Type 2 rhinovirus infection of cultured human tracheal epithelial cells: role of LDL receptor

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aspiration; common cold; airway inflammation; low-density lipoprotein receptor

Rhinoviruses (RVs) cause the majority of common colds, which often provoke wheezing in patients with asthma (18), and have also been associated with exacerbations of chronic bronchitis (14). A perspective study (17) has indicated that asthma attacks are associated with a viral infection in as many as 20–50% of the cases. Studies (12, 18) using PCR-based diagnostics have emphasized the importance of RVs by demonstrating that RVs are responsible for 80–85 and 45% of the asthma flairs in 9- to 11-yr-old children and adults, respectively, with RV being the most commonly implicated pathogen. In contrast to a variety of other respiratory pathogens (e.g., influenza virus and adenovirus), cell cytotoxicity does not appear to play a major role in the pathogenesis of RV infection (29), but the clinical and pathological features of RV infection are, to a great extent, due to the elaboration by the host of a variety of inflammatory mediators (39).

The 102 antigenically distinct serotypes of RVs are divided into two groups, major and minor, based on receptor specificity (1, 35). The major group RVs bind to intercellular adhesion molecule-1 (ICAM-1) (9, 33). Airway epithelial cells express ICAM-1, and monoclonal antibodies to anti-ICAM-1 inhibit both infection by major group RVs and cytokine production induced by major group RV infection (31, 32). Because major group RVs upregulate ICAM-1 production in airway epithelial cells (23, 32), they are suggested to amplify their infection by the overexpression of ICAM-1 on epithelial cells, resulting in a further increase in the production of inflammatory cytokines (23, 31, 32). In contrast, a recent report (11) demonstrated that the low-density lipoprotein (LDL) receptor family can function as receptors for minor group RVs in human fibroblasts. RV type 2 (RV2), a minor group RV, can also infect airway epithelial cells and produce inflammatory cytokines (31, 32). However, the role of the LDL receptor in RV2 infection of the airway epithelial cells remains uncertain.

We therefore investigated whether primary cultures of human tracheal epithelial cells can be infected with minor group RV2 through binding to a LDL receptor. We also studied whether minor group RV2 infection upregulates LDL receptor expression on the airway epithelial cells and increases the susceptibility to infection.

METHODS

Medium components. Reagents for cell culture media were obtained as follows: Eagle’s minimum essential medium...
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(MEM), Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, fetal calf serum (FCS), and ultra-low IgG FCS were from Gibco BRL (Life Technologies, Palo Alto, CA); trypsin, EDTA, dithiothreitol (DTT), Sigma type XIV protease, human placental collagen, penicillin, streptomycin, gentamicin, and amphotericin B were from Sigma (St. Louis, MO); and Ultroser G (USG) serum substitute was from Bio-Sepra (Marlborough, MA).

**Human embryonic fibroblast cell culture.** Human embryonic fibroblast cells were cultured in MEM containing 10% FCS supplemented with 5 × 10^4 U/I of penicillin and 50 mg/l of streptomycin (19) in a Roux-type bottle (Iwaki Glass, Chiba, Japan) sealed with a rubber plug. Confluence 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) were suspended in MEM containing 10% FCS and then plated in glass tubes (15 × 105 mm; Iwaki Glass) that were sealed with rubber plugs and kept stationary at a slant of 5° at 37°C.

**Human tracheal epithelial cell culture.** Tracheae for cell culture were obtained 2–8 h after death from 51 patients (age, 64 ± 5 yr; 22 women and 29 men) under a protocol passed by the Tohoku University (Sendai, Japan) Ethics Committee. Fifteen of the patients were smokers. None had a respiratory illness including bronchial asthma, and they died of acute myocardial infarction (n = 12), congestive heart failure (n = 5), malignant tumor other than lung cancer (n = 14), rupture of aortic aneurysm (n = 2), liver cirrhosis (n = 3), renal failure (n = 4), leukemia (n = 3), malignant lymphoma (n = 1), cerebral bleeding (n = 6), and cerebral infarction (n = 1).

Isolation and culture of the human tracheal surface epithelial cells were performed as previously described (20, 32, 38). In brief, the surface epithelium was scored into longitudinal strips and pulled off the submucosa. The tracheal surface epithelial cells were isolated by digestion with protease (0.4 mg/ml, Sigma type XIV) dissolved in phosphate-buffered saline (PBS) overnight at 4°C. The cells were pelleted (200 g for 10 min) and suspended in DMEM-Ham's F-12 medium containing 5% FCS (50:50 vol/vol). Cell counts were made with a hemocytometer, and estimates of viability were made with the human embryonic fibroblast cell assay described in *Detection and titration of viruses*. We found in the preliminary experiments that human embryonic fibroblast cells produce the same content of RV2 or RV14 in MEM supplemented with ultra-low IgG FCS as in MEM with γ-globulin-free calf serum (GIBCO BRL) (19, 32). The amount of specimen required to infect 50% of human embryonic fibroblast cells [50% tissue culture infective dose (TCID50)] was determined. In preliminary experiments, we found that changing the γ-globulin-free calf serum (GIBCO BRL) (19, 32) to ultra-low IgG FCS did not affect RV titration.

**Viral infection of human tracheal epithelial cells.** Medium was removed from confluent monolayers of human tracheal epithelial cells and replaced with 1 ml of DMEM-Ham's F-12 medium containing 2% USG on the first day after plating. The glass tubes were sealed with rubber plugs, kept stationary at a slant of 5°, and cultured at 37°C. The cell culture medium was supplemented with 10^5 U/I of penicillin, 100 mg/l of streptomycin, 50 mg/l of gentamicin, and 2.5 mg/l of amphotericin B.

We confirmed cilia beating on the epithelial cells and the absence of fibroblasts in the glass tubes using an inverted microscope (MIT-2, Olympus, Tokyo, Japan) as previously described (32). Under an inverted microscope, the cultured human tracheal epithelial 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 the spindle-shaped fibroblasts as shown by Winther et al. (37) but not on the tracheal epithelial 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 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 (20, 32, 38). When the cells cultured under these conditions become differentiated and form tight junctions without contamination by fibroblasts, they have values of >40 Ω·cm² for resistance and >10 μA/cm² for short-circuit current (20, 32, 38). Therefore, cultured human tracheal epithelial cells were judged as cells able to form tight junctions and were used for the following experiments when the cells on the Millicell CM inserts had a high resistance (>40 Ω·cm²) and a high short-circuit current (>10 μA/cm²). In addition, we observed whether the human tracheal epithelial cells made a dome formation to confirm that cells on a solid glass support form tight junctions. We found that the human tracheal epithelial cells made a dome formation when the cells formed confluent cell sheets as described by Widdicombe et al. (36).

**Viral stocks.** RV2 and RV14 were prepared in our laboratory from patients with common colds (19). Stocks of RV2 and RV14 were generated by infecting human embryonic fibroblast cells cultured in MEM supplemented with 2% ultra-low IgG FCS instead of γ-globulin-free calf serum (19, 32) until cytopathic effects were obvious, after which the cultures were frozen at −80°C, thawed, and sonicated. The virus-containing fluid obtained was frozen in aliquots at −80°C. The content of the viral stock solutions was determined with the human embryonic fibroblast cell assay described in *Detection and titration of viruses*. We found that the human embryonic fibroblast cells produce the same content of RV2 or RV14 in MEM supplemented with ultra-low IgG FCS as in MEM with γ-globulin-free calf serum (GIBCO BRL) (19, 32). The amount of specimen required to infect 50% of human embryonic fibroblast cells [50% tissue culture infective dose (TCID50)] was determined. In preliminary experiments, we found that changing the γ-globulin-free calf serum (GIBCO BRL) (19, 32) to ultra-low IgG FCS did not affect RV titration.

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whole volume of the medium was taken for measurement of the viral content, and the same volume of fresh medium was replaced on days 3 and 5. The whole volume of the medium was taken for measurement of the viral content on day 7. The supernatants were stored at −80°C for determination of the viral content. Cell-associated viral content was also analyzed with sonicated human tracheal epithelial cells in MEM. The viral content in the supernatant and cell-associated viral content are expressed as TCID50 units per milliliter and as TCID50 units per 10^6 cells, respectively.

**Effects of an antibody to the LDL receptor on RV infection.** Confluent human tracheal epithelial cells were incubated for 30 min at 37°C with medium alone, with medium containing a mouse anti-human monoclonal antibody to the LDL receptor (50 μg/ml; Oncogene Research Products, Cambridge, MA), or with medium containing an isotype-matched mouse IgG2b, κ control monoclonal antibody (50 μg/ml; Pharmingen, San Diego, CA). The monoclonal antibody to the LDL receptor is the IgG2b, κ isotype, and it recognizes an epitope of the ligand binding region of the LDL receptor (5). After excess antibody was washed off, the monolayers were exposed to RV2 (10^5 TCID50/ml) or RV14 (10^5 TCID50/ml) for either 15 or 60 min before the monolayers were rinsed, and fresh DMEM-Ham's F-12 medium containing 2% USG supplemented with 10^5 U/ml 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 assayed at various times after infection.

**Detection of RV RNA by RT-PCR.** Human tracheal epithelial cells cultured in glass tubes were lysed by the addition of RNAzol (0.2 ml/10^6 cells; BIOTECX, Houston, TX) and transcription of the ligand binding region of the LDL receptor (5). After excess antibody was washed off, the monolayers were exposed to RV2 (10^5 TCID50/ml) or RV14 (10^5 TCID50/ml) for either 15 or 60 min before the monolayers were rinsed, and fresh DMEM-Ham's F-12 medium containing 2% USG supplemented with 10^5 U/ml 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 assayed at various times after infection.

**Cytokine assays.** Because RV infection increased the production of various cytokines from primary cultures of human tracheal epithelial cells (32), we measured interleukin (IL)-1α, IL-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon (IFN)-α, IFN-β, and IFN-γ by specific enzyme-linked immunosorbent assays (ELISAs). Sensitivities of the assays were 25 pg/ml for the IFN-α ELISA kit (COSMO BIO, Tokyo, Japan); 10 pg/ml for the IL-1α ELISA kit (Ohtsuka, Otsu, Japan), the IL-1β ELISA kit (Ohtsuka), the IL-6 ELISA kit (Toray, Tokyo, Japan), and the IL-8 ELISA kit (Toray); 4 pg/ml for the TNF-α ELISA kit (Ohtsuka); 3 pg/ml for the TNF-γ ELISA kit (Genzyme, Cambridge, MA); 1 U/ml for the IFN-β ELISA kit (BioSource International, Camarillo, CA); and 2 pg/ml for the GM-CSF ELISA kit (Genzyme). In preliminary experiments, we found that the concentration of TNF-α in the culture medium was low (0–10 pg/ml). Therefore, we concentrated the culture medium by freeze-drying methods with a centrifugal vaporizer (Tohoku Rikakikai, Tohoku, Japan) before measuring the concentration of TNF-α.

**Northern blot analysis.** Northern blot analysis was done as previously described (27, 32). Equal amounts of total RNA (10 μg) extracted from human tracheal epithelial cells, as determined spectrophotometrically, were subjected to electrophoresis in a 1% agarose-formaldehyde gel. The gel was then transferred via capillary action onto a nylon membrane (Hybond-N+, Amersham Life Science). The LDL receptor cDNA probe (2.8 kb) was prepared by digesting the plasmid LDLr3 (American Type Culture Collection, Manassas, VA) with the restriction endonucleases HindIII and Smal (26). The membrane was hybridized with [α-32P]dCTP (3,000 Ci/ mmol; Amersham)-labeled human LDL receptor cDNA 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-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. Quantification of the autoradiographic bands was accomplished with an image analyzer (Bio Imaging Analyzer, BAS-2000; Fuji Photo Film). We used an average value from replicate cultures from the same trachea (n = 3) for analysis of the intensity of the LDL receptor or β-actin bands.

To study the effects of RV infection on the mRNA expression of the LDL receptor in human tracheal epithelial cells, cells were examined before and 8 h and 1, 3, and 5 days after RV2 infection and 1 day after RV14 infection. To determine the mechanisms responsible for the upregulation of LDL receptor mRNA expression after RV2 infection, we tested the effects of either IL-1β (200 pg/ml; Genzyme), IL-6 (100 pg/ml; Genzyme), IL-8 (100 pg/ml; Collaborative Research, Bedford, MA), or TNF-α (10 pg/ml; Genzyme) on LDL receptor mRNA expression in human tracheal epithelial
cells. The concentration of each cytokine chosen was matched to a net increase in the culture medium after RV2 infection. Cells were incubated overnight with each cytokine.

Flow cytometry analysis of cell membrane LDL receptor expression. Induction of LDL receptor expression in human tracheal epithelial cells after infection with either RV2 (10⁵ TCID₅₀/ml for 60 min) or RV14 (10⁵ TCID₅₀/ml for 60 min) was assayed by flow cytometry analysis as previously described (30). Cells in glass tubes were collected by incubation with 0.02% EDTA for 10 min at 37°C, and a volume containing 1.5 × 10⁶ cells was centrifuged for 7 min at 425 g, resuspended in ice-cold PBS-0.5% BSA, and washed twice with ice-cold PBS-0.5% BSA for 5 min at 541 g. The final pellet was resuspended in 300 μl of ice-cold PBS-0.5% BSA and divided into aliquots for 2 samples and 1 control. Then, 12.5 μl of an anti-LDL receptor antibody solution (0.5 μg/ml; Oncogene Research Products) were added to each sample and incubated for 30 min at 4°C. Subsequently, the samples were washed twice with ice-cold PBS-0.5% BSA and incubated with 100 μl of FITC-labeled goat-anti-mouse IgG (DAKO, Glostrup, Denmark) for 45 min at 4°C. The samples were then washed twice with ice-cold PBS-0.5% BSA and incubated with 5 μl of phycoerythrin-labeled mouse anti-human CD14 IgG (DAKO). The cells were washed twice more and resuspended in 500 μl of PBS-0.5 BSA-0.1% sodium azide. Controls were processed in parallel with the samples but received an equivalent volume of PBS-0.5% BSA-0.1% sodium azide instead of primary antibody. In addition, 100 μl of Set-Up bead suspension (QIFIKIT) and 100 μl of Calibration bead suspension (QIFIKIT) were processed in parallel but received equivalent volumes of PBS-0.5% BSA-0.1% sodium azide instead of the anti-LDL antibody and anti-CD14 antibody. All measurements were performed on a flow cytometer (FACSCalibur, Becton Dickinson). Subsequently, the resultant population was reviewed in a forward-scatter versus side-scatter window, and only the core of the population was used for subsequent LDL receptor determination in the FL1 (FITC) channel. The acquisition number of the final population was set at 10,000. Standardized equivalents of LDL receptor density per cell, expressed as sites per cell (S/C units), were calculated by mathematical transformation of mean fluorescence intensities into specific antibody binding capacities with a calibration curve derived from the Calibration beads (QIFIKIT, DAKO). The resultant specific antibody binding capacity represents an equivalent of LDL receptor expression (30).

Effects of neutralizing antibodies to IL-1β and TNF-α on RV2 infection and LDL receptor mRNA expression. To determine the role of endogenous IL-1β in viral infection and LDL receptor expression, confluent human tracheal epithelial cells were preincubated with a mouse anti-human IL-1β monoclonal antibody (10 μg/ml; Genzyme) or an isotype-matched mouse IgG1 control monoclonal antibody (Chemicon International) at the same concentration for 5 days (32). We also tested the effects of a mouse anti-human TNF-α monoclonal antibody (10 μg/ml; 5 days; Genzyme) on viral infection and LDL receptor expression. Viral titer in the supernatants collected for 3–5 days and the expression of LDL receptor mRNA 5 days after RV2 infection (10⁵ TCID₅₀/ml) were measured in confluent human tracheal epithelial cells preincubated with each antibody.

Effect of IL-1β on susceptibility to RV2 infection. To examine whether IL-1β increases the susceptibility to either RV2 or RV14 infection, confluent human tracheal epithelial cells were preincubated with and without IL-1β (200 pg/ml) for 24 h. The epithelial cells were then exposed to serial twofold dilutions of either RV2 or RV14 (10⁵ TCID₅₀/ml) for 1 h at 33°C before being rinsed, and fresh DMEM-Ham’s F-12 medium containing 2% USG, 10% FCS of penicillin, 100 μg/ml of streptomycin, 50 mg/l of gentamicin, and 2.5 mg/l of amphotericin B was added. The presence of RV2 or RV14 in 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 RV2 and RV14 used. This index of susceptibility to infection, defined as the minimum dose of RV that could induce infection, was compared with the susceptibility of control cells that were not incubated with IL-1β (31).

Isolation of nuclear extracts. Nuclear extracts were prepared with the methods previously described (10, 39). To isolate the nuclear extracts, all procedures were performed on ice. The human tracheal epithelial cells were washed with ice-cold calcium- and magnesium-free PBS, harvested by scraping into calcium- and magnesium-free PBS, and pelleted in a 1.5-ml microfuge tube at 1,850 g for 5 min. After these procedures were repeated once more, the pellet was suspended in one packed cell volume of lysis buffer (10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM phenylmethylsulfonyl 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 mM HEPES, 420 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1 mM PMSF), and agitated vigorously at 4°C for 15 min. After removal of the debris by centrifugation, the protein concentration of the nuclear extract was determined. The nuclear extracts were then stored at −80°C until used.

Electrophoretic mobility shift assay. Electrophoretic mobility shift assays (EMSAs) were performed as previously described (8, 10, 22, 39). The radiolabeled double-strand oligonucleotide probe for either the nuclear factor-κB (NF-κB) or Sp1 site was prepared by annealing complementary oligonucleotides and by end labeling with [γ-³²P]ATP and T4 polynucleotide kinase. The radiolabeled probes used for NF-κB and Sp1 were composed of the following sequences: 5′-AGTTGAGGGGACCTTCCCCAGGC-3′ for NF-κB (7, 20) and 5′-AATTCAGGCCGCGGGCCGTACCCCGCGGGCCGT-3′ for Sp1 (Stratagene, La Jolla, CA) (10). The labeled probes were purified by CHROMA SPIN + TE-10 (CLONTECH, Palo Alto, CA) and diluted with buffer (10 mM Tris-HCl and 1 mM EDTA) to the desired concentration. Equivalent amounts of nuclear protein were incubated with 2 μg of salmon sperm DNA and 2–5 fmol (~20,000 dpm) of the radiolabeled probe for 20 min at room temperature in 20 μl of a buffer containing 10 mM HEPES (pH 7.9), 50 mM KCl, 2 mM MgCl₂, 0.25 mM DTT, 0.25 mM PMSF, 0.1 mM EDTA, and 10% glycerol. Resolution was accomplished by electrophoresing 12 μl of the reaction solution on 4% non-denaturing polyacrylamide gels in Tris-borate-EDTA buffer (89 mM Tris·HCl, 59 mM boric acid, and 2 mM EDTA, pH 8.0) for 60 min at 150 V at room temperature. Autoradiographic detection of the hybridized probe was performed by exposure to Kodak Scientific Imaging Film for 48–72 h at −70°C.

Supershift EMSA. Supershift assays were used to determine which members of the NF-κB family were involved in RV2-induced NF-κB-DNA binding (39). In these studies, EMSAs were performed as described in Electrophoretic mobility shift assay except that rabbit polyclonal antibodies against the NF-κB subunit proteins p65, p50, c-Rel, and Rel B (Santa Cruz Biotechnology, Santa Cruz, CA) were included in the 1-h radiolabeled probe extract binding reaction at 4°C. Preimmune serum (Santa Cruz Biotechnology) was used to
control for any nonspecific effects of these antisera on SP1 activation (10).

Statistical analysis. Results are expressed as means ± SE. Statistical analysis was performed with two-way repeated-measures ANOVA. Subsequent post hoc analysis was made with Bonferroni’s method. Significance was accepted at P < 0.05; n is the number of donors from which cultured epithelial cells were used.

RESULTS

RV infection of human tracheal epithelial cells. Exposing confluent human tracheal epithelial cell monolayers to RV2 and RV14 (10^5 TCID_{50}/ml) consistently led to infection. Collection of culture medium at different times after viral exposure revealed no detectable virus 1 h after infection. Both RV2 and RV14 were detected in the 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 demonstrating that the viral titers of supernatants collected for 1–3, 3–5, and 5–7 days after infection each contained significant levels of RV2 or RV14 (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 RV2 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, 2.3 ± 0.3 log TCID_{50} units at 24 h, 2.6 ± 0.3 log TCID_{50} units at 3 days, 2.1 ± 0.3 log TCID_{50} units at 5 days, and 1.5 ± 0.3 log TCID_{50} units at 7 days (n = 7 each). The viral titers of cell-associated RV14 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 ± 0.2 log TCID_{50} units at 24 h, 2.4 ± 0.3 log TCID_{50} units at 3 days, 2.2 ± 0.3 log TCID_{50} units at 5 days, and 1.7 ± 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 24 h after RV2 infection in cells from smokers did not differ significantly from those in nonsmokers (3.1 ± 0.3 log TCID_{50} units in smokers vs. 3.2 ± 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 RV2-infected cultures. Likewise, RV2 infection did not alter the amount of lactate dehydrogenase in the supernatants (32 ± 3 IU/l before vs. 35 ± 3 IU/l 5 days after infection; P > 0.20; n = 7). RV2 infection also had no effect on cell number. Cell counts 24 h after infection were not significantly different (1.8 ± 10^6 ± 0.2 ± 10^6 in noninfected cells vs. 1.8 ± 10^6 ± 0.3 ± 10^6 in infected cells; P > 0.50; n = 7).

Detection of viral RNA by PCR. Further evidence of RV2 infection in human tracheal epithelial cells and of viral replication was provided by PCR analysis (Fig. 2). In each of three experiments, RNA extracted from control uninfected cells did not produce any detectable PCR product at 385 bp (0 h). A faint product band was observable in RNA extracted from cells 8 h after infection followed by a progressive increase in viral RNA until 3 days after infection.

![Fig. 1. Viral titers in supernatants of human tracheal epithelial cells obtained at different times after exposure to the 10^6 units of rhinovirus (RV) required to infect 50% of human embryonic fibroblast cells (50% tissue culture infective dose /ml). A: viral titers of RV2 (○) and RV14 (●) in supernatants collected at sequential times during the 1st 24 h after infection. B: viral titers of RV2 (open bars) and RV14 (solid bars) in supernatants collected for indicated times after infection. Results are means ± SE from 7 samples.](http://ajplung.physiology.org/)

![Fig. 2. Time course of replication of RV RNA from human tracheal epithelial cells after RV2 infection as detected by RT-PCR. β-actin was used as a housekeeping gene. M, 4×174/HindII fragment molecular weight marker; −, absence; +, presence. Data are representative of 3 different experiments.](http://ajplung.physiology.org/)
**Effects of RV infection on cytokine production.** 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 RV2 (A) or RV14 (B) infection. Because viral infection did not alter cell number (see RV infection of human tracheal epithelial cells), all cytokine values are reported in picograms per milliliter of supernatant. Basal secretion was quite high with IL-8 and 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 RV2 and RV14, although in terms of absolute levels, IL-1β (204 ± 18 pg/ml in RV2 and 157 ± 13 pg/ml in RV14) and IL-6 (182 ± 19 pg/ml in RV2 and 127 ± 13 pg/ml in RV14) predominated. RV2 infection did not alter the production of IL-1α (15 ± 2 pg/ml 3 days after RV2 infection vs. 14 ± 2 pg/ml 3 days after sham infection; \(P > 0.20; n = 7\)) and GM-CSF (152 ± 11 pg/ml 3 days after RV2 infection vs. 167 ± 21 pg/ml 3 days after sham infection; \(P > 0.20; n = 7\)). Of the cytokines measured, IFN-α, IFN-β, and IFN-γ were under the limit of detection of the assay in the supernatants from cells with RV2 and sham infections throughout the experiments.

In contrast to the supernatants from human tracheal epithelial cells, neither IL-1β nor TNF-α was detectable in viral stocks.

**Effects of an antibody to the LDL receptor on RV infection and cytokine production.** Incubation of cells with a monoclonal antibody to the LDL receptor completely blocked RV2 infection as assessed by the absence of detectable viral titers in the supernatants recovered 24 h after 15 min of RV2 exposure (2.1 ± 0.2 log TCID\(_{50}\) units in control cells and 0 ± 0 log TCID\(_{50}\) units with the LDL receptor antibody). Likewise, a monoclonal antibody to the LDL receptor significantly decreased viral titers in the supernatants (3.3 ± 0.2 log TCID\(_{50}\) units in control cells vs. 2.1 ± 0.2 log TCID\(_{50}\) units with the LDL receptor antibody; \(P < 0.01; n = 7\)) as well as those in cell lysates (2.3 ± 0.2 log TCID\(_{50}\) units in control cells vs. 1.1 ± 0.1 log TCID\(_{50}\) units with the LDL receptor antibody; \(P < 0.01; n = 7\) 24 h after 60 min of RV2 exposure. Treatment with the LDL receptor antibody also significantly inhibited increases in IL-1β, IL-6, IL-8, and TNF-α production induced by RV2 infection (Fig. 4A). However, an isotype-matched IgG2b, κ control monoclonal antibody failed to alter viral titers in the supernatants 24 h after 15 min of RV2 exposure (2.2 ± 0.2 log TCID\(_{50}\) units; \(P > 0.50; n = 7\)) and 60 min of RV2 exposure (3.4 ± 0.3 log TCID\(_{50}\) units; \(P > 0.50; n = 7\)). Likewise, IgG2b, κ control monoclonal antibody did not inhibit increases in IL-1β, IL-6, IL-8, and TNF-α production induced by RV2 (Fig. 4A). In contrast to RV2, viral titers in the supernatants recovered 24 h after 15 min of RV14 exposure were not altered by a monoclonal antibody to the LDL receptor (2.1 ± 0.2 log TCID\(_{50}\) units; \(P > 0.50; n = 7\) from the control value (2.2 ± 0.1 log TCID\(_{50}\) units; \(n = 7\)) and from the value of the IgG2b, κ control monoclonal antibody treatment (2.1 ± 0.1 log TCID\(_{50}\) units; \(n = 7\)). Likewise, viral titers in cell lysates 24 h after 60 min of RV14 exposure were not altered by a monoclonal antibody to the LDL receptor (2.1 ± 0.1 log TCID\(_{50}\) units; \(P > 0.50; n = 7\)) from the control value (2.2 ± 0.2 log TCID\(_{50}\) units; \(n = 7\)). Neither a monoclonal antibody to the LDL receptor nor IgG2b, κ control monoclonal antibody altered increases in the production of IL-1β, IL-6, IL-8, and TNF-α induced by RV14 infection (Fig. 4B).

**Effect of RV infection on LDL receptor expression.** The baseline expression of LDL receptor mRNA was constant for at least 5 days in confluent human tracheal epithelial cell sheets, and the coefficient of variation was small (9.7%; \(n = 22\)). Neither smoking habit nor cause of death influenced the baseline expression of LDL receptor mRNA. Exposure of human tracheal epithelial cells to RV2 (Fig. 5A) or RV14 (Fig. 5B) caused increases in LDL receptor mRNA compared with a sham exposure (control, 0 h). Human tracheal epithelial cells 24 h after RV2 (Fig. 5C) or RV14 (Fig. 5D) infection were shown to overexpress LDL receptor
mRNA fourfold compared with those 24 h after a sham exposure. IL-1β (200 pg/ml) also increased LDL receptor mRNA (0.35 ± 0.03 scan units; P < 0.01; n = 7). However, neither IL-6 (200 pg/ml), IL-8 (100 pg/ml), nor TNF-α (10 pg/ml) altered LDL receptor mRNA levels (0.24 ± 0.02 scan units with IL-6; 0.23 ± 0.03 scan units with IL-8, and 0.21 ± 0.02 scan units with TNF-α; P > 0.20; n = 7) compared with those after sham exposure (0.22 ± 0.02 scan units; n = 7).

Expression of the LDL receptor was also assayed by flow cytometry analysis. Human tracheal epithelial cells 3 and 5 days after RV2 infection were shown to increase the LDL receptor-specific fluorescence intensity compared with those after a sham exposure (Fig. 6). Human tracheal epithelial cells 5 days after RV14 infection were also shown to increase the LDL receptor-specific fluorescence intensity compared with those after a sham exposure (10.0 ± 0.7 ± 10^4 S/C units 5 days after RV14 infection vs. 6.5 ± 0.6 ± 10^4 S/C units 5 days after sham infection; P < 0.01; n = 7). IL-1β (200 pg/ml, 24 h) also increased the LDL receptor-specific fluorescence intensity (8.5 ± 0.6 ± 10^4 S/C units in IL-1β vs. 6.5 ± 0.6 ± 10^4 S/C units in control cells; P < 0.05; n = 7).

Effects of neutralizing antibodies to IL-1β and TNF-α on viral infection and LDL receptor expression. The mouse anti-human IL-1β monoclonal antibody (10 μg/ml) caused significant decreases in RV2 titers in the supernatants collected on days 3–5 (2.8 ± 0.3 log TCID_{50} units/ml; P < 0.01; n = 7) compared with those in the control supernatants (4.0 ± 0.3 log TCID_{50} units/ml; n = 7) as well as decreases in LDL receptor mRNA expression 5 days after RV2 infection (0.62 ± 0.04 scan units with IL-1β antibody vs. 0.78 ± 0.03 scan units in control cells; P < 0.05; n = 7) in human tracheal epithelial cells. In contrast, neither the mouse anti-human TNF-α monoclonal antibody (10 μg/ml) nor the mouse IgG1 control monoclonal antibody (10 μg/ml) altered viral titers of the supernatants (4.0 ± 0.3 log TCID_{50} units/ml with anti-human TNF-α antibody and 4.1 ± 0.2 log TCID_{50} units/ml with IgG1 control antibody; P > 0.50; n = 7) and LDL receptor mRNA expression (0.78 ± 0.03 scan units with anti-human TNF-α antibody and 0.79 ± 0.02 scan units with IgG1 control antibody; P > 0.50; n = 7).

The mouse anti-human IL-1β monoclonal antibody (10 μg/ml) also caused a significant reduction in LDL receptor protein expression in human tracheal epithelial cells (7.8 ± 0.6 × 10^4 S/C units; P < 0.05; n = 7) compared with that 5 days after RV2 infection alone (11.0 ± 0.7 × 10^4 S/C units; n = 7). In contrast, neither the mouse anti-human TNF-α monoclonal antibody (10 μg/ml) nor the mouse IgG1 control monoclonal antibody (10 μg/ml) altered LDL receptor protein expression (11.3 ± 0.7 × 10^4 S/C units with anti-human TNF-α antibody and 11.0 ± 0.8 × 10^4 S/C units with IgG1 control antibody; P > 0.50; n = 7).

Effect of IL-1β on the susceptibility to RV2 infection. Pretreatment of human tracheal epithelial cells for 24 h with IL-1β (200 pg/ml) increased the susceptibility of cells to RV2 infection, decreasing by twofold the minimum dose of virus necessary to cause infection (1.5 ± 0.1 log TCID_{50} units with IL-1β vs. 2.1 ± 0.1 log TCID_{50} units in control cells; P < 0.05; n = 7).

NF-κB and SP1 DNA-binding activity in human tracheal epithelial cells. Nuclear extracts from the human tracheal epithelial cells with RV2 or sham infection contained activated SP1 and NF-κB as demonstrated by the presence of a complex consisting of protein bound to a DNA fragment carrying the NF-κB site (Fig. 7A) and SP1 (Fig. 7B). The baseline intensity of NF-κB and SP1 DNA-binding activity was constant,
and increased activation of NF-κB and SP1 was present in cells from 0.5 h and continued for up to 12 h after RV2 infection (Fig. 7). The activation of NF-κB and SP1 then decreased with longer incubations. 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 (Fig. 8). The supershifting of the NF-κB band with the antibody to the p50 or p65 subunit of NF-κB was constantly observed at any time during cell culture. However, the supershifting of the NF-κB band was not observed with antibody to either p52, c-Rel, or Rel B or preimmune antiserum (Fig. 8). The band showing SP1 DNA-binding activity was completely abolished by an exogenous addition of a 50-fold excess of an unlabeled DNA fragment (data not shown).

DISCUSSION

The present study shows that minor group RV2 can infect primary cultures of human tracheal epithelial cells via binding to the LDL receptor on the epithelial...
cells as shown previously in human fibroblasts (11) and HeLa cells (16) and upregulates the production of proinflammatory cytokines and the LDL receptor in epithelial cells. These conclusions are based on the observation that RV2 titers of culture supernatants and lysates from infected cells and RV2 RNA from infected cells increased with time. RV2 infection increased the production of IL-1β, IL-6, IL-8, and TNF-α in supernatants and upregulated the expression of mRNA and protein of the LDL receptor in the cultured human tracheal epithelial cells. Furthermore, an antibody to the LDL receptor reduced RV2 titers in the culture supernatants and lysates from infected cells and RV2 infection-induced cytokine production in the culture supernatants.

Viral infection of cultured human tracheal epithelial cells and subsequent viral replication were confirmed by showing the increased viral content in the culture supernatants of infected cells with time as assessed by the cytopathic effects of this culture supernatant 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 with 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 RV2 were consistently observed when confluent monolayers were exposed to this virus. However, RV2 infection failed to influence both cell number and cell viability. This is in agreement with previous studies (31, 32, 37) showing the lack of cytotoxicity on epithelial cells in RV infection.

The specificity of the infection process for primary cultures of human tracheal epithelial cells by RV2 was confirmed by demonstrating that infection could be blocked with an antibody to the LDL receptor but not by an isotype-matched IgG2b, κ monoclonal antibody. Furthermore, a monoclonal antibody to the LDL receptor failed to block RV14 infection, a major group RV that uses ICAM-1 as its receptor (9, 33). 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 (25). The potency of inhibition by a monoclonal antibody to the LDL receptor observed in the present study is consistent with that in the previous

Fig. 7. Electrophoretic mobility shift assay (EMSA) demonstrating increases in nuclear factor (NF-κB) (A) and SP1 (B) DNA-binding activities (arrows) of human tracheal epithelial cells before (0) and at indicated times after RV2 infection.

Fig. 8. Identification of RV2-induced NF-κB bands with supershift EMSA. EMSAs were performed in the presence and absence of antisera against NF-κB family proteins, and antisera against p50, p65, p52, c-Rel, and Rel B were compared with preimmune serum. Arrows, RV2-induced NF-κB DNA-binding activities; arrowhead, supershifted bands caused by antisera against p50 and p65.
study (16) in which RV2 binding was reduced to about one-third when RV2 was present during the incubation of the cells with a soluble LDL receptor fragment. In addition, we showed that the antibody to the LDL receptor inhibited the production of IL-1β, IL-6, IL-8, and TNF-α induced by RV2 infection without an effect on RV14-induced increases in cytokine production. However, an antibody to the LDL receptor could not achieve complete inhibition of cytokine production induced by RV2 infection, which may also be due to the longer incubation times described above.

We showed that infection with either RV2 or RV14 as well as IL-1β at the experimentally measured concentration in supernatants upregulated LDL receptor expression as assessed by increases in LDL receptor mRNA with Northern blot analysis and LDL protein with flow cytometry analysis. IL-1β increased the susceptibility to RV2 infection by only twofold despite increases in susceptibility to RV14 infection by >10-fold (32), suggesting that an endogenous IL-1β may upregulate LDL receptor expression less than ICAM-1 expression in the cultured human tracheal epithelial cells. These findings are consistent with previous reports (10, 13, 24) that IL-1 and TNF-α increase ICAM-1 expression by >10-fold in pulmonary epithelial cells, but they increase LDL receptor mRNA expression by only 5- and 6-fold in hepatocellular carcinoma cells and vascular endothelial cells. The anti-IL-1β antibody significantly inhibited RV2 infection and RV2 infection-induced increases in mRNA expression and protein of the LDL receptor. However, the inhibitory effects of the anti-IL-1β antibody on LDL receptor expression and RV2 infection were smaller than those on ICAM-1 expression and RV14 infection (32). Therefore, the role of endogenous IL-1β in LDL receptor expression and RV2 infection may be minor. Other factors regulating RV2 infection need to be clarified in the future.

Increased activation of NF-κB was present in cells from 0.5 h after RV2 infection in the present study. The time course of NF-κB activation is consistent with previous reports (23, 39) in airway epithelial cells caused by infection with RV14 or RV16. NF-κB increases the expression of genes for many cytokines, enzymes, and adhesion molecules including ICAM-1 (4, 23, 39). Therefore, RV2-induced cytokine production may be associated with the activation of NF-κB in human tracheal epithelial cells. In contrast, another transcription factor, SP1, but not NF-κB, is reported to mediate the TNF-α-induced LDL receptor expression and sterol-induced repression of LDL receptor expression (7, 10, 15). We observed that RV2 infection also induces activation of SP1 in human tracheal epithelial cells. Therefore, LDL receptor expression by RV2 infection may be mediated, in part, through activation of SP1. A variety of intracellular signals such as several kinases and intracellular calcium are suggested to activate NF-κB (3) and SP1 (21). Because stimulation of the LDL receptor with LDL induces protein kinase C activation in smooth muscle cells (28), protein kinase C may, in part, relate to the activation of NF-κB and SP1 in airway epithelial cells, although we did not examine the relationship between the LDL receptor and the intracellular signals. Further studies are needed to clarify the mechanisms.

RV infection induces local production of cytokines, known to mediate the acute-phase reactions of airway inflammation (31, 32, 39). The species of cytokine and time course of cytokine production differ between cell types. For example, RV14 infection increases production of IL-6, IL-8, and GM-CSF in the airway epithelial cell line BEAS-2B (31) and that of IL-6 in A549 alveolar epithelial type II-like cells (39). In the present study, RV2 induced the production of IL-1β, IL-6, IL-8, and TNF-α in human tracheal epithelial cells, which is consistent with a previous report (32). Although an increase in TNF-α production after infection was subtle and in IL-8 was <30% from the baseline, there was a large amount of IL-1β production from human tracheal epithelial cells, 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 production, prostaglandin E2 synthesis, and degranulation from neutrophils (25). IL-1β also causes increases in ICAM-1 expression on both epithelial and vascular endothelial cells (2, 6, 34). Upregulation of ICAM-1 on HeLa cells (33) and human tracheal epithelial cells (32) is shown to be associated with the increased binding of major group RVs.

Both RV2 and RV14 infections increased the expression of mRNA and protein of the LDL receptor in the human tracheal epithelial cells. The physiological and pathological role of the LDL receptor expression in the airway epithelial cells is still uncertain. However, upregulation of the LDL receptor on human fibroblasts is associated with increased binding of RV2 (11), suggesting an increase in susceptibility to minor group RV infection. Therefore, both major and minor subgroup RVs may cause a predisposition to other serotypes of minor group RV infection through increasing the expression of its receptor, the LDL receptor. 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 have shown that infection of minor group RV2 induces LDL receptor expression. RV2 infection upregulated the production of cytokines that regulate the acute-phase reaction of airway inflammation. Furthermore, an antibody to the LDL receptor inhibited RV2 infection and the production of cytokines in response to RV2 infection. These findings suggest that minor group RV2 can be infectious through binding to the LDL receptor in primary cultures of human tracheal epithelial cells. Because major group RV14 also induced LDL receptor expression, both major and minor RVs may amplify their own and the other group’s RV infection by overexpression of the LDL receptor and ICAM-1 (23, 32) on epithelial cells. These processes may be rele-
vant to airway inflammation and exacerbations of asthma induced by minor group RVs.

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REFERENCES


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