Rhinovirus infection of primary cultures of human tracheal epithelium: role of ICAM-1 and IL-1β

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PERSPECTIVE STUDIES have indicated that asthma at-tractive epithelium: role of ICAM-1 and IL-1β

Rhinovirus infection of primary cultures of human tracheal epithelium: role of ICAM-1 and IL-1β. Am. J. Physiol. 273 (Lung Cell. Mol. Physiol. 17): L749–L759, 1997.—Exacerbations of asthma are often associated with respiratory infection caused by rhinoviruses. To study the effects of rhinovirus infection on respiratory epithelium, a primary target for respiratory viruses, human rhinovirus (HRV)-2 and HRV-14 were infected to primary cultures of human tracheal epithelial cells. Viral infection was confirmed by showing that viral titers of supernatants and lysates from infected cells increased with time and by polymerase chain reaction. HRV-2 and HRV-14 infections upregulated the expression of ICAM-1 mRNA, the major rhinovirus receptor, on epithelial cells, and they increased the production of interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF-α) in supernatants. Antibodies to ICAM-1 inhibited HRV-14 infection of epithelial cells and decreased the production of cytokines after HRV-14 infection, but they did not alter HRV-2 infection-induced production of cytokines. IL-1β upregulated ICAM-1 mRNA expression and increased susceptibility to HRV-14 infection, whereas other cytokines failed to alter ICAM-1 mRNA expression. Furthermore, a neutralizing antibody to IL-1β significantly decreased viral titers of supernatants and ICAM-1 mRNA expression after HRV-14 infection, but a neutralizing antibody to TNF-α was without effect. Immunohistochemical studies revealed that both HRV-14 infection and IL-1β increased ICAM-1 expression on cultured epithelial cells. These findings imply that HRV-14 infection upregulated ICAM-1 expression on epithelial cells through increased production of IL-1β, thereby increasing susceptibility to infection. These events may be important for amplification of airway inflammation after viral infection in asthma.

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

Eagle's minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, fetal calf serum (FCS), and γ-globulin-free calf serum (GGFCS) were from Gibco-BRL. Life Technologies, Palo Alto, CA; trypsin, EDTA, dithiothreitol, Sigma type XIV protease, human placental collagen, penicillin, streptomycin, gentamicin, and amphotericin B were from Sigma Chemical, St. Louis, MO; and Ultroser G serum substitute (USG) was from BioSepra, Marlborough, MA.

Human embryonic fibroblast cell culture. Human embryonic fibroblast cells were cultured in the Roux type bottle (Iwaki Garasu, Chiba, J apan) sealed with a rubber plug in MEM containing 10% FCS supplemented with 5 × 10^5 U/l penicillin and 50 mg/l streptomycin (20). Confluency was achieved at 7 days, at which time the cells were collected by trypsinization (0.05% trypsin, 0.02% EDTA). Cells (1.5 × 10^5 cells/ml) suspended in MEM containing 10% FCS were then plated in glass tubes (15 × 105 mm; Iwaki Garasu), sealed with rubber plugs, and cultured at 37°C.

METHODS

Media components. Reagents for cell culture media were obtained as follows: Eagle's minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, fetal calf serum (FCS), and γ-globulin-free calf serum (GGFCS) were from Gibco-BRL. Life Technologies, Palo Alto, CA; trypsin, EDTA, dithiothreitol, Sigma type XIV protease, human placental collagen, penicillin, streptomycin, gentamicin, and amphotericin B were from Sigma Chemical, St. Louis, MO; and Ultroser G serum substitute (USG) was from BioSepra, Marlborough, MA.
Human tracheal epithelial cell culture. Tracheas for cell culture were obtained 3–6 h after death from 51 patients (mean age, 64 ± 4 yr; 22 female, 29 male) under a protocol passed by the Tohoku University Ethics Committee. Twenty-four of the patients were smokers. None had a respiratory illness, and they died of acute myocardial infarction (n = 13), congestive heart failure (n = 3), malignant tumor other than lung cancer (n = 14), rupture of aortic aneurysm (n = 4), liver cirrhosis (n = 3), renal failure (n = 3), leukemia (n = 3), malignant lymphoma (n = 1), cerebral bleeding (n = 6), and cerebral infarction (n = 1). Tracheas were rinsed in ice-cold and sterile phosphate-buffered saline (PBS) to remove mucus and debris, opened longitudinally along the anterior surface, and mounted in a stretched position in a dissection tray. The surface epithelium was scored into longitudinal strips and was pulled off of the submucosa (34). The tissue strips were rinsed four times in PBS containing 5 mM dithiotreitol and then two times in PBS alone. The tissue strips were placed into the conical tubes (Costar, Cambridge, MA) containing protease (0.4 mg/ml; Sigma type XIV) dissolved in PBS. The strips were stored overnight in the refrigerator at 4°C. The enzyme was then competitively inhibited by the addition of FCS to a final concentration of 2.5%, and small sheets of cells were dislodged from the epithelial strips by vigorous agitation. The denuded strips were removed, and the sheets of cells remaining were dispersed by repeated aspiration in a 10-ml pipette.

Cells were pelleted (200 g, 10 min) and suspended in DMEM-Ham’s F-12 containing 5% FCS (50:50 vol/vol). Cell counts were made using a hemocytometer, and estimates of viability were done using trypan blue and by measuring the amount of lactate dehydrogenase (LDH) in the medium as previously reported (2). The cells were then plated at 5 × 10^6 viable cells/ml in glass tubes coated with human placental collagen (34). This medium was then replaced by DMEM-Ham’s F-12 containing 2% USG on the first day after plating. The enzyme was then competitively inhibited by the addition of FCS to a final concentration of 2.5%, and small sheets of cells were dislodged from the epithelial strips by vigorous agitation. The denuded strips were removed, and the sheets of cells remaining were dispersed by repeated aspiration in a 10-ml pipette.

We screened 16 kinds of viruses (e.g., influenza types A, B, and C; parainfluenza virus, adenovirus, rhinovirus, and respiratory syncytial virus) in supernatants of cultured human tracheal epithelial cells before rhinovirus infection using the method described previously (20) and used the epithelial cell sheets without contamination by any viruses. Furthermore, we confirmed cilia beating on the epithelial cells and the absence of fibroblasts in glass tubes using the inverted microscope (MIT-2; Olympus, Tokyo, Japan). Finally, to determine whether cultured cells can form tight junctions, we performed parallel cultures of human tracheal epithelial 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 using Ussing chamber methods (34). When cells cultured under these conditions became differentiated and form tight junctions without contamination of fibroblasts, they have values of > 40 Ω·cm² for resistance and > 10 µA/cm² for short-circuit current (34). Therefore, cultured human tracheal epithelial cells were judged as cells able to form tight junctions and were used for the following experiments when cells on Millicell CM inserts had high resistance (> 40 Ω·cm²) and high short-circuit current (> 10 µA/cm²).

Viral stocks. HRV-2 and HRV-14 were prepared in our laboratory from the patients with common colds (20). Stocks of HRV-14 and HRV-2 were generated by infecting human embryonic fibroblast cells cultured in glass tubes in 1 ml of MEM supplemented with 2% GGFCS, 50 U/ml penicillin, and 50 µg/ml streptomycin at 33°C. The cells were incubated for several days in glass tubes in 1 ml of MEM supplemented with 2% GGFCS until 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 viral stock solutions was determined using the human embryonic fibroblast cell assay described below.

Detection and titration of viruses. Rhinoviruses 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. 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 previously (20). The amount of specimen required to infect 50% of human embryonic fibroblast cells (TCID₅₀) was determined.

Viral infection of human tracheal epithelial cells. Medium was removed from confluent monolayers of human tracheal epithelial cells and was replaced with 1 ml of DMEM-Ham’s F-12 containing 2% USG. Rhinovirus was added at a concentration of 10² TCID₅₀/ml. After a 1-h incubation at 33°C, the viral solution was removed, and the cells were rinsed once with 1 ml of PBS. The cells were then fed with DMEM-Ham’s F-12 containing 2% USG supplemented with 10 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin, and 2.5 mg/l amphotericin B.

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Effects of antibodies to ICAM-1 on rhinovirus infection. Confluent human tracheal epithelial cells were incubated for 30 min at 37°C with medium alone, with medium containing either of the two mouse monoclonal anti-human antibodies to ICAM-1 (84H10, 100 µg/ml; Immunotech, Marseille, France) or RR1 (100 µg/ml; a gift from Boehringer Ingelheim, Ridgefield, CT; see Ref. 14), or with medium containing an isotype-matched mouse immunoglobulin G1 (IgG1) control monoclonal 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 HRV-2 (10⁵ TCID₅₀/ml) or HRV-14 (10⁵ TCID₅₀/ml) for either 15 or 60 min before rinsing and adding fresh DMEM-Ham’s F-12 containing 2% USG supplemented with 10 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml gentamicin, and 2.5 mg/l amphotericin B. The viral content of this medium was then assessed at various times after infection.

Detection of rhinovirus RNA and cytokine mRNA by reverse transcription-polymerase chain reaction. Human tracheal epithelial cells cultured in the glass tubes were lysed by the addition of RNazol (0.2 ml/10⁵ cells; B IOTEXC, Houston, TX) and were transferred into 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 and mixed with an equal volume of isopropanol. Pellets of RNA were obtained by centrifugation at 12,000 g for 15 min at 4°C, dissolved in a water, and stored at −80°C before use.

Polymerase chain reaction (PCR) was performed as previously described (4, 10). Briefly, 2 µg of RNA from each aliquot of human tracheal epithelial cells were dissolved in a 100-µl buffer containing the reagents for the reverse transcriptase (RT) reaction with the following composition: 50 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 8.3), 75 mM KCl, 3 mM MgCl₂, 10 mM dithiothreitol, 5 U/µl Moloney murine leukemia virus RT (GIBCO-BRL Life Technologies), 0.5 mM deoxynucleoside 5’-triphosphate (dNTP; Takara, Ohtsu, Japan), 1.5 mM MgCl₂, 10 mM dithiothreitol, 5 U/µl Moloney murine leukemia virus RT (GIBCO-BRL Life Technologies), 0.5 mM deoxynucleoside 5’-triphosphate (dNTP; Takara), 5 µM random hexamers (Pharmacia Biotech, Uppsala, Sweden). The RT reaction was performed for 60 min at 37°C, followed by 95°C for 10 min. The resulting cDNA was frozen at −80°C until use 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 dNTP, and 1.25 units Taq polymerase (Takara). Primer pairs for the rhinovirus and cytokines were present at 2 ng/µl. Sequences of the PCR primer pairs used in these experiments are shown in Table 1. The PCR was performed in an automated thermal cycler (MJ Research, Watertown, MA), and 10 µl of the reaction were removed at 30 cycles for each sample.

Table 1. Polymerase chain reaction primer sequences

<table>
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<tr>
<th>Target Sequence</th>
<th>Ref. No.</th>
<th>Base Pair</th>
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HRV, human rhinovirus; IL, interleukin; GM-CSF, granulocyte macrophage colony-stimulating factor; TNF-α, tumor necrosis factor-α; IFN-γ, interferon-γ.
(100 pg/ml; Genzyme), IL-8 (100 pg/ml; Collaborative Research, Bedford, MA), or TNF-α (10 pg/ml; Genzyme) on ICAM-1 mRNA expression in human tracheal epithelial cells. The concentration of each cytokine chosen was matched to a net increase in the culture medium after HRV-14 infection, and cells were incubated overnight with each cytokine.

Effects of neutralizing antibodies to IL-1β and TNF-α on HRV-14 infection and ICAM-1 mRNA expression. To determine the role of endogenous IL-1β in viral infection and ICAM-1 expression, confluent human tracheal epithelial cells were preincubated using a monoclonal mouse anti-human IL-1β (10 µg/ml; Genzyme) or an isotype-matched mouse IgG1 control monoclonal antibody (Chemicon International), at the same concentration for 5 days. We also tested the effects of a monoclonal mouse anti-human TNF-α (10 µg/ml; 5 days; Genzyme) on viral infection and ICAM-1 expression. Viral titers in the supernatants collected during 3–5 days and the expression of ICAM-1 mRNA 5 days after HRV-14 infection (10^2 TCID50/ml) were measured in confluent human tracheal epithelial cells preincubated with each antibody.

Effects of IL-1β on susceptibility to HRV-14 infection. To examine whether IL-1β increases the susceptibility to HRV-14 infection, confluent human tracheal epithelial cells were preincubated with or without IL-1β (200 pg/ml) for 24 h. The epithelial cells were then exposed to serial 10-fold dilutions of HRV-14 for 1 h at 33°C. The presence of HRV-14 in supernatants collected during 1–3 days after infection was determined using the human embryonic fibroblast cell assay described above to assess whether infection occurred at each dose of HRV-14 used. This index of susceptibility to infection, defined as the minimum dose of HRV-14 that could induce infection, was compared with the susceptibility of control cells that were not preincubated with IL-1β (27).

Immunohistochemical analysis. Immunohistochemical analysis for ICAM-1 expression in human tracheal epithelial cells was done as described previously (21). Human tracheal epithelial cells were cultured on Vitrogen gels on Millicell cells was done as described previously (21). Human tracheal epithelial cells were cultured on Vitrogen gels on Millicell inserts (0.45 µm pore size and 0.6 cm² area; Millipore) and cells were incubated overnight with each cytokine.

Immunohistochemical analysis. Immunohistochemical analysis for ICAM-1 expression in human tracheal epithelial cells was done as described previously (21). Human tracheal epithelial cells were incubated with each cytokine. In addition, we tested the effects of either IL-1β (200 pg/ml; Ohtsuka), IL-6 (100 pg/ml; Genzyme), IL-8 (100 pg/ml; Collaborative Research), or TNF-α (10 pg/ml; Genzyme) on ICAM-1 expression in human tracheal epithelial cells. Cells were incubated overnight with each cytokine. In addition, we tested a 10 ng/ml concentration of IL-1β as a positive control (29).

Statistical analysis. Results are expressed as means ± SE. Statistical analysis was performed using a one-way analysis of variance (ANOVA), and multiple comparisons were made using Bonferroni’s method. Significance was accepted at P < 0.05; n refers to the number of donors from which cultured epithelial cells were used.

Fig. 1. Viral titers in supernatants of human tracheal epithelial cells obtained at different times after exposure to 10^6 TCID50/ml of human rhinovirus (HRV)-14 (○) and HRV-2 (●). TCID50 is the amount of specimen required to infect 50% of human embryonic fibroblasts. A: viral titers in supernatants collected at sequential times during the first 24 h after infection. B: viral titers of HRV-14 (open bars) and HRV-2 (filled bars) in supernatants collected during 1–3 days, 3–5 days, and 5–7 days after infection. Results are reported as means ± SE from 7 samples.
RESULTS

Rhinovirus infection of human tracheal epithelial cells. Exposing confluent human tracheal epithelial cell monolayers to HRV-2 or HRV-14 (10^5 TCID₅₀/ml) consistently led to infection. Collection of culture medium at differing times after viral exposure revealed no detectable virus at 1 h after infection. Both HRV-2 and HRV-14 were detected in culture medium 6 h after infection, and the viral content progressively increased between 6 and 24 h after infection (Fig. 1A). Evidence of
continuous viral production was obtained by the demonstration that the viral titers of supernatants collected during 1–3 days, 3–5 days, and 5–7 days after infection each contained significant levels of HRV-2 or HRV-14 (Fig. 1B). Analysis of the levels of cell-associated virus (the virus detectable in sonicates of the human tracheal epithelial cells) followed a similar time course to that observed in the medium. The viral titers of cell-associated HRV-14 were 0.0 ± 0.0 log TCID_{50} units at 1 h, 0.1 ± 0.1 log TCID_{50} units at 6 h, 0.8 ± 0.1 log TCID_{50} units at 12 h, 2.2 ± 0.3 log TCID_{50} units at 24 h, 2.5 ± 0.3 log TCID_{50} units at 3 days, 2.2 ± 0.3 log TCID_{50} units at 5 days, and 1.6 ± 0.3 log TCID_{50} units at 7 days (n = 7 each). In both cell supernatants and lysates, viral titer levels increased significantly with time (P < 0.05 in each case by ANOVA). Viral titers at 24 h after HRV-14 infection in cells from smokers did not differ significantly from those in nonsmokers (3.2 ± 0.3 log TCID_{50} units in smokers vs. 3.3 ± 0.3 log TCID_{50} units in nonsmokers; P > 0.50). Human tracheal cell viability, as assessed by the exclusion of trypan blue, was consistently >96% in HRV-14-infected culture. Likewise, HRV-14 infection did not alter the amount of LDH in the supernatants (29 ± 3 IU/l before vs. 33 ± 3 IU/l 5 days after infection; P > 0.20, n = 7). HRV-14 infection also had no effect on cell numbers. Cell counts 24 h after infection were not significantly different (1.7 ± 0.1 × 10^6 in noninfected cells vs. 1.7 ± 0.2 × 10^6 in infected cells; P > 0.50, n = 7).

Detection of viral RNA by PCR. Further evidence of HRV-14 infection of human tracheal epithelial cells and of viral replication was provided by PCR analysis (Fig. 2). In each of three experiments, RNA extracted from uninfected cells did not produce any detectable PCR product at 381 bp (0 h). A faint product was observable in RNA extracted from cells 6 h after infection followed by a progressive increase in viral RNA until 3 days after infection.

Effects of rhinovirus infection on cytokine production. Human tracheal epithelial cells were screened for mRNA expression of various cytokines. PCR analysis revealed mRNA expression for IL-1β, IL-6, IL-8, TNF-α, and GM-CSF before and after cells were exposed to HRV-14 (10^5 TCID_{50}/ml). However, mRNA for IL-4, IL-5, IL-10, and interferon-γ were not detectable in human tracheal epithelial cells before and after HRV-14 infection in all seven experiments (data not shown). Figure 3 shows the time course of IL-1β, IL-6, IL-8, and TNF-α production in supernatants of human tracheal epithelial cells after HRV-14 (Fig. 3A) or HRV-2 (Fig. 3B) infection. Because viral infection did not alter cell numbers (see above), all cytokine values are reported in picograms per milliliter of supernatant. Basal secretion was quite high with IL-8, relatively high with IL-6, but low or negligible with IL-1β and TNF-α. However, secretion of IL-1β, IL-6, IL-8, and TNF-α all increased in response to both HRV-2 and HRV-14, although, in terms of absolute levels, IL-1β (195 ± 15 pg/ml in HRV-2 and 145 ± 15 pg/ml in HRV-14) and IL-6 (185 ± 22 pg/ml in HRV-2 and 120 ± 14 pg/ml in HRV-14) predominated. Of the cytokines measured, GM-CSF...
Effects of antibodies to ICAM-1 on rhinovirus infection and cytokine production. Incubation of cells with both mouse monoclonal antibodies to ICAM-1 completely blocked HRV-14 infection, as assessed by the absence of detectable viral titers in the supernatants recovered 24 h after 15 min of HRV-14 exposure (2.2 ± 0.2 log TCID50 units in control, 0 ± 0 log TCID50 units in 84H10, and 0 ± 0 log TCID50 units in RR1). Likewise, viral titers 24 h after 60 min of HRV-14 exposure were significantly decreased by 84H10 (1.9 ± 0.3 log TCID50 units; P < 0.01, n = 7) and RR1 (1.7 ± 0.3 log TCID50 units; P < 0.01, n = 7) from control values (3.3 ± 0.1 log TCID50 units; n = 7). These treatments also significantly inhibited increases in IL-1β, IL-6, IL-8, and TNF-α production induced by HRV-14 infection (Fig. 4A). However, an isotype-matched IgG1 control monoclonal antibody failed to alter viral titers in the supernatants 24 h after 15 min of viral exposure (2.3 ± 0.2 log TCID50 units; P > 0.50, n = 7) and 60 min of viral exposure (3.3 ± 0.2 log TCID50 units; P > 0.50, n = 7). Likewise, IgG1 control monoclonal antibody did not inhibit increases in IL-1β, IL-6, IL-8, and TNF-α production induced by HRV-14 infection (Fig. 4A). In contrast to HRV-14, viral titers in the supernatants recovered 24 h after 15 min of HRV-2 exposure were not altered by 84H10 (2.4 ± 0.2 log TCID50 units; P > 0.50, n = 7) and RR1 (2.3 ± 0.2 log TCID50 units; P > 0.50, n = 7) from control values (2.3 ± 0.2 log TCID50 units; n = 7) and from values of IgG1 control monoclonal antibody treatment (2.2 ± 0.3 log TCID50 units; n = 7). Neither 84H10, RR1, nor IgG1 control monoclonal antibody altered increases in production of IL-1β, IL-6, IL-8, and TNF-α induced by HRV-2 infection (Fig. 4B).

Effect of rhinovirus infection on ICAM-1 mRNA expression. The baseline expression of ICAM-1 was constant in confluent human tracheal epithelial cell sheets, and the coefficient of variation was small (9.8%; n = 22). Neither smoking habit nor cause of death influenced the baseline expression of ICAM-1 mRNA. Exposure of human tracheal epithelial cells to HRV-14 (Fig. 5A) or HRV-2 (Fig. 5B) caused increases in ICAM-1 mRNA compared with sham exposure (control). Human tracheal epithelial cells 5 days after HRV-14 (Fig. 5C) or HRV-2 (Fig. 5D) infection were shown to overexpress ICAM-1 mRNA twofold compared with those 5 days after a sham exposure. IL-1β (200 pg/ml) increased ICAM-1 mRNA to the levels similar to those induced by rhinovirus infection expressed as the intensity of the ICAM-1/β-actin bands (0.49 ± 0.03 scan units; P < 0.01, n = 7). However, neither IL-6 (100 pg/ml), IL-8 (100 pg/ml), nor TNF-α (100 pg/ml) altered the levels (0.23 ± 0.03 scan units in IL-6, 0.22 ± 0.03 scan units in IL-8, and 0.21 ± 0.03 scan units in TNF-α; P > 0.20, n = 7) compared with sham exposure (0.21 ± 0.02 scan units; n = 7).

Effects of neutralizing antibodies to IL-1β and TNF-α on viral infection and ICAM-1 mRNA expression. The monoclonal mouse anti-human IL-1β (10 µg/ml) significantly decreased HRV-14 titers of supernatant collected during 3–5 days (Fig. 6A) and inhibited ICAM-1 mRNA expression in human tracheal epithelial cells (Fig. 6B). In contrast, neither the monoclonal mouse

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**Fig. 5.** Northern blot analysis demonstrating increases in ICAM-1 mRNA levels of human tracheal epithelial cells 1, 3, and 5 days after HRV-14 infection (A) and 5 days after HRV-2 infection (B) compared with sham infection (control). β-Actin was used as a housekeeping gene. C and D: expression of ICAM-1 mRNA in human tracheal epithelial cells 5 days after HRV-14 infection (C) and HRV-2 infection (D; filled bars). Open bars, sham infection (control). ICAM-1 mRNA is normalized to constitutive expression of β-actin mRNA. Results are reported as means ± SE from 7 samples. Significant differences from corresponding control values are indicated by **P < 0.01.**

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and IL-1α levels were not changed by HRV-14 infection (140 ± 10 pg/ml before vs. 165 ± 18 pg/ml after in GM-CSF and 12 ± 2 pg/ml before vs. 13 ± 2 pg/ml after in IL-1α; P > 0.20, n = 7).

In contrast to supernatants of human tracheal epithelial cells, neither IL-1β nor TNF-α was detectable in viral stocks.
The minimum dose of virus necessary to cause infection of cells to HRV-14 infection, decreasing by 10-fold the supernatant (Fig. 6) monoclonal antibody (10 µg/ml) altered viral titers of (Fig. 6B anti-human TNF-α (10 µg/ml) or mouse IgG1 control antibodies to IL-1β (IL-1β; 10 µg/ml; filled bars), TNF-α (TNF-α; 10 µg/ml; stippled bars), or mouse IgG1 monoclonal antibody (IgG1; 10 µg/ml; hatched bars) and absence of an antibody (control; open bars). ICAM-1 mRNA is normalized to constitutive expression of β-actin mRNA. Results are reported as means ± SE from 7 samples. Significant differences from HRV-14 infection alone are indicated by **P < 0.01.

Fig. 6. HRV-14 titers in supernatants collected during 3–5 days (A) and expression of ICAM-1 mRNA (B) in human tracheal epithelial cells 5 days after HRV-14 infection in the presence of neutralizing antibodies to IL-1β (a-IL-1β; 10 µg/ml; filled bars), TNF-α (a-TNF-α; 10 µg/ml; stippled bars), or mouse IgG1 monoclonal antibody (IgG1; 10 µg/ml; hatched bars) and absence of an antibody (control; open bars). ICAM-1 mRNA is normalized to constitutive expression of β-actin mRNA. Results are reported as means ± SE from 7 samples. Significant differences from HRV-14 infection alone are indicated by **P < 0.01.

Effects of IL-1β on susceptibility to HRV-14 infection. Pretreatment of the human tracheal epithelial cells for 24 h with IL-1β (200 pg/ml) increased the susceptibility of cells to HRV-14 infection, decreasing by 10-fold the minimum dose of virus necessary to cause infection (1.1 ± 0.1 log TCID50 units in IL-1β vs. 2.2 ± 0.2 log TCID50 units in control; P < 0.01, n = 7).

Immunohistochemical analysis. Figure 7 shows ICAM-1 expression in the human tracheal epithelial cells, which was detected as a red color. HRV-14 infection (Fig. 7B) increased ICAM-1 expression in both apical and basolateral sides compared with sham infection, which caused staining only on the basolateral side (Fig. 7A). Treatment with IL-1β at either 200 pg/ml (Fig. 7C) or 10 ng/ml (Fig. 7D) mimicked HRV-14 infection, with a stronger staining observed in the latter. In contrast, treatment with either IL-6, IL-8, or TNF-α failed to alter ICAM-1 expression in the human tracheal epithelial cells (not shown).

DISCUSSION

Primary cultures of human airway epithelial cells are thought to be important for characterizing viral infection in the airway and for advancing our knowledge of airway inflammation (8). Respiratory viral infection of primary cultures of human nasal epithelial cells were reported previously, but these cultures contained a significant number of fibroblasts (32), making it difficult to interpret the results obtained. We report rhinovirus infection of primary cultures of the human tracheal epithelial cells that do not contain other cell types such as fibroblasts (34). However, it should be noted here that this model system has significant differences from airway epithelial cells in vivo.

Viral infection of cultured human tracheal epithelial cells and subsequent viral replication were confirmed by showing the increased viral content in the culture medium of infected cells with time, assessed by the cytopathic effects of this medium on human embryonic fibroblast cells, and by showing that the cytopathic effects of human tracheal epithelial cell lysates also increased with time after infection. Viral replication was also detected by PCR of viral RNA after reverse transcription into DNA. A progressive increase in viral RNA, observed until 3 days after infection, was detected by a pronounced band on PCR compared with the absence of any signal in RNA extracted from uninfected cells. Infections of human tracheal epithelial cells with HRV-2 and HRV-14 were consistently observed when confluent monolayers were exposed to virus. However, HRV-14 infection failed to influence both cell numbers and cell viability. This is in agreement with previous studies showing the lack of cytotoxicity on epithelial cells in rhinovirus infection (27, 32).

Infection of the respiratory epithelium by viruses has been shown to cause increased production of cytokines (19, 27). Subauste et al. (27) demonstrated that infection of BEAS-2B cells with HRV-14 induced an increased production of IL-6, IL-8, and GM-CSF. The present study is in agreement with a previous study showing the enhanced production of IL-6 and IL-8 within 24 h after infection (27). However, infection of human tracheal epithelial cells with HRV-2 and HRV-14 also caused increases in the production of IL-1β and TNF-α, which differed significantly from HRV-14 infection of BEAS-2B cells (27). Although an increase in TNF-α production after infection was subtle and that in IL-8 was <30% from the baseline, there was a large amount of IL-1β production from human tracheal epithelial cells in the present study, which was maximal at 3 days and was sustained up to 5 days after infection. IL-1β is a potent inflammatory cytokine that induces growth and differentiation of T and B lymphocytes, other cytokine productions, prostaglandin E2 synthesis, and degranulation from neutrophils (1). IL-1β also
causes increases in ICAM-1 expression on both epithelial and vascular endothelial cells (1, 5, 29). Increases in ICAM-1 expression on vascular endothelial cells promote the adhesion of neutrophils, monocytes, and lymphocytes to these cells (5). Likewise, upregulation of ICAM-1 on HeLa cells is associated with the increased binding of major group rhinoviruses (28).

The specificity of the infection process for primary cultures of human tracheal epithelial cells by HRV-14 was confirmed by demonstrating that infection could be blocked using antibodies to ICAM-1 but not by an isotype-matched IgG1 monoclonal antibody. Furthermore, antibodies to ICAM-1 failed to block HRV-2 infection, a minor group of rhinoviruses that do not use ICAM-1 as its receptor. However, inhibition became less consistent at longer incubation times (e.g., 1 h), presumably because of the high affinity of the virus for its receptor and of the requirement for very few viral particles to enter the cell to induce infection (27). In addition, we showed that antibodies to ICAM-1 significantly inhibited the production of IL-1β, IL-6, IL-8, and TNF-α induced by HRV-14 but that HRV-2-induced effects on cytokine production were not altered by antibodies to ICAM-1. However, antibodies to ICAM-1 could not achieve complete inhibition of cytokine production induced by HRV-14 infection, which may be also due to longer incubation times described above. Furthermore, we showed that rhinovirus infection and IL-1β at the experimentally measured concentration in supernatants upregulated ICAM-1 expression assessed by increases in ICAM-1 mRNA using Northern blot analysis, whereas an increase in ICAM-1 mRNA was two times the control and relatively small compared with the study on respiratory syncytial virus infection of human pulmonary type II-like epithelial (A549) cells (22).

Similar to ICAM-1 mRNA levels, however, both HRV-14 infection and IL-1β increased ICAM-1 expression on epithelial cells as shown by immunohistochemical analysis, and IL-1β increased susceptibility to HRV-14 infection. Furthermore, enhancement of ICAM-1 mRNA expression by HRV-14 infection was almost entirely blocked by the antibody to IL-1β, and the anti-IL-1β antibody significantly inhibited HRV-14 infection, confirming the role of the endogenous IL-1β in viral infection and ICAM-1 expression in the present study. Although TNF-α is reported to upregulate the expression of ICAM-1 on epithelial cells (27), the present study failed to show the expression. Furthermore, the antibody to TNF-α did not alter HRV-14 infection or ICAM-1 mRNA expression. Lack of TNF-α-induced effects on ICAM-1 mRNA expression is probably due to a small amount of TNF-α production from human tracheal epithelial cells in response to HRV-14 infection. Although IL-1α is reported to be induced by A549 cells in response to respiratory syncytial virus infection (22), HRV-14 did not alter IL-1α production in the present study. The discrepancy may be explained by differences in the species of virus and cultured cells.

Upregulation of ICAM-1 expression on epithelial cells and the production of cytokines from these cells in response to rhinovirus infection may be relevant to the pathogenesis of airway inflammation associated with colds and mechanisms of viral exacerbations of asthma. IL-6 induces antibody production in B cells and T cell activation and differentiation (1). IL-8 is a major chemoattractant for neutrophils and stimulates neutrophils to cause enzyme release and production of reactive oxygen metabolites (12). Neutrophils and lymphocytes are shown to be predominant cell types in the nasal mucosa during rhinovirus infection (13). Like-

Fig. 7. ICAM-1 expression in human tracheal epithelial cells cultured for 2 days after sham infection (A; control) or HRV-14 infection (B) and that treated with either 200 pg/ml (C) or 10 ng/ml (D) concentration of IL-1β. Cells expressing ICAM-1 are stained as red and are indicated by arrows (bar = 20 µm and magnification = ×100).
wise, upregulation of ICAM-1 could increase susceptibility to major group rhinoviruses (7, 28) and could lead cells adjacent to infected cells to infection when viruses are released from the cells originally infected. Furthermore, chronic antigen challenge is shown to increase ICAM-1 expression on epithelial cells. Rhinovirus infection induces an increased production of cytokines that regulate the acute phase reaction of airway inflammation. Of these, IL-1β is able to upregulate ICAM-1 expression and to increase the susceptibility to HRV-14 infection, and the antibody to IL-1β inhibited both viral infection and ICAM-1 expression. Furthermore, antibodies to ICAM-1 reduce the production of cytokines induced by HRV-14 infection. These findings suggest that rhinoviruses secrete a cytokine that by the overexpression of ICAM-1 on epithelial cells through the production of IL-1β, resulting in a further increase in the production of inflammatory cytokines. A recent report has also demonstrated a similar IL-1α-dependent autocrine mechanism in respiratory syncytial virus infection of A549 cells (22). Thus these processes may be relevant to airway inflammation induced by respiratory viruses and viral exacerbations of asthma.

In summary, we have shown, for the first time, that rhinovirus is infectious in primary cultures of human tracheal epithelial cells. Rhinovirus infection upregulates ICAM-1 expression on epithelial cells. Rhinovirus infection induces an increased production of cytokines that regulate the acute phase reaction of airway inflammation. Of these, IL-1β is able to upregulate ICAM-1 expression and to increase the susceptibility to HRV-14 infection, and the antibody to IL-1β inhibited both viral infection and ICAM-1 expression. Furthermore, antibodies to ICAM-1 reduce the production of cytokines induced by HRV-14 infection. These findings suggest that rhinoviruses per se amplify their infection by the overexpression of ICAM-1 on epithelial cells through the production of IL-1β, resulting in a further increase in the production of inflammatory cytokines. A recent report has also demonstrated a similar IL-1α-dependent autocrine mechanism in respiratory syncytial virus infection of A549 cells (22). Thus these processes may be relevant to airway inflammation induced by respiratory viruses and viral exacerbations of asthma.

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