Infection of human respiratory submucosal glands with rhinovirus: effects on cytokine and ICAM-1 production

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Yamaya, Mutsuo, Kiyohisa Sekizawa, Tomoko Suzuki, Norihiro Yamada, Masayuki Furukawa, Satoshi Ishizuka, Katsutoshi Nakayama, Masanori Terajima, Yoshio Numazaki, and Hidetada Sasaki. Infection of human respiratory submucosal glands with rhinovirus: effects on cytokine release and ICAM-1 production. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L362–L371, 1999.—To further understand the early biochemical events that occur in infected surface epithelium, we developed for the first time a model in which a respiratory submucosal gland cell population can be infected with rhinovirus (RV). Viral infection was confirmed by demonstrating with PCR that viral titers in supernatants and lysates from infected cells increased with time. Infection by RV14 upregulated the expression of intercellular adhesion molecule-1 (ICAM-1) mRNA, the major RV receptor, on submucosal gland cells, and it increased production of interleukin (IL)-1α, IL-1β, IL-6, IL-8, tumor necrosis factor-α, and granulocyte-macrophage colony-stimulating factor in supernatants. Antibodies to ICAM-1 inhibited RV infection of submucosal gland cells and decreased the production of cytokines after RV infection. Both IL-1α and IL-1β upregulated ICAM-1 mRNA expression and increased susceptibility to RV infection, whereas other cytokines failed to alter ICAM-1 mRNA expression. Furthermore, neutralizing antibodies to IL-1α and IL-1β significantly decreased the viral titers in supernatants and ICAM-1 mRNA expression after RV infection, but a neutralizing antibody to tumor necrosis factor-α was without effect. These findings suggest that respiratory submucosal gland cells play an important role in the initial stages of inflammation and provide useful insights into the pathogenesis of RV infection.

Intercellular adhesion molecule-1; asthma; common cold; airway inflammation; interleukin-1; polymerase chain reaction

RHINOVIRUSES (RVs) are the major cause of the common cold, the most common acute infectious illness in humans (10). Furthermore, 80% of asthma exacerbations in school-aged children and half of all asthma exacerbations in adults are associated with viral upper respiratory infection, and the majority of viruses isolated are RVs (18, 23).

Intercellular adhesion molecule-1 (ICAM-1), the receptor for the major group of RVs (13), is expressed by airway epithelial cells (26, 32). Likewise, during colds, RV has been detected in a limited number of shed nasal epithelial cells by indirect immunofluorescence (34) as well as in nasal epithelial cells from biopsies of infected subjects by in situ hybridization (3). These findings indicate that the nasal epithelium is an important site of RV infection in humans. Furthermore, the literature (9, 11, 16) suggests RV infection in the lower respiratory tract in humans. In contrast to a variety of other respiratory pathogens (e.g., influenza and adenovirus), cytotoxicity of epithelial cells does not appear to play a major role in the pathogenesis of RV infections (8, 10, 15). Instead, it is believed that the manifestations of RV-induced pathogenesis are the result of virus-induced mediators of inflammation (37).

The submucosal glands produce airway secretions 40 times more than goblet cells (28). The airway submucosal glands may be the target of RV infection because nasal discharge in the common cold contains both watery and mucus secretions (6, 14). Nasal discharge in RV infection is reduced by a parasympatholytic agent and an antihistamine, but the inhibitory effects are not complete and an antihistamine agent failed to improve nasal obstruction (6, 14). Therefore, RV infection itself may also relate to airway submucosal edema and secretions of the submucosal glands. Histological examination showed RV infection-induced infiltration of leukocytes in the airway mucosa and submucosa (12). Therefore, cells in the submucosa may be involved in RV infection and its infection-induced inflammation. Infection of epithelial cells with RV induces production of several cytokines such as interleukin (IL)-1, IL-6, IL-8, tumor necrosis factor (TNF)-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF) (31, 32). These cytokines are known to mediate a wide variety of proinflammatory and immunoregulatory effects (1) and may play an important role in the pathogenesis of RV infections. However, the role of submucosal gland cells in RV infection has not been investigated.

To further understand the cellular events involved in the pathogenesis of RV respiratory tract infection, studies were undertaken to determine whether human respiratory submucosal gland cells can be infected with RV and whether infection of these cells with RV leads to increased production of proinflammatory cytokines and increases in ICAM-1 expression in human submucosal gland cells.

MATERIALS AND METHODS

 Medium components. Reagents for cell culture medium were obtained as follows: Eagle’s minimum essential medium

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Human embryonic fibroblast cell culture. Human embryonic fibroblast cells were cultured in MEM containing 10% FCS and supplemented with 5 × 10^5 UI of penicillin and 50 mg/ml of streptomycin in Roux-type bottles (Iwaki Garasu, Chiba, Japan) that were sealed with rubber plugs (25). Confluency was achieved at 7 days, at which time the cells were collected by trypsinization (0.05% trypsin and 0.02% EDTA). The cells (1.5 × 10^6 cells/ml) suspended in the trypsinizing flask were again exposed to enzymatic detachment and incubated at 37°C in 5% CO2-medium, fetal calf serum (FCS), and Gentamicin (G). Growth factors on the first day of plating contained 0.1% USG and growth factors on the first day of plating. The cell culture medium was supplemented with 10^5 UI of penicillin, 100 mg/ml of streptomycin, 50 mg/ml of gentamicin, and 2.5 mg/l of amphotericin B. All culture vessels were coated with human placental collagen (20 µg/cm²) (36). The absence of fibroblasts in the glass tubes was confirmed with an inverted microscope (Mit-2, Olympus, Tokyo, Japan). Under an inverted microscope, the cultured human tracheal submucosal gland cells had a cuboidal or round-shaped appearance of confluent sheets. In contrast, the fibroblasts had a spindle-shaped appearance. We found in the preliminary experiments that RV infection caused cytopathic effects on spindle-shaped fibroblasts as shown by Winther et al. (35) but not on submucosal gland cells with a cuboidal or round-shaped appearance. Furthermore, to determine whether cultured cells can form tight junctions, we performed parallel cultures of human tracheal submucosal gland cells on Millicell CM inserts (0.45 µm pore size and 0.6 cm² area; Millipore Products Division, Bedford, MA) to measure electrical resistance and short-circuit current with Ussing chamber methods (36). When cells cultured under these conditions formed tight junctions without contamination by fibroblasts, they have values of >40 Ω·cm² for resistance and >2 µA/cm² for short-circuit current (36). Therefore, cultured tracheal submucosal gland cells able to form tight junctions without contamination by fibroblasts were used for the following experiments when the cultured cells in the tubes had a cuboidal or round-shaped appearance and the cells on the Millicell CM inserts had high resistance (>40 Ω·cm²) and high short-circuit current (>2 µA/cm²).

To determine whether cultured cells possess immunologic characteristics of submucosal gland cells, the expression of serous and mucous gland cell secretory antigen was examined by using immunocytochemical methods as previously described (5, 30). The cells cultured on glass coverslips were fixed with 4% paraformaldehyde and 0.5% Triton X-100 in 0.1 M phosphate buffer for 15 min at room temperature and were then washed with PBS. Immunocytochemistry with a monoclonal antibody directed against mucous (A1F8) or serous (B1D8) gland cells of the original tissues (30) was performed with a modification of a biotin-avidin procedure (5). The specimens were counterstained with methyl green.

Viral stocks. RV14 was prepared in our laboratory from patients with the common cold (25, 32). Stocks of RV14 were generated by infecting human embryonic fibroblast cells cultured in glass tubes in 1 ml of MEM supplemented with 2% GGFCS, 50 U/ml of penicillin, and 50 µg/ml of streptomycin at 33°C. The cells were incubated for several days in glass tubes in 1 ml of MEM supplemented with 2% GGFCS until the cytopathic effects were obvious, after which the cultures were frozen at −80°C, thawed, and sonicated. The virus-containing fluid so obtained was frozen in aliquots at −80°C. The content of the viral stock solution was determined with the human embryonic fibroblast cell assay described in Detection and titration of viruses.

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 or lysates in MEM supplemented with 2% GGFCS. The glass tubes were then incubated at 33°C for 7 days, and the cytopathic effects of the viruses on human embryonic fibro-
blast cells were observed with an inverted microscope (MIT, Olympus) as previously reported (25, 32). The amount of specimen required to infect 50% of human embryonic fibroblast cells [50% tissue culture infectious dose (TCID50)] was determined. Furthermore, to confirm that the cytopathic effects on human embryonic fibroblast cells were caused by RV infection, RNA was extracted from culture supernatants of human tracheal submucosal gland cells in each subject and RV RNA was detected with the methods described in Detection of RV RNA and cytokinemRNA by RT-PCR.

Viral infection of human tracheal submucosal gland cells. The medium was removed from confluent monolayers of human tracheal submucosal gland cells and replaced with 1 mL of F-12-DMEM supplemented with 0.1% USG and growth factors. RV was added at a concentration of 10^5 TCID50 units/mL. After a 1-h incubation at 33°C, the viral solution was removed, and the cells were rinsed one time with 1 ml of PBS. The cells were then fed with F-12-DMEM containing 0.1% USG and growth factors supplemented with 10^5 U/l of penicillin, 100 mg/l of streptomycin, 50 mg/l of gentamicin, and 2.5 mg/l of amphotericin B and cultured at 33°C with rolling in an incubator (HDR-6T, Hirasawa, Tokyo, Japan) (32). The supernatants were removed at various times after infection and were stored at −80°C for the determination of viral content. Cell-associated viral content was also analyzed with sonicated human tracheal submucosal gland cells. The viral content in the supernatant and the cell-associated viral content are expressed as TCID50 units per milliliter and TCID50 units per 10^6 cells, respectively.

Effects of antibodies to ICAM-1 on RV infection. Confluent human tracheal submucosal gland cells were incubated for 30 min at 37°C with medium alone, with medium containing either of two mouse monoclonal anti-human antibodies to ICAM-1 (84H10 (100 µg/ml; Immunotech, Marseilles, France) or RR1 (100 µg/ml; a gift from Boehringer Ingelheim, Ridgefield, CT)), or with medium containing an isotype-matched mouse IgG1 control antibody (100 µg/ml; Chemicon International). Both 84H10 and RR1 are IgG1 isotypes and recognize the ICAM-1 functional domain. After excess antibodies were washed off, the monolayers were exposed to RV14 (10^5 TCID50 units/ml) for 60 min while being rinsed and fresh F-12-DMEM containing 0.1% USG and growth factors supplemented with 10^5 U/l of penicillin, 100 mg/l of streptomycin, 50 mg/l of gentamicin, and 2.5 mg/l of amphotericin B was added. The viral content of this medium was then assessed at various times after infection.

Detection of RV RNA and cytokine mRNA by RT-PCR. Human tracheal submucosal gland cells cultured in glass tubes were lysed by the addition of RNAzol (0.2 ml/10^6 cells; BIOTECX, Houston, TX) and were transferred to Eppendorf tubes. The cell homogenates were mixed with a 10% volume of chloroform, shaken vigorously for 15 s, placed on ice for 15 min, and centrifuged at 12,000 g for 15 min at 4°C. The upper aqueous phase containing RNA was collected, mixed with 20 µg of glycogen (Boehringer Mannheim) as a carrier, and then mixed with an equal volume of isopropanol. Pellets of RNA were obtained by centrifugation at 12,000 g for 15 min at 4°C and dissolved in water.

PCR was performed as previously described (4, 19). Briefly, 2 µg of RNA from each aliquot of human tracheal submucosal gland cells were dissolved in 100 µl of buffer containing the following reagents for the RT reaction: 50 mM Tris·HCl (pH 8.3), 75 mM KCl, 3 mM MgCl2, 10 mM dithiothreitol, 5 µl of Moloney murine leukemia virus RT ( Gibco BRL), 0.5 mM deoxynucleoside 5'-triphosphate (Takara, Ohtsu, Japan), 1 U/µl of ribonuclease inhibitor (Promega, Madison, WI), and 5 µM random hexamers (Pharmacia Biotech, Uppsala, Sweden). The RT reaction was performed for 60 min at 37°C followed by 10 min at 95°C. The resulting cDNA was frozen at −80°C until used in the PCR. For each sample, 5 µl of RT mixture were added to a 45-µl PCR mixture consisting of 10 mM Tris·HCl buffer (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 0.2 mM deoxynucleoside 5'-triphosphate, and 1.25 U of Taq polymerase (Takara). Primer pairs for the RV and cytokines were present at 2 ng/µl. Sequences of the PCR primer pairs used in these experiments are described elsewhere (32). The PCR was performed in an automated thermal cycler (MJ Research, Watertown, MA), and 10 µl of the reaction mixture from each sample were removed at 30 cycles. The samples were separated on a 2% agarose gel (FMC Bioproducts, Rockland, ME) and stained for 30 min in 1 µg/ml of ethidium bromide. The DNA bands were visualized on an ultraviolet (UV) illuminator and were photographed with type 667 positive/negative film (Polaroid, Cambridge, MA).

For the RV and each cytokine, mRNA expression in the human tracheal submucosal gland cells and RV RNA content in the culture supernatants were examined before and 1, 3, and 5 days after RV14 infection.

Cytokine assays. In the preliminary study, we found that the mRNAs of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and GM-CSF were expressed in cultured human tracheal submucosal gland cells before and after RV14 infection. To determine the effects of RV14 infection on the production of cytokines, we measured the amount of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and GM-CSF released from human tracheal submucosal gland cells into the culture medium before and after RV14 infection. The proteins of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and GM-CSF were measured by specific enzyme-linked immunosorbent assays (ELISAs). Sensitivities of the assay were 10 pg/ml for the IL-1α, IL-1β (both from Ohtsuka, Tokushima, J apan), IL-6, and IL-8 (both from Toray, Tokyo, J apan) ELISA kits, 4 pg/ml for the TNF-α ELISA kit (Ohtsuka), and 2 pg/ml for the GM-CSF ELISA kit (Genzyme, Cambridge, MA). We used an average value of replicate cultures from the same trachea (n = 3 donors) for the analysis of cytokine production.

To determine the effects of antibodies to ICAM-1 on RV infection, confluent human tracheal submucosal gland cells were incubated for 30 min with medium alone, with medium containing either 84H10 (100 µg/ml) or RR1 (100 µg/ml) antibody, or medium containing an isotype-matched mouse IgG1 control monoclonal antibody (100 µg/ml) or RR1 (100 µg/ml) antibody, or medium containing an isotype-matched mouse IgG1 control monoclonal antibody (100 µg/ml; Chemicon International) at 37°C before RV14 infection. To confirm that increases in the cytokines induced by RV14 infection were due to the effects of RV14 infection and not a contaminant present in the viral stock, the ability of UV-inactivated virus to induce increases in the cytokines was also examined. UV inactivation was performed as previously described (17).

Northern blot analysis. Northern blot analysis was done as previously described (29). Equal amounts of total RNA (10 µg)
extracted from human tracheal submucosal gland cells, as determined spectrophotometrically, were subjected to electrophoresis in 1% agarose-formaldehyde gels. The gel was then transferred via capillary action onto a nylon membrane (Hybond-N+, Amersham Life Science). The membrane was hybridized with [α-32P]dCTP (3,000 Ci/mmol; Amersham)–labeled human ICAM-1 cDNA (1.8-kb XbaI fragment; British Biotechnology, Oxon, UK) with a random-primer labeling kit (Random Primer, Takara). Hybridization with a radiolabeled probe was performed overnight at 42°C. After high-stringency washing was performed (1× saline-sodium citrate and 0.1% sodium dodecyl sulfate, 60°C), autoradiographic detection of the hybridized probe was performed by exposing Kodak scientific imaging film for 48–72 h at −70°C. Quantitation of autoradiographic bands was accomplished with an image analyzer (Biolmage analyzer BAS-2000; Fuji photo film) and was expressed as the intensity of the ICAM-1 band to that of the β-actin band. We used an average value of replicate cultures from the same trachea (n = 3 donors) for analysis of the intensity of the ICAM-1 band to that of the β-actin band.

To study the effects of RV14 infection on mRNA expression of ICAM-1 in human tracheal submucosal gland cells, the cells were examined 1, 3, and 5 days after RV14 infection.

To determine the mechanisms responsible for the upregulation of ICAM-1 mRNA expression after RV14 infection, we tested the effects of either IL-1α (200 pg/ml; Genzyme), IL-1β (200 pg/ml; Ohtsuka), IL-6 (300 pg/ml; Genzyme), IL-8 (10 ng/ml; Collaborative Research), TNF-α (10 pg/ml; Genzyme), or GM-CSF (200 pg/ml; Genzyme) on ICAM-1 mRNA expression in human tracheal submucosal gland cells. The experimentally measured concentrations in the supernatants were used for each cytokine.

Effects of neutralizing antibodies to IL-1 and TNF-α on RV14 infection and ICAM-1 mRNA expression. To determine the role of endogenous cytokines in viral infection and ICAM-1 expression, confluent human tracheal submucosal gland cells were preincubated with a monoclonal mouse anti-human IL-1α (10 µg/ml; Genzyme), an anti-human IL-1β (10 µg/ml; Genzyme), an anti-human TNF-α (10 µg/ml; Genzyme), or an isotype-matched mouse IgG1 control (10 µg/ml; Chemicon International) antibody for 5 days. Viral titers in the supernatants collected for 3–5 days and the expression of ICAM-1 mRNA 5 days after RV14 infection (105 TCID50 units/ml) were measured in confluent human tracheal submucosal gland cells preincubated with each antibody.

Effects of IL-1 on susceptibility to RV14 infection. To examine whether IL-1α and IL-1β increase the susceptibility to RV14 infection, confluent human tracheal submucosal gland cells were preincubated with and without IL-1α (200 pg/ml) or IL-1β (200 pg/ml) for 24 h. The submucosal gland cells were then exposed to serial 10-fold dilutions of RV14 for 60 min at 33°C. The presence of RV14 in the supernatants collected for 1–3 days after infection was determined with the human embryonic fibroblast cell assay described in Detection and titration of viruses to assess whether infection occurred at each dose of RV14 used. This index of susceptibility to infection, defined as the minimum dose of RV14 that could induce infection, was compared with the susceptibility of control cells that were not preincubated with IL-1α or IL-1β (31).

Statistical analysis. Results are expressed as means ± SE; n is the number of donors from which cultured submucosal gland cells were used. Statistical analysis was performed with a two-way repeated-measures ANOVA. Bonferroni’s test was used to estimate the level of significance of differences between means. For all analyses, values of P < 0.05 were assumed to be significant.

RESULTS

Immunocytochemistry. Figure 1 shows immunocytochemistry of confluent monolayers of human tracheal submucosal gland cells. Nearly all cells (>95%) reacted with the monoclonal antibody A1F8 directed against mucous cells (Fig. 1B) and with the monoclonal anti-
Trypan blue was consistently gland cell viability as assessed by the exclusion of in each case by ANOVA. Human tracheal submucosal titer levels increased significantly with time (Fig. 2). In both cell supernatants and lysates, viral similar time course to that observed in the medium after infection (Fig. 2). Evidence of continuous viral production was obtained by demonstrating that the viral titers in supernatants collected for 1–3, 3–5, and 5–7 days after infection each contained significant levels of RV14 (Fig. 2). Analysis of the levels of cell-associated virus (the virus detectable in sonicates of the human tracheal submucosal gland cells) followed a similar time course to that observed in the medium (Fig. 2). In both cell supernatants and lysates, viral titer levels increased significantly with time (P < 0.05 in each case by ANOVA). Human tracheal submucosal gland cell viability as assessed by the exclusion of trypan blue was consistently >96% in RV14-infected cultures. Likewise, RV14 infection did not alter the amount of lactate dehydrogenase in the supernatants (31 ± 3 IU/l before vs. 34 ± 3 IU/l 5 days after infection; P > 0.20; n = 7). RV14 infection also had no effect on cell numbers. Cell counts 24 h after infection were not significantly different (6.0 ± 0.4 × 10^5 in noninfected cells vs. 6.0 ± 0.3 × 10^5 in infected cells; P > 0.50; n = 7).

Detection of viral RNA by PCR. Further evidence of RV14 infection of human tracheal submucosal gland cells, of viral replication, and of viral release into the supernatants was provided by PCR analysis (Fig. 3). In each of three experiments, RNA extracted from control uninfected cells did not produce any detectable PCR product at 381 bp (0 h). A clear product band was observed when RNA extracted from cells 8 h after the infection period was used (Fig. 3A). Likewise, in each of three experiments, RNA extracted from control uninfected culture supernatants did not produce any detectable PCR product at 381 bp (0 h). A clear product band was observed when supernatants extracted 8 h after the infection period were used (Fig. 3B). The time course of the viral titers in supernatants and lysates detected by the cytopathic effects of viruses on human embryonic fibroblast cells was similar to that of the intensity of the RV RNA product extracted from supernatants and infected cells detected by PCR analysis.

Effect of RV infection on cytokine production. Human tracheal submucosal gland cells were screened for mRNA expression of various cytokines. PCR analysis revealed mRNA expression for IL-1α, IL-1β, IL-6, IL-8, TNF-α, and GM-CSF before and after cells were exposed to RV14 (10^5 TCID₅₀ units/ml). However, mRNAs for IL-4, IL-5, IL-10, and interferon-γ were not detectable in human tracheal submucosal gland cells before and after RV14 infection in all seven experiments (data not shown). Figure 4 shows the time course of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and GM-CSF production in supernatants from human tracheal submucosal gland cells after RV14 infection. Because viral infection did not alter cell numbers (see RV infection of human tracheal submucosal gland cells), all cytokine values are reported in picograms per milliliter of supernatant. Basal secretion was quite high with IL-8 but low or negligible with IL-1α, IL-1β, IL-6, TNF-α, and GM-CSF. However, secretion of IL-1α, IL-1β, IL-6, IL-8, TNF-α, and GM-CSF all increased in response to RV14.

Effects of anti-ICAM-1 on RV infection and cytokine production. Incubation of cells with both mouse monoclonal antibodies to ICAM-1 completely blocked RV14

Fig. 1

Fig. 2. Viral titers in supernatants of human tracheal submucosal gland cells were obtained at sequential times during 1st 24 h and for 1–3, 3–5, and 5–7 days after exposure to 10^5 50% tissue culture infectious dose (TCID₅₀) units/ml of RV14 (○). Viral titers in lysates of human tracheal submucosal gland cells were obtained at sequential times during 1st 24 h and 3, 5, and 7 days after infection (●). Results are means ± SE from 7 samples.
infection as assessed by the absence of detectable viral titers in the supernatants recovered 24 h after 60 min of viral exposure (4.1\( \pm \) 0.3 log TCID\(_{50}\) units in control, 0\( \pm \) 0 log TCID\(_{50}\) units in 84H10-treated, and 0\( \pm \) 0 log TCID\(_{50}\) units in RR1-treated cells). These treatments also completely inhibited increases in IL-1\( \alpha \), IL-1\( \beta \), IL-6, IL-8, TNF-\( \alpha \), and GM-CSF production induced by RV14 infection (Fig. 5).

Neither viral titers in the supernatants (4.2\( \pm \) 0.2 log TCID\(_{50}\) units; \( P < 0.50; n = 7 \)) nor virally induced cytokine production (Fig. 5) were altered by an isotype-matched IgG1 control monoclonal antibody. Likewise, exposure to UV-inactivated virus resulted in the production of only 16.1\( \pm \) 4.1 pg/ml of IL-1\( \alpha \), 17.8\( \pm \) 3.9 pg/ml of IL-1\( \beta \), 15.9\( \pm \) 4.4 pg/ml of IL-6, 4,660\( \pm \) 961 pg/ml of IL-8, 3.6\( \pm \) 1.0 pg/ml of TNF-\( \alpha \), and 18.8\( \pm \) 5.1 pg/ml of GM-CSF. Thus UV inactivation failed to increase cytokine production compared with a sham infection (\( P > 0.20; n = 7 \)).

Effects of neutralizing antibodies to cytokines on RV infection and ICAM-1 mRNA expression. The combination of monoclonal mouse anti-human IL-1\( \alpha \) (10 µg/ml) and anti-human IL-1\( \beta \) (10 µg/ml) antibodies significantly decreased RV14 titers in supernatants collected for 3–5 days (Fig. 7A) and significantly inhibited ICAM-1 mRNA expression in human tracheal submucosal gland cells (Fig. 7B). In contrast, neither the monoclonal mouse anti-human TNF-\( \alpha \) (10 µg/ml) nor the mouse IgG1 control monoclonal antibody (10 µg/ml) altered the viral titers in the supernatant (Fig. 7A) and ICAM-1 mRNA expression (Fig. 7B).

Effects of cytokine pretreatment on susceptibility to RV14 infection. Pretreatment of the human tracheal submucosal gland cells for 24 h with IL-1\( \alpha \) (200 pg/ml) and IL-1\( \beta \) (200 pg/ml) increased the susceptibility of cells to RV14 infection, decreasing by 10-fold the minimum dose of virus necessary to cause infection (1.1\( \pm \) 0.1 log TCID\(_{50}\) units for IL-1\( \alpha \)-treated and 1.1\( \pm \) 0.1 log TCID\(_{50}\) units for IL-1\( \beta \)-treated cells vs. 2.1\( \pm \) 0.2 log TCID\(_{50}\) units for control cells; \( P < 0.01; n = 7 \)).

DISCUSSION
To understand the cellular and molecular events involved in the host response to RVs, culture systems of human respiratory epithelial cells have been used (15,
These studies demonstrated that RV triggers the release of inflammatory mediators that play an important role in the pathogenesis of RV infections. To gain further insights into the early biochemical events that occur in infected epithelial cells, we developed for the first time a culture system in which human respiratory submucosal gland cells can be infected with RV. We judged cultured human tracheal submucosal gland cells as cells able to form tight junctions without contamination by fibroblasts and used them for the experiments when the cultured cells in the tubes had a cuboidal or round-shaped appearance and the cells on Millicell CM inserts had high electrical resistance and high short-circuit current (36). The RV infection caused cytopathic effects on spindle-shaped fibroblasts but not on submucosal gland cells with a cuboidal or round-shaped appearance, which is consistent with previous observations on airway epithelial cells and fibroblasts (25, 31, 32, 35). Furthermore, >95% of passaged cells reacted with antibodies against gland cells (30, 36), although there was cell-to-cell variability in the staining. Therefore, cultured human tracheal submucosal glands may make confluent sheets of a heterogeneous collection of gland cells. However, significant contamination by fibroblasts is unlikely in the present study.

The primary target of RV in human infection is the nasal mucosa (3, 6, 14, 34). However, small-particle aerosols of RV have been shown to produce tracheobronchitis (9, 11). Cultures of sputum from children with wheezy bronchitis were more often positive for RV than nasal swabs taken at the same time (16). These findings suggest that RV infects the lower respiratory tract in humans.

Airway mucosal epithelial cells secrete RV into the mucosal and serosal sides (26, 32), and RV infection affects the barrier function of airway epithelium (26). Therefore, RVs released into the submucosa or RVs that penetrate across the airway mucosa might be infected to submucosal glands. The infected airway submucosal glands may release RV and inflammatory cytokines into the submucosal space and upregulate ICAM-1 production, thereby resulting in the activation of lymphocytes and monocytes to secrete interferon-γ (12) and in the accumulation of lymphocytes and eosinophils in the airway (11). Histamine release from human peripheral blood leukocytes is activated by infection of respiratory viruses (7). Therefore, RV replication, cytokine release, and ICAM-1 induction in submucosal glands may play a role in the initial stages in inflammation.

Viral infection of cultured human tracheal submucosal gland cells and subsequent viral replication were confirmed by showing the increased viral content in the culture medium of infected cells with time as assessed...
The specificity of the infection process for human tracheal submucosal gland cells by RV14 was confirmed by demonstrating that infection could be blocked with antibodies directed against the functional binding site of ICAM-1, the major RV receptor, but not by an isotype-matched IgG1 monoclonal antibody. In addition, we showed that antibodies to ICAM-1 completely inhibited virally induced cytokine production, whereas both an isotype-matched IgG1 and a UV-inactivated virus failed to inhibit them, suggesting that cytokine production requires active RV infection.

In recent years, it has become apparent that respiratory epithelial cells play a much more active role in regulating airway inflammation and that the stimulation of biochemical pathways in infected epithelial cells could induce a series of events that contribute to the pathogenesis of viral infections. Respiratory syncytial viruses cause an increase in IL-8 mRNA expression in the nasal epithelium (4) and production of IL-6, IL-8, and GM-CSF from a human bronchial epithelial cell line (BEAS-2B) (24). Likewise, RV infection induces production of IL-6, IL-8, and GM-CSF from BEAS-2B cells (31) and of IL-1β, IL-6, IL-8, and TNF-α from primary cultures of human tracheal epithelial cells.

by the cytopathic effects of this medium on human embryonic fibroblast cells and by showing that the cytopathic effects of human tracheal submucosal gland cell lysates also increased with time after infection. RV can be distinguished from enterovirus, which also produces “entero-like” cytopathic effects on human embryonic fibroblasts, by inactivation of RV after treatment with acid (25). Viral replication in the human tracheal submucosal gland cells and release of RV into the culture supernatants were also detected by PCR of viral RNA after RT into DNA. Experimental conditions clearly demonstrated that viral RNA increased 8 h after infection as indicated by a pronounced band on PCR compared with the absence of any signal immediately after infection. The lack of any PCR signal immediately after infection, together with the inability to detect virus with the human embryonic fibroblast assay in either the supernatants or lysates of infected cells until 8 h postinfection, implies that very little virus is initially taken up during the infection process. However, RV14 infection failed to influence both cell growth rate and cell viability. This is in agreement with previous studies (8, 15, 31, 32, 35) showing the lack of cytotoxicity on epithelial cells in RV infection. The lack of cytotoxicity in this model facilitates studies of virally induced biochemical changes in respiratory submucosal gland cells because the possibility that any such changes are occurring as a result of cell death can be ruled out.

Fig. 6. Northern blot analysis demonstrating increases in ICAM-1 mRNA levels in human tracheal submucosal gland cells 1, 3, and 5 days after RV14 infection compared with a sham infection (control; top). β-Actin was used as a housekeeping gene. Bottom: expression of ICAM-1 mRNA in human tracheal submucosal gland cells 5 days after RV14 infection (solid bar) or a sham infection (open bar). ICAM-1 mRNA was normalized to a constitutive expression of β-actin mRNA (ICAM-1/β-actin). Results are means ± SE from 7 samples. *** Significant difference from a sham infection; P < 0.001.

Fig. 7. RV14 titers in supernatants collected for 3–5 days (A) and expression of ICAM-1 mRNA (B) in human tracheal submucosal gland cells 5 days after RV14 infection in presence of neutralizing antibodies to IL-1α (a-IL-1α; 10 µg/ml), IL-1β (a-IL-1β; 10 µg/ml), or TNF-α (a-TNF-α; 10 µg/ml) or mouse IgG1 monoclonal antibody (IgG1; 10 µg/ml) and in absence of an antibody (control). Results are means ± SE from 7 samples. Significant difference from RV14 infection alone: ** P < 0.01; *** P < 0.001.
(32). In addition to the enhanced production of IL-1α, IL-6, IL-8, and TNF-α as previously observed in human tracheal epithelial cells with RV14 infection (32), infection of human tracheal submucosal gland cells with RV14 also caused increases in IL-1α and GM-CSF production.

The cytokines produced from submucosal gland cells in response to viral stimulation have biological properties that are of interest with respect to the pathogenesis of colds and asthma. IL-8 is a potent chemoattractant for and activator of neutrophils (2) and also has chemotactic activity for lymphocytes (20), the two predominant cell types in the nasal mucosa during RV infection (21). IL-6 cannot only induce B-cell differentiation and antibody production but is also capable of stimulating T-cell activation (1). Likewise, GM-CSF can prime both neutrophils and eosinophils for enhanced activation to chemical stimuli (22). Increased epithelial expression of IL-8, IL-6, and GM-CSF has been reported in asthmatic airways, and, therefore, virally induced production of these cytokines may be relevant to viral exacerbations of asthma.

Infection of submucosal gland cells with RV14 was found to upregulate the expression of ICAM-1 on these cells. It has been shown that exposure of epithelial cells to cytokines such as IL-1α, IL-1β, and TNF-α can upregulate the expression of ICAM-1 on these cells (27, 31, 32). Furthermore, in HeLa cells, upregulation of ICAM-1 by these cytokines is associated with increased binding of major group RVs (33). In this study, virally induced enhancement of ICAM-1 expression was mimicked by IL-1α and IL-1β but not by IL-6, IL-8, TNF-α, and GM-CSF at the experimentally measured concentrations in the supernatants. Furthermore, a combination of antibodies to IL-1α and IL-1β almost entirely blocked increases in ICAM-1 expression induced by RV14 infection and significantly inhibited RV14 infection. Finally, both IL-1α and IL-1β increased susceptibility to infection with RV14 infection, consistent with their ability to increase the expression of ICAM-1. These results confirmed the role of endogenous IL-1 in viral infection and ICAM-1 expression in submucosal gland cells. In contrast to IL-1, TNF-α failed to alter both susceptibility to RV14 infection and ICAM-1 expression and anti-TNF-α was without an effect on virally induced enhancement of ICAM-1 expression. This is in agreement with a previous study by Terajima et al. (32) in primary cultures of human tracheal epithelial cells. Inflammatory conditions such as asthma, smoking, and ozone exposure in which ICAM-1 expression is increased on submucosal gland cells may cause a predisposition to RV infection through the increased expression of the major group RV receptors. The RV infection would enhance airway inflammation by recruiting neutrophils and, potentially, other inflammatory cells, causing increased mediator release and exacerbation of the underlying reactive airway diseases.

In summary, we demonstrate that a pure population of human respiratory submucosal gland cells can be infected with RV. Infection of submucosal gland cells with RV induces biochemical changes in the infected cells as evidenced by the increased production of cytokines that could play a role in the recruitment and activation of inflammatory cells into the airway of infected individuals. Of these cytokines, IL-1 is an autocrine mechanism of enhanced ICAM-1 expression in RV-infected submucosal gland cells. This model system provides a valuable tool for RV infection of submucosal gland cells and should yield useful insights into the initial stages of inflammation and host immune responses in the microenvironment of the respiratory mucosa associated with the pathogenesis of RV infections.

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