) and 0.1 mol/L NaCl [24]. Subsequently, the bacteria were washed 3 times with PBS and resuspended to a final concentration of 10\(^8\) cfu/mL. In preliminary experiments, the numbers of *S. pneumoniae* detected by Gram staining and by fluorescent labeling were equivalent. We also found in the preliminary experiments that *S. pneumoniae* labeled with FITC can be grown in the growth medium described above.

**Adherence of bacteria to human tracheal epithelial cells.** The number of *S. pneumoniae* adhering to human tracheal epithelial cells was counted with methods described elsewhere [13, 18, 24]. To examine the effects of RV infection on the adherence of *S. pneumoniae* to the cells, the epithelial monolayers in the 6-well plates were further incubated in control DMEM–Ham’s F-12 medium containing 2% USG for 24 h after RV infection (exposure to 10\(^5\) TCID\(_{50}\) units/mL for 60 min). The epithelial cell monolayers were then rinsed twice with PBS, and FITC-labeled *S. pneumoniae* (10\(^8\) cfu/mL; 20 µL/well) suspended in 2 mL of DMEM–Ham’s F-12 medium containing 2% USG were added to the cells in 6-well tissue culture plates. The mixtures were incubated for 30 min at 37\(^\circ\)C and subsequently rinsed 5 times with fresh DMEM–Ham’s F-12 medium containing 2% USG to remove nonadherent bacteria. Adherent FITC-labeled *S. pneumoniae* were counted visually with an inverted microscope (IX70; Olympus) equipped for fluorescence with a DM500 filter (Olympus). Adherence was expressed as the number of adhered bacteria per 100 human tracheal epithelial cells counted in a 200× field. During the bacterial adherence experiments, the cell culture medium was not supplemented with any antibiotic.

**RNA preparation.** mRNA was isolated from monolayers of human tracheal epithelial cells cultured in 6-well plates as described elsewhere [25] using a Fast Trak 2.0 Kit (Invitrogen), according to the instruction manual. The amounts of mRNA were determined spectrophotometrically, and stock tubes were stored at \(-80^\circ\text{C}\).

**Northern blot analysis.** Northern blot analysis was done as described elsewhere [21, 26, 27]. Equal amounts of mRNA (2 µg) extracted from human tracheal epithelial cells were subjected
Figure 2. Effects of incubation period after rhinovirus type 14 (RV-14) infection on the no. of *Streptococcus pneumoniae* adhering to human tracheal epithelial cells after infection with RV-14 (10^5 TCID_{50} units/mL; 60 min; closed columns) or respiratory syncytial virus (RSV; 10^4 TCID_{50} units/mL; 60 min; hatched columns), or sham infection (open columns). The cells were further incubated in the fresh medium for 3, 6, 24, or 72 h after infection with RV-14 or RSV. Fluorescein isothiocyanate (FITC)–labeled *S. pneumoniae* (10^6 cfu/mL) were added to the medium and coincubated with the cells for 30 min. Adherence is expressed as the no. of adhered *S. pneumoniae* per 100 cells. Results are reported as from 7 samples from mean ± SEM RV-14 infection and from 5 samples for RSV infection. Data were analyzed using the Wilcoxon matched-pairs test. *P < .05 and **P < .01 vs. sham infection (control).

To electrophoresis on a 1% agarose-formaldehyde gel. The gel was then transferred via capillary action onto a nylon membrane (Hybond N; Amersham Life Sciences). The membrane was hybridized with [α-^32P]dCTP-labeled (3000 Ci/mmole [α-^32P]dCTP; Amersham) human PAF-R cDNA (1-kb EcoRI and SmaI fragment; Riken DNA Bank) with a random-primer labeling kit (Random Primer; Takara), and autoradiographic detection of the hybridized probe was performed by exposure to Kodak Scientific Imaging film for 48–72 h at −70°C. Quantification of autoradiographic bands was accomplished with an image analyzer (Bioimaging Analyzer BAS-2000; Fuji Photo Film).

**Flow cytometric analysis of the cell-membrane PAF-R.** The effects of RV-14 infection on PAF-R protein expression in human tracheal epithelial cells were assayed by flow cytometric analysis as described elsewhere [18, 28, 29]. Cells were removed from the 6-well tissue culture plate by incubation with Cell Dissociation Solution (Sigma) at 37°C for 10 min, collected, and washed with cold PBS. The cells were then incubated with an anti–PAF-R monoclonal antibody at a 1:200 dilution (1 mg/mL; Cayman Chemical) at 4°C for 30 min. For negative controls, mouse serum (1:200; Dako) was used instead of the first antibody. The cells were then washed twice and incubated with FITC-conjugated goat anti–mouse IgG (DAKO) diluted to 1:25 at 4°C for 20 min, extensively washed, and resuspended in ice-cold PBS at a cell concentration of 10^6 cells/mL and analyzed by a flow cytometer (FACSCalibur; Becton Dickinson). Expression of PAF-R was determined by subtracting the mean fluorescence intensity measured with the control monoclonal antibody from that measured with the antibody to PAF-R.

**Isolation of nuclear extracts.** Nuclear extracts were prepared using methods described elsewhere [18, 30, 31]. The human tracheal epithelial cells in 6-well dishes were washed with ice-cold PBS, harvested by scraping into FCS, and pelleted in a 1.5-mL microfuge tube at 1850 g for 5 min. The pellet was suspended in 1 packed-cell volume of lysis buffer (10 mmol/L HEPES [pH 7.9], 10 mmol/L KCl, 0.1 mmol/L EDTA, 1 mmol/L DTT, and 0.5 mmol/L phenylmethyl-sulfonyl fluoride [PMSF]) and incubated for 15 min. Membrane lysis was accomplished by adding 25 μL of 10% Nonidet P-40, followed by vigorous agitation. The nuclei were then collected by centrifugation, resuspended in 50 μL of extract buffer (20 mmol/L HEPES, 420 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L DTT, and 0.5 mmol/L phenylmethyl-sulfonyl fluoride [PMSF]) and incubated vigorously at 4°C for 15 min. The nuclear extracts were stored at −70°C until use.

**Electrophoretic mobility shift assays (EMSA).** EMSAs were performed as described elsewhere [18, 30, 31]. Radiolabeled double-stranded oligonucleotide probes for the NF-κB or the promoter-specific transcription factor 1 (SP-1) site...
were prepared by annealing complementary oligonucleotides and end-labelling with $[^{32}P]$ATP and T4 polynucleotide kinase. The radiolabeled probes used for NF-$\kappa$B and SP-1 were composed of the following sequences: $5'$-GATCGAGGGGAC-TTTCCCTAGC-3' for NF-$\kappa$B (Stratagene) [32, 33] and $5'$-AATTACGGGGCGGGCAGTACCGGGGCGGT-3' for SP-1 (Stratagene) [34].

To study the effects of RV-14 infection on the activation of NF-$\kappa$B and SP-1, nuclear extracts from human tracheal epithelial cells were isolated before and 30, 60, and 120 min after RV-14 infection ($10^5$ TCID$_{50}$ units/mL; 60 min). We also studied the effects of IL-1$\alpha$ (10 ng/mL), IL-1$\beta$ (10 ng/mL), and TNF-$\alpha$ (10 ng/mL) on the activation of NF-$\kappa$B and SP-1 as a positive control.

**Supershift EMSAs.** Supershift assays were used to determine which members of the NF-$\kappa$B family were involved in RV-14 infection–induced binding of DNA by NF-$\kappa$B. In these studies, EMSAs were performed as described above, except that rabbit polyclonal antibodies against the NF-$\kappa$B subunit proteins p65, p50, c-Rel, and Rel B (Santa Cruz Biotechnology) were included in the 1-h radiolabeled probe–extract binding reaction at 4°C [18, 30, 31]. Preimmune serum (Santa Cruz Biotechnology) was used to control for any nonspecific effects of these antisera.

**Statistical analysis.** Results are expressed as mean ± SEM. Within-group comparisons were tested using the Wilcoxon matched-pairs test, and between-group comparisons were tested using the Mann-Whitney $U$ test. $P<.05$ was considered to be significant. "n" refers to the number of donors from whom cultured epithelial cells were used.

**RESULTS**

**Effects of RV-14 infection on adherence of S. pneumoniae to human tracheal epithelial cells.** Adherence of S. pneumoniae to cultured human tracheal epithelial cells was observed consistently. The number of epithelial cells observed in each 200× field was 696 ± 61 (196 monolayers) and did not differ significantly among the tracheas (28 tracheas; $P>.20$). The number of cells of the confluent cultured epithelial cell sheets also did not differ significantly between the tracheas from nonsmokers and those from smokers (data not shown). Measurement of the number of S. pneumoniae adhering to the cells at differing times after the addition of S. pneumoniae to the medium revealed no significant change in the numbers of S. pneumoniae at 10 min after the addition of S. pneumoniae. Adhered S. pneumoniae were observed at 20 min, and the number of adhering S. pneumoniae progressively increased between 20 and 150 min after the addition of S. pneumoniae (figure 1A). Adherence of S. pneumoniae to the epithelial cells was constant, and the coefficient of variation of the number of adherent S. pneumoniae at 30 min was small (7.9%; $n = 20$). The number of S. pneumoniae adhering to the cells increased significantly with time ($P<.05$; figure 1A).

Similarly, the measurement of the number of S. pneumoniae adhering to human tracheal epithelial cells at differing concentrations of S. pneumoniae revealed no significant number of S. pneumoniae at 10$^5$ cfu/mL. Adherence of S. pneumoniae was observed at 10$^6$ cfu/mL, and the number of adhering S. pneumoniae progressively increased between additions of 10$^5$ cfu/mL and 10$^6$ cfu/mL of S. pneumoniae into the culture medium (figure 1B). The number of S. pneumoniae adhering to the cells increased in a concentration-dependent fashion ($P<.05$).

Exposing confluent human tracheal epithelial cell monolayers to RV-14 ($10^5$ TCID$_{50}$ units/mL) consistently led to infection. Collection of culture medium at different times after RV-14 infection revealed no detectable virus at 1 h after infection. RV-14 was detected in culture medium 12 h after infection, and the virus content progressively increased between 12 and
Figure 4. Effects of an inhibitor of a platelet-activating factor receptor (PAF-R), Y-24180 (10^{-5} mol/L; closed columns; A) and pretreatment with PAF (10^{-5} mol/L; hatched columns; B) on the no. of *Streptococcus pneumoniae* adhering to cultured human tracheal epithelial cells after infection with rhinovirus type 14 (RV-14) or respiratory syncytial virus (RSV). The cells were incubated in control medium for 6 h after infection with RV-14 (10^5 TCID_50 units/mL; 60 min) or RSV (10^4 TCID_50 units/mL; 60 min) and further incubated with either Y-24180 (30 min) or PAF (10 min). *S. pneumoniae* were then coincubated with the cells for 30 min. Open columns, untreated cells. Adherence is expressed as the no. of adhered *S. pneumoniae* per 100 cells. Results are reported as means ± SEM from 7 samples for RV-14 infection and from 5 samples for RSV infection. Within-group comparisons were tested using the Wilcoxon matched-pairs test, and between-group comparisons were tested using the Mann-Whitney U test. *P < .05 and **P < .01, vs. medium alone (control); + , vs. infection with RV-14.

72 h after infection, as described elsewhere [21]. RV-14 titers in culture supernatants were 1.1 ± 0.1 log TCID_50 units/mL (n = 7) at 12 h, 2.2 ± 0.2 log TCID_50 units/mL (n = 7) at 24 h, and 3.1 ± 0.2 log TCID_50 units/mL (n = 7) at 24–72 h after RV-14 infection. RSV titers in culture supernatants were 2.0 ± 0.1 log TCID_50 units/mL (n = 5) at 24 h.

Measurement of the number of *S. pneumoniae* adhering to human tracheal epithelial cells at different times after RV-14 infection (10^5 TCID_50 units/mL; 60 min) revealed no significant increase in the number of *S. pneumoniae* at 3 h (figure 2). However, an increase in the number of *S. pneumoniae* was observed at 6 h, and the number of adhering *S. pneumoniae* progressively increased between 6 h and 24 h after RV-14 infection (figure 2). The adherence of *S. pneumoniae* was not equal, and some clumps of *S. pneumoniae* were observed. The bacteria adhered to a subset of epithelial cells, but not to all of the cells. An increase in the number of *S. pneumoniae* was also observed at 6 h after RSV infection, and the number of adhering *S. pneumoniae* progressively increased between 6 and 24 h after infection (figure 2).

Incubation of cells with a mouse monoclonal antibody to intercellular adhesion molecule (ICAM)–1 (100 µg/mL; 84H10; Immunotech), a receptor for RV-14 [27], significantly inhibited adherence of *S. pneumoniae* induced by RV-14 infection, whereas an isotype-matched mouse IgG1 control antibody (100 µg/mL; Chemicon International) did not change the number of *S. pneumoniae* induced by RV-14 infection (P > .20; figure 3). 84H10 is an IgG1 isotype and recognizes the ICAM-1 function domain.

To confirm that the increases in the number of *S. pneumoniae* adhering to human tracheal epithelial cells induced by RV-14 infection were the result of the effects of RV-14 infection and not a contaminant present in the virus stock, the ability of UV-inactivated virus [35] to induce increases in the number of *S. pneumoniae* adhering to the cells was also examined. RV-14 was not detected in culture medium 24 h after infection with UV-inactivated RV-14, and UV-inactivated RV-14 did not change the number of *S. pneumoniae* (figure 3), compared with medium alone.

Human tracheal cell viability, assessed by the exclusion of trypan blue, was consistently >96% in RV-14–infected cultures. Similarly, RV-14 infection did not alter the amount of lactate dehydrogenase in the supernatants (14 ± 1 IU/L before vs. 15 ± 1 IU/L 24 h after infection; P > .50; n = 7). RV-14 infection also had no effect on cell numbers. Cell counts at 24 h after RV-14 infection were not significantly different from those after sham infection (4.1 ± 10^6 ± 0.3 × 10^6 sham-infected cells vs. 4.0 ± 10^6 ± 0.3 × 10^6 RV-14–infected cells; P > .50; n = 7).
Effects of interleukin (IL)–1α (10 ng/mL), IL-1β (10 ng/mL), and tumor necrosis factor (TNF)–α (10 ng/mL) on the no. of *Streptococcus pneumoniae* adhering to cultured human tracheal epithelial cells in the presence (closed columns) or absence (open columns) of an inhibitor of the platelet-activating factor receptor (PAF-R), Y-24180 (10⁻⁵ mol/L). The cells were incubated in control medium containing either IL-1α, IL-1β, or TNF-α for 4 h and further incubated with Y-24180 for 30 min. *S. pneumoniae* were then coincubated with the cells for 30 min. Adherence is expressed as the no. of adhered *S. pneumoniae* per 100 cells. Results are reported as mean ± SEM from 7 samples. Within-group comparisons were tested using the Wilcoxon matched-pairs test, and between-group comparisons were tested using the Mann-Whitney U test. *P < .05*, vs. medium alone (control); + , vs. treatment with each cytokine.

**Effects of the PAF-R inhibitor Y-24180 and PAF on RV-14 infection–induced adherence of *S. pneumoniae*.** A PAF-R inhibitor, Y-24180 (10⁻³ mol/L; 30 min; figure 4A) [36], or PAF (10⁻⁵ mol/L; 10 min; figure 4B) [13] alone did not affect the adherence of *S. pneumoniae* to the cultured human tracheal epithelial cells under control conditions. However, both Y-24180 (10⁻³ mol/L; figure 4A) and PAF (10⁻⁵ mol/L; 10 min; figure 4B) significantly inhibited adherence of *S. pneumoniae* induced by infection with RV-14 or RSV. 

IL-1α (10 ng/mL; 4 h), IL-1β (10 ng/mL; 4 h), and TNF-α (10 ng/mL; 4 h) [13] all increased the number of *S. pneumoniae* adhering to cells, compared with medium alone (figure 5). Y-24180 (10⁻⁵ mol/L) significantly reduced the number of *S. pneumoniae* induced by these cytokines (figure 5).

The culture supernatants of human embryonic fibroblast cells without RV-14 infection did not contain significant amounts of IL-1β and TNF-α, and the levels of IL-1β and TNF-α in the supernatants of cultured human tracheal epithelial cells were not altered from baseline (data not shown). Furthermore, supernatants of the epithelial cells did not contain significant levels of IL-1β or TNF-α at 6 h after RV-14 infection (data not shown).

**Effects of RV-14 infection on PAF-R expression.** The baseline expression of PAF-R mRNA was constant in confluent human tracheal epithelial cell sheets, and the coefficient of variation was small (7.8%; n = 7). Neither smoking nor cause of death influenced the baseline expression of PAF-R mRNA. Infection of the cells with RV-14 (10⁵ TCID₅₀ units/mL; 60 min) caused increases in PAF-R mRNA (figure 6A). At 4 h after RV-14 infection, the cells were shown to overexpress PAF-R mRNA, compared with cells at 4 h after sham infection (control; figure 6B).

Expression of PAF-R was also assayed by flow cytometric analysis. At 6 h after RV-14 infection, the cells were shown to increase PAF-R–specific fluorescence intensity, compared with
cells after sham infection (figure 7A, 7C, and 7D). Similarly, IL-1β (10 ng/mL; 4 h) increased PAF-R–specific fluorescence intensity in the cells (figure 7B and 7D).

**NF-κB DNA-binding activity in human tracheal epithelial cells.** Nuclear extracts from the human tracheal epithelial cells with RV-14 or sham infection contained activated NF-κB, as demonstrated by the presence of a complex consisting of a protein bound to a DNA fragment carrying NF-κB and SP-1 (figure 8). The baseline intensity of NF-κB and SP-1 DNA-binding activity was constant, and increased activation of NF-κB and SP-1 was present in cells from 30 min after RV-14 infection (figure 8). Similarly, IL-1α (10 ng/mL), IL-1β (10 ng/mL), and TNF-α (10 ng/mL) all increased activation of NF-κB at 0.5 and 1 h (data not shown). In contrast, IL-1α (10 ng/mL), IL-1β (10 ng/mL), and TNF-α (10 ng/mL) did not increase the activation of SP-1 at any time (data not shown). The specificity of the NF-κB binding was confirmed by supershift EMSA, in which antibodies to the p50 or p65 subunit of NF-κB ablated NF-κB bands (figure 9). The supershifting of the NF-κB band with the antibody to the p50 or p65 subunit of NF-κB was constantly observed at all times during the cell culture. However, the supershifting of the NF-κB band was not observed with antibody to p52, c-Rel, Rel B, or preimmune antiserum (figure 9).

**Effects of the NF-κB inhibitor PDTC on adherence of S. pneumoniae.** To examine the relationship between NF-κB activation and the adherence of *S. pneumoniae* to cultured human tracheal epithelial cells after RV-14 infection, we studied the effects of a specific NF-κB inhibitor, PDTC [37], on the adherence of *S. pneumoniae*. The cells were pretreated with PDTC (10⁻⁴ mol/L) for 90 min and exposed to RV-14 (10⁵ TCID₅₀ units/mL; 60 min) containing PDTC (10⁻⁴ mol/L). The cells were further cultured in fresh DMEM–Ham’s F-12 medium containing 2% USG and PDTC (10⁻⁴ mol/L) for 6 h. The cell monolayers were then coincubated with FITC-labeled *S. pneumoniae* (10⁶ cfu/mL) for 30 min. PDTC alone did not affect the adherence of *S. pneumoniae* to the cells in the control condition (45 ± 4 cfu/100 cells for NF-κB inhibitor vs. 44 ± 4 cfu/100 cells for control).
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DISCUSSION

The results of the present study suggest that RV-14 infection increases adherence of S. pneumoniae by inducing surface expression of PAF-R, a receptor for S. pneumoniae [9, 13], in cultured human tracheal epithelial cells. These conclusions are based on the observation that RV-14 infection increased the number of S. pneumoniae adhering to the cells and increased the expression of the protein and mRNA of PAF-R in the cells, whereas UV-inactivated RV-14 did not increase the number of adhered S. pneumoniae. Both a monoclonal antibody to ICAM-1, a receptor for RV-14 [38], and a specific inhibitor of PAF-R, Y-24180, reduced the number of adhered S. pneumoniae induced by RV-14 infection. In the absence of the RV-14 infection, time course and concentration dependency on the number of S. pneumoniae adhered to the human tracheal epithelial cells were consistent with another report that involved resting human lung cells [9–13]. Because Y-24180 had no effect on the number of S. pneumoniae adhering to human tracheal epithelial cells in culture medium alone, as described elsewhere [18], S. pneumoniae may adhere to the nonactivated human tracheal epithelial cells via binding to receptors other than PAF-R, such as N-acetylgalactosamine β-1–4-galactose and N-acetylgalactosamine β-1–3-galactose, as was shown in the resting lung cells [9–13]. In contrast, inflammatory activation induced by RV-14 infection may enhance the adherence of S. pneumoniae to human tracheal epithelial cells through the up-regulation of PAF-R expression [9, 13]. Because pretreatment with PAF also inhibited RV-14 infection–induced adherence of S. pneumoniae, S. pneumoniae may bind near the active site of PAF-R, as has been shown in vascular endothelial cells and lung cells [13].

In the present study, the location of S. pneumoniae in cultured human tracheal epithelial cells was not observed. However, in previous reports [13, 24], 2%–3% of S. pneumoniae internalized into the cultured endothelial cells within 30 min of incubation. Therefore, S. pneumoniae might begin to internalize into the cultured human tracheal epithelial cells, because we cocultured S. pneumoniae with the epithelial cells for 30 min after RV-14 infection.

RV-14 increased activation of NF-κB and SP-1 from 30 min after infection in the present study. Two different promoters, transcript 1 and 2, have been demonstrated to express mRNA of PAF-R, and both transcripts have been shown in the lung [39]. Transcript 1 has consensus sequences for transcription

**Figure 8.** Electrophoretic mobility shift assay demonstrating the time course of NF-κB (A) and promoter-specific transcription factor 1 (SP-1; B) DNA-binding activity of human tracheal epithelial cells before (0 min) and 30, 60, and 120 min after infection with rhinovirus type 14 (RV-14; 10⁵ TCID₅₀ units/mL; 60 min). NF-κB and SP-1 DNA-binding activity is highlighted by arrows. Data are representative of 3 different experiments.
The adherence of *S. pneumoniae* was not equal, and some clumps of *S. pneumoniae* were observed. The bacteria adhered to a subset of epithelial cells but not to all the cells. These findings suggest that RV-14 might infect a small cluster of epithelial cells. It has been reported elsewhere that at most 15%–20% of primary epithelial cells can be infected by RV [41].

Respiratory viral infection induces tracheal epithelial damage in ferrets and humans, including desquamation of ciliated and nonciliated cells [5, 42]. It has been suggested that respiratory viral infection may reduce bacterial clearance in the lung [7]. Thus, viral infection may affect the airway mucociliary clearance and local antibacterial defenses, resulting in the development of bacterial proliferation [7]. Furthermore, infection with viruses, including RV, stimulates the alveolar and bronchial epithelial cells and increases the production of various cytokines and ICAM-1 [15, 16, 21]. Therefore, viral infection not only damages the lung cells but also causes the stimulation of airway and lung epithelial cells. It has been suggested that *S. pneumoniae* may adhere to lung cells that have been activated by inflammatory cytokines and acid exposure via binding to PAF-R [14, 18]. In the present study, RV-14 infection increased PAF-R expression in human tracheal epithelial cells. Therefore, increased adherence of *S. pneumoniae* via binding of PAF-R may be one of the mechanisms responsible for airway pneumococcal infection that occurs after RV-14 infection [5, 17].

Infection with *S. pneumoniae* after respiratory viral infection is associated with the severity of illness and more frequent hospitalization [1]. Concomitant viral-bacterial infection, including infection with *S. pneumoniae* and RV, was observed in patients with community-acquired pneumonia in one study [5]. Examination of bacteriologic flora revealed the presence of *S. pneumoniae* in secretions in the upper respiratory tract of patients infected with various viruses, including RV [3–5]. Furthermore, superinfection with influenza virus and bacteria, including *S. pneumoniae*, has also been suggested in the normal host with pneumonia [43]. RV infection–induced increases in adherence of *S. pneumoniae* may promote the development of pneumonia.

In summary, we have demonstrated that the number of *S. pneumoniae* adhering to human tracheal epithelial cells and the expression of PAF-R, a receptor for *S. pneumoniae*, in cells increased after RV-14 infection. A specific inhibitor of PAF-R, Y-24180, and pretreatment with PAF inhibited the adherence of *S. pneumoniae* to cells that was induced by RV-14 infection. Infection with RSV also increased the number of *S. pneumoniae* adhering to the epithelial cells, and a specific inhibitor of PAF-R and pretreatment with PAF inhibited adherence. Therefore, infection with respiratory viruses, including RV and RSV, may induce adherence of *S. pneumoniae* to human tracheal epithelial cells.
cells via binding to PAF-R. In addition to the impairment of bacterial clearance [7] caused by airway epithelial damage [6, 42], increased adherence of S. pneumoniae to airway epithelial cells through binding to PAF-R may be important in the development of bacterial infection. A specific inhibitor for PAF-R may have protective effects against infection by S. pneumoniae after respiratory viral infection.

Acknowledgments

We thank Grant Crittenden, for English correction, and Akira Ohmi, Michiko Okamoto, Minako Tada, and Fusako Chiba, for technical assistance.

References