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# Effects of dexamethasone on rhinovirus infection in cultured human tracheal epithelial cells

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**Suzuki, Tomoko, Mutsuo Yamaya, Kiyohisa Sekizawa, Norihiro Yamada, Katsutoshi Nakayama, Satoshi Ishizuka, Masahito Kamanaka, Tetsushi Morimoto, Yoshio Numazaki, and Hidetada Sasaki.** Effects of dexamethasone on rhinovirus infection in cultured human tracheal epithelial cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* 278: L560–L571, 2000.—To examine the effects of glucocorticoid on rhinovirus (RV) infection, primary cultures of human tracheal epithelial cells were infected with either RV2 or RV14. Viral infection was confirmed by demonstrating that viral RNA in infected cells and viral titers of supernatants and lysates from infected cells increased with time. RV14 infection upregulated the expression of mRNA and protein of intercellular adhesion molecule-1 (ICAM-1), the major RV receptor, on epithelial cells, and it increased the production of interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor-α in supernatants. Dexamethasone reduced the viral titers of supernatants and cell lysates, viral RNA of infected cells, and susceptibility of RV14 infection in association with inhibition of cytokine production and ICAM-1 induction. In contrast to RV14 infection, dexamethasone did not alter RV2 infection, a minor group of RVs. These results suggest that dexamethasone may inhibit RV14 infection by reducing the surface expression of ICAM-1 in cultured human tracheal epithelial cells. Glucocorticoid may modulate airway inflammation via reducing the production of proinflammatory cytokines and ICAM-1 induced by rhinovirus infection.

asthma; common cold; airway inflammation; intercellular adhesion molecule-1

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 using PCR-based diagnostics have emphasized the importance of rhinoviruses (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 (12, 18). 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 (27), 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 (35).

Glucocorticoids have anti-inflammatory actions including the inhibition of cytokine release, reduction in the number of circulating inflammatory cells, reduction in vascular permeability, and inhibition of the release of arachidonic acid metabolites (26). Glucocorticoids also inhibit expression of intercellular adhesion molecule-1 (ICAM-1), a major RV receptor, on airway epithelial cells (32). Farr et al. (7) showed that steroid prophylaxis may suppress nasal inflammation and cold symptoms during the first 2 days in experimental RV infection. However, it remains uncertain whether glucocorticoids inhibit RV infection of the respiratory tract epithelium, a primary target for respiratory viruses.

We therefore investigated whether glucocorticoids have an inhibitory effect on RV infection in cultured human tracheal epithelial cells and determined the mechanisms responsible for glucocorticoid inhibition of RV infection.

## METHODS

*Medium 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); dexamethasone, hydrocortisone-water soluble, trypsin, EDTA, dithiothreitol, Sigma type XIV protease, human placental collagen, penicillin, streptomycin, gentamicin, and amphotericin B were from Sigma (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 MEM containing 10% FCS supplemented with $5 \times 10^4$ U/l of penicillin and 50 mg/l of streptomycin in a Roux-type bottle (Iwaki Garasu, Chiba, Japan) sealed with a rubber plug (19). Confluency was achieved at 7 days, at which time the cells were collected by trypsinization (0.05% trypsin-0.02% EDTA). Cells ($1.5 \times 10^5$ 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.

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*Human tracheal epithelial cell culture.* Tracheae for cell culture were obtained 3–6 h after death from 77 patients (age 68 ± 5 yr; 37 women and 40 men) under a protocol passed by the Tohoku University (Sendai, Japan) Ethics Committee. Thirty-three of the patients were smokers. None had a respiratory illness including bronchial asthma, and they died of acute myocardial infarction ($n$ = 20), congestive heart failure ($n$ = 5), malignant tumor other than lung cancer ($n$ = 23), rupture of aortic aneurysm ($n$ = 5), liver cirrhosis ($n$ = 3), renal failure ($n$ = 4), leukemia ($n$ = 5), malignant lymphoma ($n$ = 3), cerebral bleeding ($n$ = 7), and cerebral infarction ($n$ = 2).

Isolation and culture of the human tracheal surface epithelial cells were performed as previously described (34). 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) at 4°C overnight. 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 hemacytometer, and estimates of viability were done with trypan blue and by measuring the amount of lactate dehydrogenase in the medium as previously reported (31). The cells were then plated at $5 \times 10^5$ viable cells/ml in glass tubes coated with human placental collagen (34). This medium was replaced by DMEM-Ham's F-12 medium containing 2% USG on the first day after the cells were plated. The glass tubes were sealed with rubber plugs and cultured at 37°C. The cell culture medium was 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.

To examine whether the culture medium contained hydrocortisone, we measured the hydrocortisone levels in the culture medium containing 2% USG with a cortisol RIA kit (Amersham, Arlington Heights, IL) using the method previously described (30). Cortisol levels in this medium were under the limit of detection of the assays ($<3 \times 10^{-8}$ M).

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). 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 with Ussing chamber methods (34). When the cells cultured under these conditions become differentiated and form tight junctions without contamination by fibroblasts, they have values of $>40\ \Omega \cdot cm^2$ for resistance and $>10\ \mu A/cm^2$ 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 the cells on the Millicell CM inserts had a high resistance ($>40\ \Omega \cdot cm^2$) and a high short-circuit current ($>10\ \mu A/cm^2$). We observed whether the human tracheal epithelial cells made a dome formation to confirm that the cells on the solid glass support form tight junctions, and we found that human tracheal epithelial cells made a dome formation when the cells made confluent cell sheets as described by Widdicombe et al. (33).

To further exclude fibroblast contamination, the cells were examined with cytokeratin staining. The cells cultured in glass tubes were collected by trypsinization and cultured on slide glasses overnight at 37°C in a $CO_2$ incubator. The cells were then fixed with 4% paraformaldehyde and 0.05% Triton X-100 in 0.1 M phosphate buffer for 15 min at room temperature. After being rinsed with PBS, the cells were incubated with 10% skim milk to block nonspecific binding of the first and second antibodies. The sections were overlaid with either LeukoStat anti-keratin antibody (MAK-5, Triton, Alameda, CA), anti-vimentin antibody (DAKO, Santa Barbara, CA), or an isotype-matched mouse monoclonal antibody with an irrelevant specificity (Chemicon International) in PBS containing 1% bovine serum albumin. Anti-keratin antibody and anti-vimentin antibody label epithelial cells and fibroblast cells, respectively (8, 28). After an overnight incubation at 4°C and a wash with PBS, the sections were incubated with biotinylated horse anti-mouse immunoglobulins (Vector Laboratories) diluted with PBS for 1 h at room temperature followed by incubation with the avidin-biotin peroxidase complexes (Vectastain ABC Kit, Vector Laboratories) for 1 h at room temperature. The sections were developed by exposure to substrate with 3,3′-diaminobenzidine tetrahydrochloride (Dojindo, Kumamoto, Japan) and were counterstained with methyl green.

*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 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 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. Content of the viral stock solutions 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 in MEM supplemented with 2% GGFCS. The glass tubes were then incubated at 33°C for 7 days, and the cytopathic effects of viruses on human embryonic fibroblast cells were observed with an inverted microscope (MIT, Olympus) as previously reported (19). The amount of specimen required to infect 50% of the human embryonic fibroblast cells ($TCID_{50}$) was determined.

We examined in the preliminary experiments whether dexamethasone affects RV2 and RV14 titration in human embryonic fibroblasts. We performed titration of RV2 or RV14 using DMEM-Ham's F-12 medium containing 2% USG and either RV2 or RV14 at concentrations ranging from $10^1$ to $10^5$ $TCID_{50}$ units/ml supplemented with dexamethasone (1 µM). The medium containing RV2 or RV14 supplemented with dexamethasone (1 µM) was diluted 10-fold with MEM supplemented with 2% GGFCS and added to the human embryonic fibroblasts in glass tubes to observe the cytopathic effects. We found that dexamethasone did not influence the cytopathic effects on human embryonic fibroblasts at any concentration of RV2 and RV14. Furthermore, to confirm that glucocorticoid does not influence the RV titration in human embryonic fibroblasts, we studied the effects of another glucocorticoid, hydrocortisone, and found that hydrocortisone (1 µM) did not influence the cytopathic effects on human embryonic fibroblasts induced by RV2 and RV14 as did dexamethasone.

*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 medium containing 2% USG. RV was added at a concentration of $10^5$ $TCID_{50}$ 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 DMEM-Ham's F-12 medium containing 2% USG supple-

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mented 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-6-T, Hirasawa, Tokyo, Japan). To measure the time course of viral release for the first 24 h, we used four separate cultures from each trachea, and the culture supernatants were collected at either 1, 6, 12, or 24 h after RV infection. Furthermore, to measure the viral release over either 1–3, 3–5, or 5–7 days, human tracheal epithelial cells cultured in the tubes were rinsed with PBS and fresh DMEM-Ham's F-12 containing 2% USG was added at 24 h after RV infection. The whole volume of medium was then taken for measurement of viral content, the same volume of fresh medium was replaced on *days 3* and *5*, and the whole volume of medium was taken on *day 7*. The supernatants were stored at −80°C for the determination of viral content. Furthermore, cell-associated viral content was analyzed with sonicated human tracheal epithelial cells in MEM. Viral content in the supernatant and cell-associated viral content are expressed in $TCID_{50}$ units per milliliter and $TCID_{50}$ units per $10^6$ cells, respectively.

*Detection of RV RNA with RT-PCR.* RNA extraction from the human tracheal epithelial cells cultured in glass tubes and PCR were performed as previously described (12, 31). Briefly, 2 µg of RNA from each aliquot of human tracheal epithelial 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 $MgCl_2$, 10 mM dithiothreitol, 5 U/µl of Moloney murine leukemia virus RT (GIBCO BRL), 0.5 mM deoxynucleoside 5′-triphosphate (Takara, Ohtsu, Japan), 1 U/µl of RNase 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 $MgCl_2$, 0.2 mM deoxynucleoside 5′-triphosphate, and 1.25 U of *Taq* polymerase (Takara). A primer pair for the RV was present at 2 ng/µl. Sequences of the PCR primer pair used in these experiments are described elsewhere (31). 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 photographed with type 667 positive/negative film (Polaroid, Cambridge, MA).

RV RNA content in the cultured human tracheal epithelial cells was examined before and 8, 24, 72, and 120 h after RV14 infection in the presence and absence of dexamethasone (1 µM).

*Cytokine assays.* Because RV14 infection increased the production of various cytokines from primary cultures of human tracheal epithelial cells (31), we measured interleukin (IL)-1α, IL-1β, IL-6, IL-8, tumor necrosis factor (TNF)-α, interferon (IFN)-α, IFN-β, and IFN-γ by specific enzyme-linked immunosorbent assays (ELISAs). Sensitivities of the assays were 25 pg/ml for IFN-α ELISA kit (COSMO BIO, Tokyo, Japan); 10 pg/ml for IL-1α (Ohtsuka, Tokushima, Japan), IL-1β (Ohtsuka), IL-6 (Toray, Tokyo, Japan), and IL-8 (Toray) ELISA kits; 4 pg/ml for TNF-α ELISA kit (Ohtsuka); 3 pg/ml for IFN-γ ELISA kit (Genzyme); and 1 U/ml for IFN-β ELISA kit (BioSource International, Camarillo, CA). 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-dry methods with a centrifugal vaporizer (Tokyo Rikakikai, Tokyo, Japan) before measuring the concentration of TNF-α. After the culture medium was freeze-dried, the pellet was dissolved in 200 µl of water, and the concentration of TNF-α was measured. The value was normalized according to the medium volume.

We used an average value of replicate cultures ($n = 3$) from the same trachea for the analysis of cytokine production.

*Northern blot analysis.* Northern blot analysis was done as previously described (24, 31). Equal amounts of total RNA (10 µg) extracted from human tracheal epithelial cells, as determined spectrophotometrically, were subjected to electrophoresis on a 1% agarose-formaldehyde gel. The gel was then transferred via capillary action onto a nylon membrane (Hybond $N^+$, Amersham Life Sciences). The membrane was hybridized with [α-$^{32}$P]dCTP (3,000 Ci/mmol; Amersham)-labeled human ICAM-1 cDNA (1.8-kb *Xba* I fragment; British Bio-technology, Oxon, UK) with a random-primer labeling kit (Random Primer, Takara). Hybridization with a radiolabeled probe was performed overnight at 42°C. After a high-stringency wash was performed (1× standard saline-sodium citrate-0.1% sodium dodecyl sulfate, 60°C), autoradiographic detection of the hybridized probe was performed by exposure to Kodak Scientific Imaging film for 48–72 h at −70°C. Quantification of autoradiographic bands was accomplished with an image analyzer (Bio Imaging Analyzer BAS-2000; Fuji Photo Film).

*Effects of glucocorticoids on RV infection.* Confluent human tracheal epithelial cells were incubated at 37°C for 3 days with medium containing dexamethasone (1 µM) or the vehicle for dexamethasone. Dexamethasone was dissolved in 100% ethanol to give stock solutions of 1 mM. These stock solutions were diluted in the culture medium as necessary for each experiment. The monolayers were then exposed to either RV14 ($10^5$ $TCID_{50}$ units/ml) or RV2 ($10^5$ $TCID_{50}$ units/ml) for 60 min before being rinsed and the addition of fresh DMEM-Ham's F-12 medium containing 2% USG, $10^5$ U/l of penicillin, 100 mg/l of streptomycin, 50 mg/l of gentamicin, and 2.5 mg/l of amphotericin B supplemented with either dexamethasone (1 µM) or the vehicle for dexamethasone. The whole volume of medium was taken for measurement of viral content, and the same whole volume of fresh medium containing either dexamethasone (1 µM) or the vehicle for dexamethasone was replaced at various times after infection. We also studied the effects of another glucocorticoid, hydrocortisone (1 µM), on the viral content of the supernatants and cell lysates of human tracheal epithelial cells infected with RV14 ($10^5$ $TCID_{50}$ units/ml) or RV2 ($10^5$ $TCID_{50}$ units/ml). Hydrocortisone (hydrocortisone-water soluble, Sigma) was dissolved in water and diluted in the culture medium. Cortisol levels in the cell lysates of human tracheal epithelial cells treated with hydrocortisone (1 µM) were under the limit of detection of the assays ($<3 \times 10^{-8}$ M).

To study the concentration-response effects of glucocorticoids on RV14 infection, concentration-response curves to dexamethasone were obtained with concentrations ranging from 10 nM to 10 µM. To determine the minimum preincubation period needed to cause inhibition of RV14 infection, preincubation with dexamethasone (1 µM) was performed for various times from 6 h to 3 days. The cells were then cultured in fresh medium containing the vehicle for dexamethasone. To see whether viral replication recovered when dexamethasone was removed, the cells were preincubated with dexamethasone (1 µM) for 3 days. The cells were then exposed to RV14 ($10^5$ $TCID_{50}$ units/ml) and cultured in fresh medium containing the vehicle for dexamethasone.

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*Effects of dexamethasone on susceptibility to RV infection.* To examine whether glucocorticoids decrease the susceptibility to RV14 infection, confluent human tracheal epithelial cells were preincubated with either dexamethasone (1 μM) or the vehicle for dexamethasone for 3 days. The epithelial cells were then exposed to serial 10-fold dilutions of RV2 or RV14 for 60 min at 33°C before being rinsed and the addition of fresh DMEM-Ham's F-12 medium containing 2% USG, $10^5$ U/l of penicillin, 100 mg/l of streptomycin, 50 mg/l of gentamicin, and 2.5 mg/l of amphotericin B. The presence of RV2 or RV14 in the supernatants collected over 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 or 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 dexamethasone (29).

*Effects of dexamethasone on cytokine production.* Because the maximal production of cytokines was obtained on *day 3* for IL-1β, on *day 1* for IL-6, on *day 1* for IL-8, and on *day 3* for TNF-α after exposure of human tracheal epithelial cells to RV14 (31), production of either IL-1β, IL- 6, IL-8, or TNF-α in the culture medium was measured at the maximal production of each cytokine after RV14 infection ($10^5$ $TCID_{50}$ units/ml) in the presence of either dexamethasone (1 μM) or the vehicle for dexamethasone. The amounts of IL-1α, IFN-α, IFN-β, and IFN-γ released from human tracheal epithelial cells into the culture medium were measured before and 1, 3, and 5 days after RV14 infection.

To confirm that increases in cytokine production 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 cytokine production was also examined. UV inactivation was performed as previously described (11).

*Effects of dexamethasone on ICAM-1 mRNA expression.* To study the effects of glucocorticoids on ICAM-1 mRNA expression, human tracheal epithelial cells treated with either dexamethasone (1 μM) or the vehicle for dexamethasone were exposed to RV14 ($10^5$ $TCID_{50}$ units/ml) for 60 min. The mRNA expression of ICAM-1 in the cells was then examined with Northern blot analysis before and 12, 24, and 72 h after RV14 infection. ICAM-1 mRNA was normalized to a constitutive expression of β-actin mRNA. We used an average value of replicate cultures ($n = 3$) from the same trachea for analysis of the intensity of the ICAM-1/β-actin bands.

*Flow cytometry analysis of cell membrane ICAM-1.* The effects of dexamethasone (1 μM) on RV14 infection ($10^5$ $TCID_{50}$ units/ml for 60 min)-induced changes in ICAM-1 expression in human tracheal epithelial cells were assayed by flow cytometry analysis as previously described (5, 22). Cells in the glass tubes were collected by incubation with 0.02% EDTA for 10 min at 37°C and incubated with a fluorescein isothiocyanate-conjugated monoclonal antibody to ICAM-1 (1 μg/50 μl; DAKO) or an isotype-matched control antibody (DAKO) at 4°C for 30 min. Cells were then extensively washed and resuspended in ice-cold PBS at a cell concentration of $10^6$/ml and analyzed by a flow cytometer (FACS Calibur, Becton Dickinson). A total of 10,000 cells were analyzed by gating on a uniform cell population on a two-parameter histogram of forward versus side scatter. The fluorescence histograms were overlaid to determine significant differences from negative control antibodies. Expression of ICAM-1 was determined by subtracting the mean fluorescence intensity measured with the control monoclonal antibody from that measured with the antibody to ICAM-1.

*Effects of neutralizing antibodies to IL-1β on ICAM-1 mRNA expression.* To determine the role of endogenous IL-1β in ICAM-1 expression, confluent human tracheal epithelial cells were incubated with a mouse anti-human IL-1β (10 μg/ml; Genzyme) or an isotype-matched mouse IgG1 control (Chemicon International) monoclonal antibody at the same concentration for either 1, 3, or 5 days after RV14 infection ($10^5$ $TCID_{50}$ units/ml for 60 min). We also tested the effects of a monoclonal mouse anti-human TNF-α antibody (10 μg/ml, 5 days; Genzyme) on ICAM-1 mRNA expression.

*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. For all analysis, values of $P < 0.05$ were assumed to be significant; $n$ is the number of donors from which cultured epithelial cells were used.

## RESULTS

*Immunocytochemical analysis.* Figure 1 shows the immunocytochemistry of the cells isolated from monolayers of human tracheal epithelial cells in the tubes. Nearly all cells (>95%) reacted with the anti-keratin antibody (Fig. 1*A*), but they did not react with the anti-vimentin antibody (Fig. 1*B*). As a positive control, we stained human embryonic fibroblast cells with the anti-vimentin antibody and obtained positive signals (Fig. 1*C*).

*Effects of glucocorticoids on RV infection of human tracheal epithelial cells.* Exposing confluent human tracheal epithelial cell monolayers to RV2 and RV14 ($10^5$ $TCID_{50}$ units/ml) consistently led to infection. Collection of culture medium at differing times after viral exposure revealed no detectable virus 1 h after infection. Both RV2 and RV14 were detected in culture medium 6 h after infection, and the viral content progressively increased between 6 and 24 h after infection (Fig. 2, *A* and *B*). Evidence of continuous viral production was obtained by demonstrating that the viral titers of supernatants collected over 1–3, 3–5, and 5–7 days after infection each contained significant levels of RV2 or RV14 (Fig. 2, *C* and *D*). In the supernatants, viral titer levels increased significantly with time ($P < 0.05$ in each case by ANOVA). RV14 infection of human tracheal epithelial cells was constant, and the coefficient of variation of the viral titers in the supernatants over 1–3 days was small (8.7%; $n = 56$). Treatment of cells with dexamethasone (1 μM) significantly decreased the viral titers of RV14 in the supernatants from 12 h after infection (Fig. 2, *A* and *C*), but dexamethasone (1 μM) was without effect on the viral titers of RV2 in the supernatants throughout the experiments (Fig. 2, *B* and *D*). Another glucocorticoid, hydrocortisone (1 μM), also decreased the viral titers of RV14 but not of RV2. Viral titers of RV14 and RV2 in the supernatants over 1–3 days after infection were 3.1 ± 0.1 and 4.1 ± 0.2 log $TCID_{50}$ units, respectively, in hydrocortisone (1 μM) compared with 4.2 ± 0.2 and 4.1 ± 0.3 log $TCID_{50}$ units, respectively, in the control supernatants ($P < 0.05$ for RV14 and $P > 0.50$ for RV2; $n = 7$).

The viral titers of the cell lysates also contained significant levels of RV2 and RV14. The viral titers of

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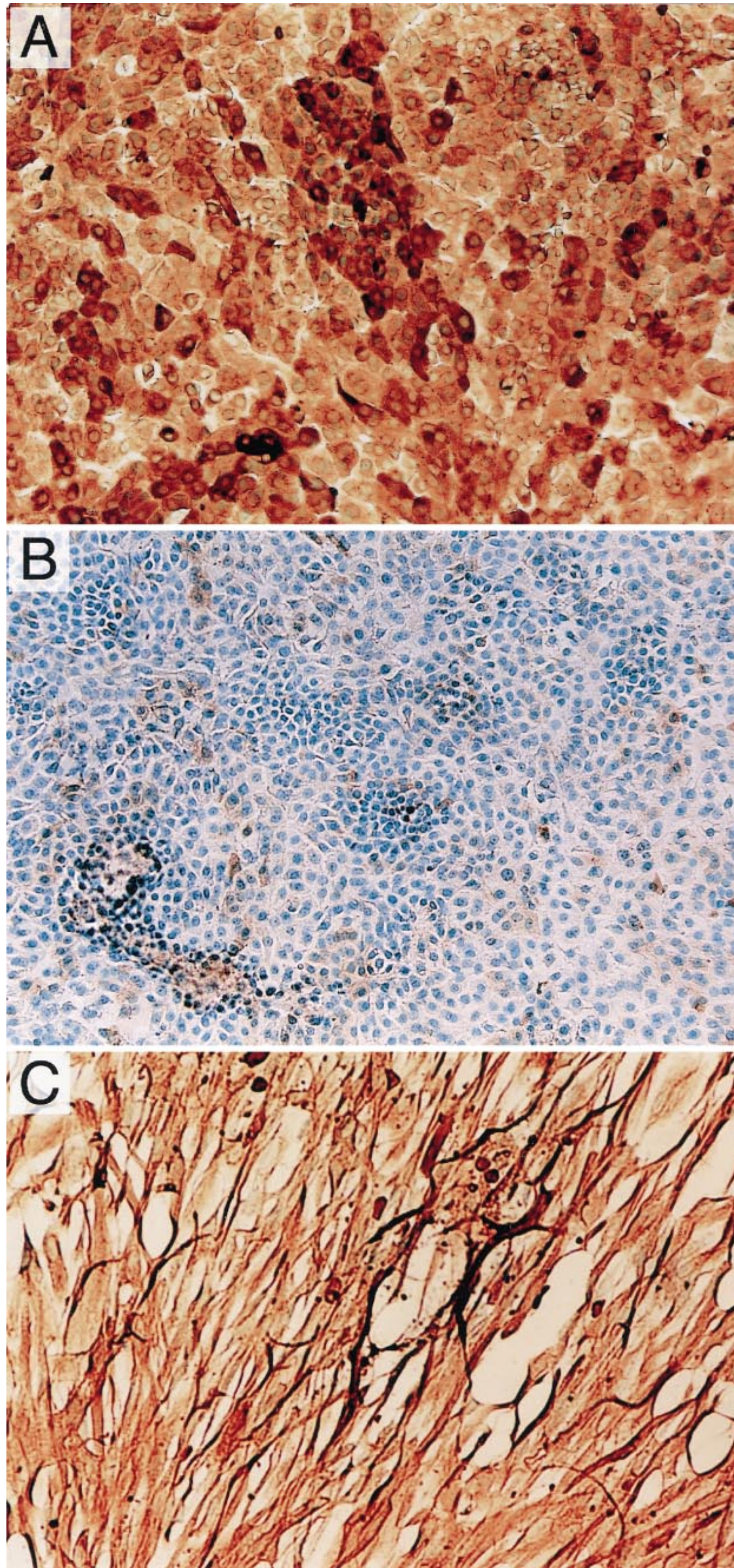


Fig. 1. Immunocytochemistry of cultured human tracheal epithelial cells with monoclonal antibodies directed against epithelial (anti-keratin antibody; *A*) and fibroblast (anti-vimentin antibody; *B*) cells and of cultured human embryonic fibroblast cells stained with anti-vimentin antibody as a positive control (*C*). Original magnification, ×100.

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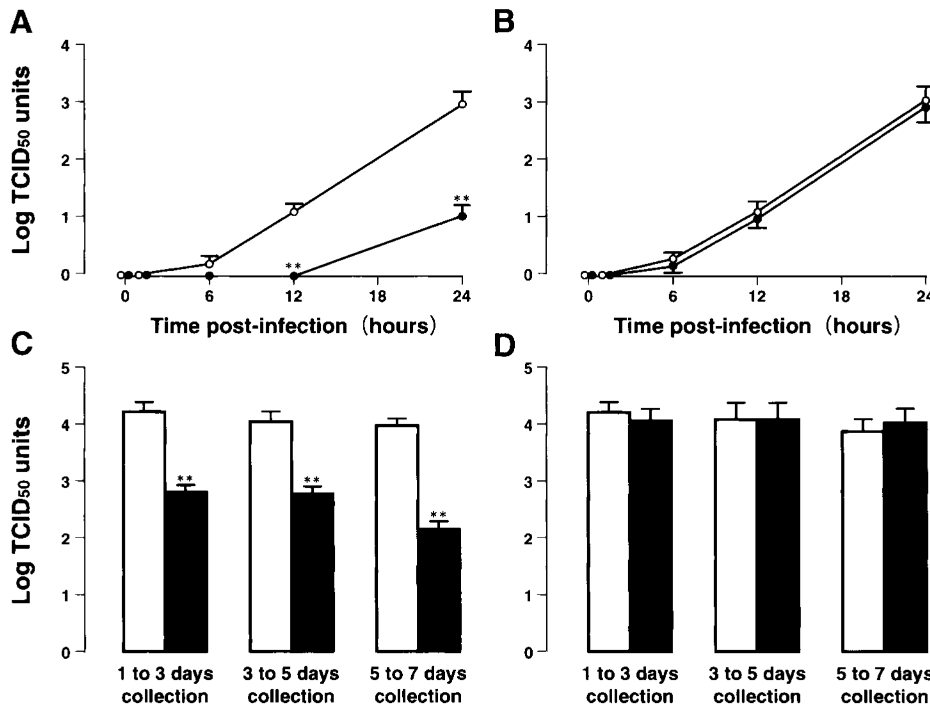


Fig. 2. Viral titers in supernatants of human tracheal epithelial cells obtained at different times after exposure to $10^5$ units of virus required to infect 50% of cells ($TCID_{50}$)/ml of rhinovirus (RV) 14 (*A* and *C*) or RV2 (*B* and *D*) in presence (●) and absence (○) of dexamethasone (1 μM). Viral titers in supernatants were collected at sequential times during 1st 24 h after infection (*A* and *B*). Viral titers in supernatants were collected at indicated times after infection (*C* and *D*). Results are means ± SE from 7 samples. ** Significant difference from viral infection alone, $P < 0.01$.

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, 2.1 ± 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 1.8 ± 0.2 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 ± 0.2 log $TCID_{50}$ units at 7 days ($n = 7$ each). In the cell lysates, viral titer levels also increased significantly with time ($P < 0.05$ in each case by ANOVA). Dexamethasone (1 μM) significantly decreased the cell-associated viral titers of RV14. The viral titers of cell-associated RV14 in dexamethasone-treated human tracheal epithelial cells were 0.0 ± 0.0 log $TCID_{50}$ units at 1 h, 0.0 ± 0.0 log TCID50 units at 6 h, 0.0 ± 0.0 log TCID50 units at 12 h ($P < 0.01$), 1.1 ± 0.1 log $TCID_{50}$ units at 24 h ($P < 0.01$), 1.2 ± 0.1 log $TCID_{50}$ units at 3 days ($P < 0.01$), 1.0 ± 0.1 log $TCID_{50}$ units at 5 days ($P < 0.01$), and 0.9 ± 0.1 log $TCID_{50}$ units at 7 days ($P < 0.01$; $n = 7$ each). Hydrocortisone (1 μM) also decreased the cell-associated viral titers of RV14. The cell-associated viral titers of RV14 3 days after infection were 1.1 ± 0.1 log $TCID_{50}$ units in hydrocortisone (1 μM) compared with 2.4 ± 0.2 log $TCID_{50}$ units in control supernatants ($P < 0.01$; $n = 7$). However, neither dexamethasone (1 μM) nor hydrocortisone (1 μM) decreased the cell-associated viral content of RV2. The cell-associated viral titers of RV2 3 days after infection were 2.5 ± 0.3 log $TCID_{50}$ units in dexamethasone (1 μM) and 2.4 ± 0.2 log $TCID_{50}$ units in hydrocortisone (1 μM) compared with 2.4 ± 0.3 log $TCID_{50}$ units in the control supernatants ($P > 0.50$; $n = 7$).

The inhibitory effects of dexamethasone on RV14 infection were concentration dependent, and the maximum effect was obtained at 1 μM (Fig. 3). Likewise, the inhibitory effects of dexamethasone (1 μM) on RV14 infection were dependent on the preincubation period, and the minimum preincubation time to cause inhibition of RV14 infection was obtained at 1 day (Fig. 4*A*). When the cells were pretreated with dexamethasone (1 μM) for 3 days and dexamethasone was then removed, the viral titers of RV14 in the supernatants became the same as those in cells without dexamethasone pretreatment 5 days after infection (Fig. 4*B*).

Human tracheal cell viability, assessed by the exclusion of trypan blue, was consistently >96% in the RV-infected and dexamethasone-treated cultures. Likewise, RV14 infection did not alter the amount of lactate

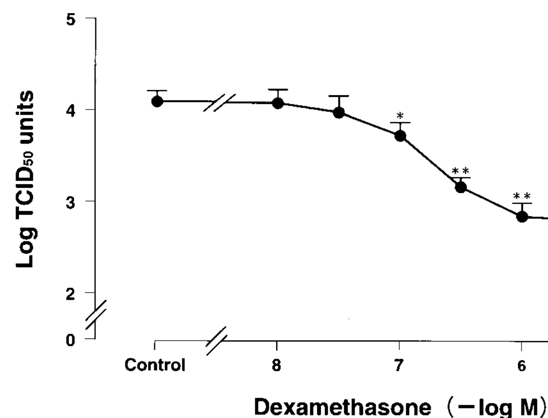


Fig. 3. Concentration-response effects of dexamethasone on RV14 infection in human tracheal epithelial cells. Viral titers in supernatants were collected over 1–3 days after RV14 infection ($10^5$ $TCID_{50}$ units/ml). Results are means ± SE from 7 samples. Significant difference from corresponding control value: * $P < 0.05$; ** $P < 0.01$.

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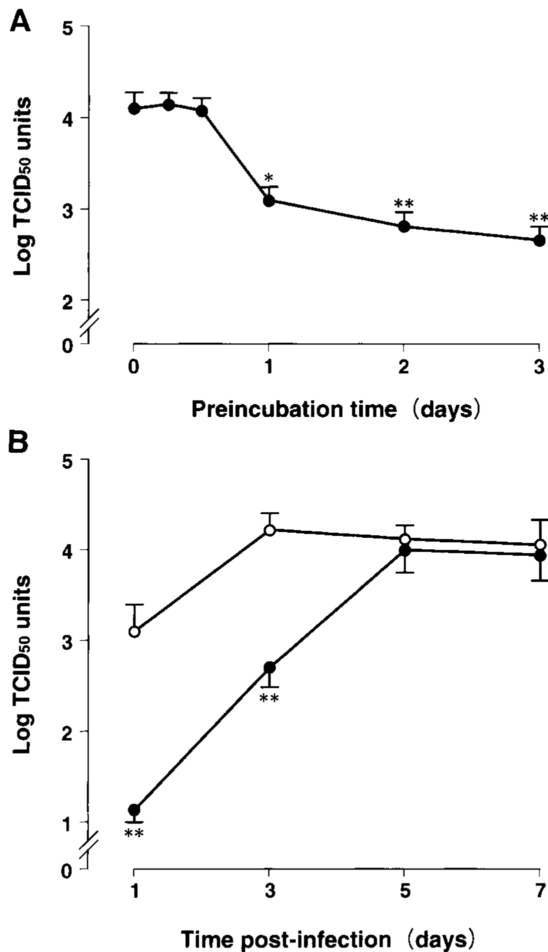


Fig. 4. *A*: effects of preincubation time of dexamethasone (1 μM) on viral titers in supernatants collected over 1–3 days after exposure to $10^5$ $TCID_{50}$ units/ml of RV14. Cells were then cultured in medium containing vehicle for dexamethasone. Results are means ± SE from 7 samples. Significant difference from control value (*day 0*): $*P < 0.05$; $**P < 0.01$. *B*: viral titers in supernatants 1, 3, 5, and 7 days after exposure to $10^5$ $TCID_{50}$ units/ml of RV14 with (●) and without (control; ○) dexamethasone preincubation (1 μM, 3 days). Cells were then cultured in medium containing vehicle for dexamethasone. Results are means ± SE from 7 samples. **Significant difference from corresponding control value, $P < 0.01$.

dehydrogenase in the supernatants (30 ± 3 IU/l before vs. 31 ± 3 IU/l 5 days after infection; $P > 0.50$; $n = 7$). Both RV14 infection ($10^5$ $TCID_{50}$ units/ml, 5 days) and dexamethasone treatment (1 μM; 5 days) also had no effect on cell numbers when RV14 infection and dexamethasone treatment were performed after the cells had made confluent sheets in the tubes. The cell counts after RV14 infection ($1.8 \pm 0.2 \times 10^6$; $P > 0.50$; $n = 7$) and those with dexamethasone treatment ($1.8 \pm 0.1 \times 10^6$; $P > 0.50$; $n = 7$) were not significantly different from those in the control supernatants ($1.8 \pm 0.1 \times 10^6$). Furthermore, dexamethasone did not change the baseline electrical resistance and short-circuit current of the cultured human tracheal epithelial cells. The baseline electrical resistance and short-circuit current in the presence and absence of dexamethasone (1 μM, 3 days) were 152 ± 29 and 145 ± 25 Ω·cm², respectively, and 49 ± 8 and 51 ± 9 μA/cm², respectively ($P > 0.50$ by paired *t*-test; $n = 7$). In contrast, dexamethasone reduced the transient increases in short-circuit current induced by isoproterenol (10 μM) and bradykinin (1 μM) after the addition of amiloride (10 μM). The increases in short-circuit current in response to isoproterenol (10 μM) and bradykinin (1 μM) were 1.4 ± 0.2 and 1.2 ± 0.1 μA/cm², respectively, in dexamethasone compared with 13.2 ± 1.2 and 6.6 ± 0.5 μA/cm², respectively, in the control supernatants ($P < 0.01$ by paired *t*-test; $n = 7$).

*Effects of dexamethasone on viral RNA by PCR.* Further evidence of the inhibitory effects of dexamethasone on RV14 infection and viral replication in human tracheal epithelial cells was provided by PCR analysis (Fig. 5). In each of three experiments, RNA extracted from control uninfected cells did not produce any detectable PCR product at 381 bp (0 h). A 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. Dexamethasone (1 μM) decreased the intensity of the product band of RV14 RNA (Fig. 5).

*Effects of dexamethasone on susceptibility to RV14 infection.* Treatment of the human tracheal epithelial cells with dexamethasone (1 μM) decreased the susceptibility of the cells to RV14 infection, increasing by 10-fold the minimum dose of virus necessary to cause infection (Fig. 6*A*). In contrast to RV14 infection, the minimum dose of RV2 infection was not affected by dexamethasone (1 μM; Fig. 6*B*).

*Effects of dexamethasone on cytokine production.* Because both viral infection and dexamethasone treatment did not alter cell numbers (see *Effects of glucocorticoids on RV infection of human tracheal epithelial cells*), all cytokine values are reported in picograms per milliliter of supernatant. Figure 7 shows that secretion of IL-1β, IL-6, IL-8, and TNF-α all increased in response to RV14. However, the increases in cytokine production in cells with UV-inactivated RV14 infection were similar to those in cells with a sham infection. Dexamethasone decreased RV14 infection-induced increases in the production of IL-1β, IL-6, IL-8, and

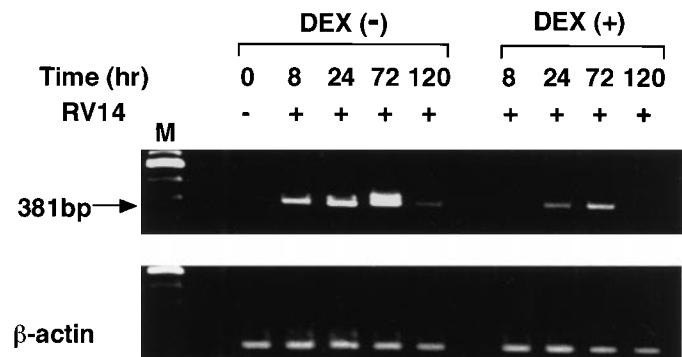


Fig. 5. Time course of replication of rhinovirus RNA from human tracheal epithelial cells after RV14 infection in presence of dexamethasone [DEX (+); 1 μM] or vehicle for dexamethasone [control; DEX (−)] as detected by RT-PCR. β-Actin was used as a housekeeping gene. M, ΦX174-*Hinc* II fragment molecular-size marker. Data are representative of 3 different experiments.

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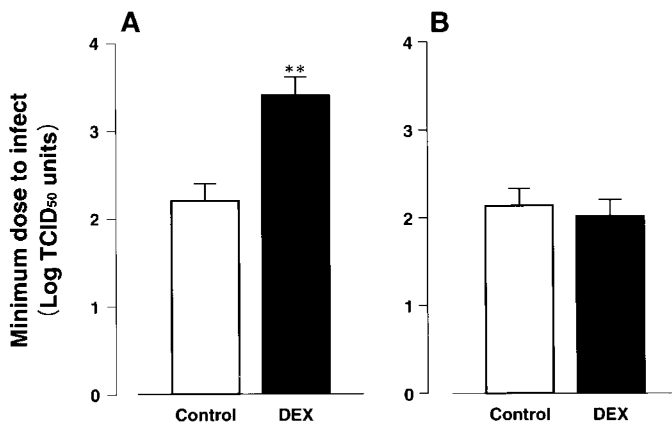


Fig. 6. Effects of DEX (1 μM) on susceptibility to infection by RV14 (*A*) or RV2 (*B*) in human tracheal epithelial cells. Results are means ± SE from 7 samples. **Significant difference from viral infection alone (control): $P < 0.01$.

TNF-α as well as in basal secretions of IL-1β and IL-8 (Fig. 7). In contrast, RV14 infection did not alter IL-1α production (13 ± 2 pg/ml 3 days after RV14 infection vs. 12 ± 2 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 RV14 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 dexamethasone on ICAM-1 expression.* The baseline expression of ICAM-1 mRNA was constant in confluent human tracheal epithelial cell sheets, and the coefficient of variation was small (8.8%; $n = 19$). Neither smoking habits nor cause of death influenced the baseline expression of ICAM-1 mRNA. Exposure of the cells to RV14 caused increases in ICAM-1 mRNA in the absence of dexamethasone (Fig. 8*A*). Human tracheal epithelial cells 3 days after RV14 infection were shown to overexpress ICAM-1 mRNA twofold compared with that in cells 3 days after a sham exposure (control; Fig. 8*C*). Dexamethasone (1 μM) inhibited the increases in ICAM-1 mRNA induced by RV14 infection (Fig. 8*B*) and significantly decreased ICAM-1 mRNA 3 days after RV14 infection as well as the baseline ICAM-1 mRNA expression (Fig. 8*C*). In contrast, dexamethasone did not inhibit β-actin mRNA before and after RV14 infection.

The monoclonal mouse anti-human IL-1β antibody (10 μg/ml) significantly inhibited ICAM-1 mRNA expression induced by RV14 infection in human tracheal epithelial cells. The levels of ICAM-1 mRNA were 0.35 ± 0.03 scan units in control cells and 0.24 ± 0.02 scan units in anti-human IL-1β antibody on *day 1* ($P < 0.05$; $n = 7$), 0.41 ± 0.03 scan units in control cells and 0.25 ± 0.03 scan units in anti-human IL-1β antibody on *day 3* ($P < 0.05$; $n = 7$), and 0.48 ± 0.03 scan units in control cells and 0.28 ± 0.02 scan units in anti-human IL-1β antibody on *day 5* ($P < 0.01$; $n = 7$). In contrast, neither the mouse anti-human TNF-α (10 μg/ml) nor mouse IgG1 control (10 μg/ml) monoclonal antibody altered ICAM-1 mRNA expression. The levels of ICAM-1 mRNA in the anti-human TNF-α and control antibodies were 0.36 ± 0.04 and 0.35 ± 0.04 scan units on *day 1*, 0.41 ± 0.04 and 0.40 ± 0.04 scan units on *day 3*, and 0.50 ± 0.04 and 0.49 ± 0.04 scan units on *day 5*, respectively ($P > 0.50$; $n = 7$).

Expression of ICAM-1 was also assayed by flow cytometric analysis. Human tracheal epithelial cells 3 days after RV14 infection were shown to increase ICAM-1-specific fluorescence intensity compared with

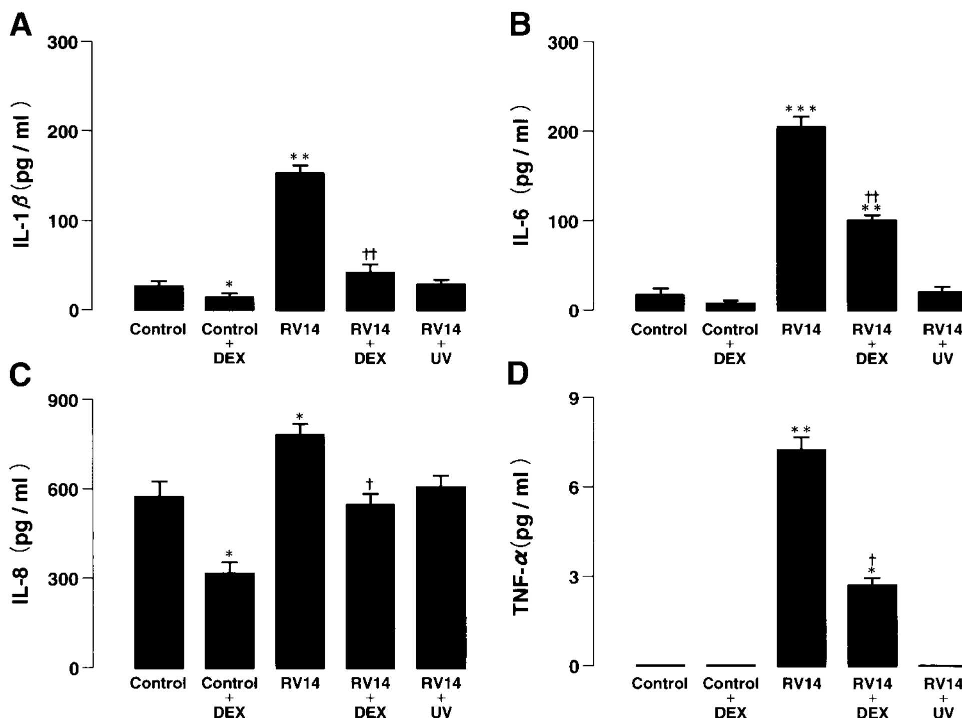


Fig. 7. Effect of DEX (1 μM) on release of cytokines [interleukin (IL)-1β (*A*), IL-6 (*B*), and IL-8 (*C*), and tumor necrosis factor (TNF)-α (*D*)] into supernatants after RV14, ultraviolet (UV)-inactivated RV14, or sham (control) infection. Effects of DEX were examined at maximal production of each cytokine after RV14 infection. Results are means ± SE from 7 samples. Significant difference from corresponding control value: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Significant difference from RV14 infection alone: $\dagger P < 0.05$ and $\dagger\dagger P < 0.01$.

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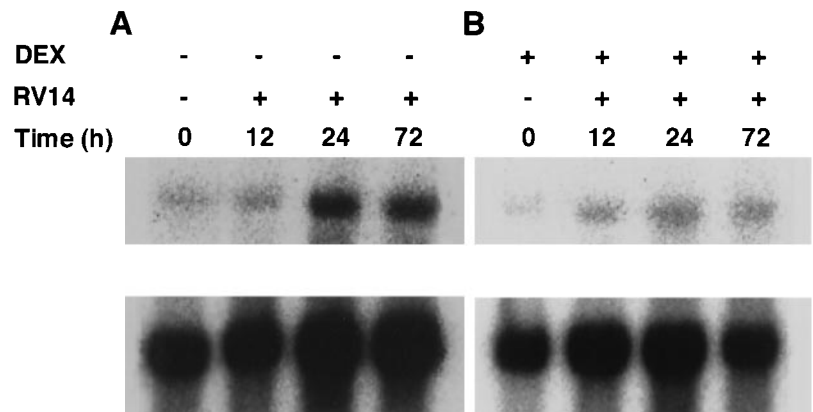
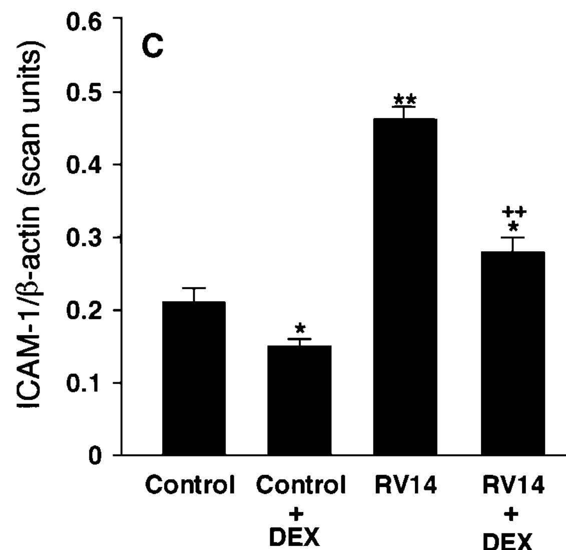



Fig. 8. Northern blot analysis for intercellular adhesion molecule (ICAM)-1 mRNA levels in human tracheal epithelial cells before (0) and 12, 24, and 72 h after RV14 infection in absence (*A*) and presence (*B*) of DEX (1 μM). β-Actin was used as a housekeeping gene. C: effects of DEX (1 μM) on expression of ICAM-1 mRNA in human tracheal epithelial cells 3 days after RV14 or sham (control) infection. ICAM-1 mRNA was normalized to a constitutive expression of β-actin mRNA. Results are means ± SE from 7 samples. Significant difference from corresponding control value: $*P < 0.05$; $**P < 0.01$. ++Significant difference from RV14 alone, $P < 0.01$.




that in cells 3 days after a sham exposure (Fig. 9, *A*, *B*, and *E*). Dexamethasone (1 μM) decreased the baseline ICAM-1-specific fluorescence intensity (Fig. 9, *C* and *E*) and reversed increases in the intensity induced by RV14 infection (Fig. 9, *D* and *E*). The degree of reduction in ICAM-1-specific fluorescence intensity by dexamethasone was larger after than before RV14 infection in the human tracheal epithelial cells (72 ± 6% after RV14 infection vs. 43 ± 4% before RV14 infection; $P < 0.05$ by paired *t*-test; $n = 7$; Fig. 9*E*).

## DISCUSSION

The present study suggests that dexamethasone inhibits RV14 infection by reducing the surface expression of ICAM-1 in cultured human tracheal epithelial cells. These conclusions are based on the observation that dexamethasone reduces the titers of RV14 in the culture medium in a concentration-dependent fashion, reduces the cell-associated viral titer and viral RNA in cultured human tracheal epithelial cells, and decreases the susceptibility of cells to RV14 infection, increasing by 10-fold the minimum dose of virus necessary to cause infection (29). Furthermore, dexamethasone does not alter infection of RV2, a minor group of RVs that does not use ICAM-1 as its receptor (10). The minimum preincubation time of dexamethasone to cause inhibition of RV14 infection in the present study is coincident with the preincubation time of dexamethasone decreasing ICAM-1 protein levels in monocytic U-937 cells (32). Finally, when the cells were preincubated with dexamethasone for 3 days and dexamethasone was then removed from the culture medium after RV14 infection, the viral titers in the supernatant became the same on *day 5* after RV14 infection as the titers without any dexamethasone treatment. Therefore, the mechanism by which dexamethasone inhibits RV14 infection in human tracheal epithelial cells is most likely via reducing the expression of ICAM-1, a surface receptor for a major group of RVs (9), on the cells. Although the effects of glucocorticoid on the replication of RVs have not been well studied, glucocorticoid enhances replication of the mouse mammary tumor virus, an RNA-containing virus, at the level of viral RNA transcription (21). Neither dexamethasone nor hydrocortisone altered RV2 release into culture supernatants in the present study, suggesting that glucocorticoid does not influence RV2 replica-

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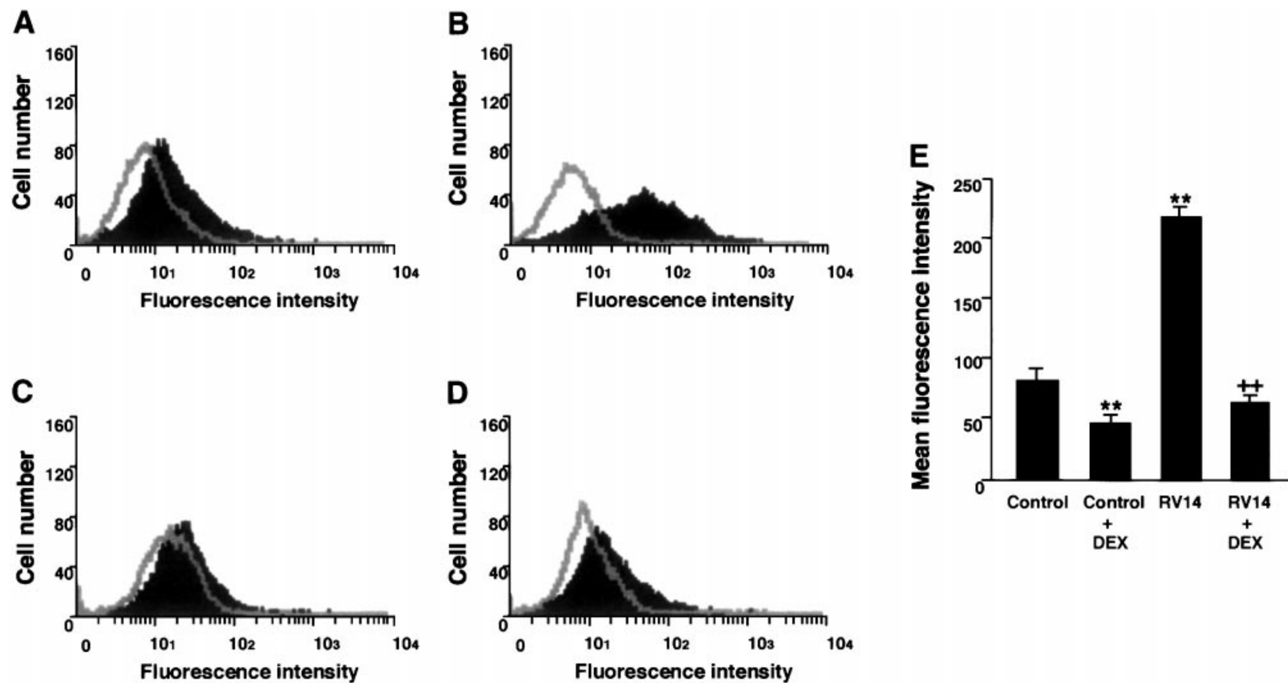


Fig. 9. Flow cytometry analysis demonstrating increases in ICAM-1 expression in human tracheal epithelial cells 3 days after RV14 (*B*) or sham (*A*) infection and inhibition of ICAM-1 expression 3 days after RV14 (*D*) or sham (*C*) infection by DEX (1 μM). *E*: effects of DEX on ICAM-1 fluorescence intensity in human tracheal epithelial cells 3 days after RV14 or sham infection. Results are means ± SE from 7 samples. **Significant difference from sham infection, $P < 0.01$. ++Significant difference from RV14 infection alone, $P < 0.01$.

tion in human tracheal epithelial cells. However, it is not ruled out that dexamethasone may affect replication of RV14 in human tracheal epithelial cells. Further study is needed to examine the precise mechanisms.

Epithelial cells in the human airway express ICAM-1 on their surface, which is the site of attachment for 90% of the ~100 RV serotypes (9, 27). ICAM-1 interacts physiologically with leukocyte function-associated antigen-1, expressed on leukocytes, and thus plays a vital role in the recruitment and migration of immune effector cells to sites of local inflammation. Recent studies (20, 31) have shown that RV infection upregulates ICAM-1 expression on airway epithelial cells, an effect that would facilitate viral cell attachment and entry. Increases in ICAM-1 expression on human tracheal epithelial cells induced by RV14 infection in the present study are in accord with those previous studies.

RV infection induces the local production of cytokines, known to mediate the acute-phase reactions of airway inflammation (29, 31, 35). These epithelial cell-derived mediators affect epithelial cell expression of ICAM-1 in an autocrine/paracrine fashion. For example, treatment of bronchial epithelial cell-conditioned medium with antibodies to TNF-α and IL-1β, but not antibodies to IL-8 and regulated on activation normal T cells expressed and secreted (RANTES), reduced their ability to increase ICAM-1 expression and inflammatory cell adherence (1). IL-8 has also been implicated in the autocrine/paracrine enhancement of ICAM-1 expression on colonic epithelial cells (14). In the present study, we observed that RV14 infection increased production of IL-1β, IL-6, IL-8 and TNF-α in cultured human tracheal epithelial cells. Of these, IL-1β, but not TNF-α, upregulated ICAM-1 expression in cultured human tracheal epithelial cells as previously reported (31). A lack of TNF-α-induced effects on ICAM-1 expression may be due to a small amount of TNF-α production from human tracheal epithelial cells in response to RV14 infection.

Glucocorticoid therapy effectively relieves asthmatic airway inflammation and inhibits inflammatory cytokine production in the cells of the lung (4). Glucocorticoids are shown to inhibit granulocyte-macrophage colony-stimulating factor expression in both human tracheal epithelial cells and human lung tissue (6, 13) and repress IL-1β-induced granulocyte-macrophage colony-stimulating factor production (2) and TNF-α-mediated secretion of IL-6 (15) in BEAS-2B cells. Likewise, glucocorticoids inhibit IL-1β-induced induction of ICAM-1 in A549 human adenocarcinoma cells (23). The present study also shows that dexamethasone inhibits production of IL-1β, IL-6, IL-8 and TNF-α as well as of ICAM-1 expression induced by RV14 infection in primary cultures of human tracheal epithelial cells. Because an antibody to IL-1β, but not an antibody to TNF-α, repressed RV14-induced increases in ICAM-1 expression, the inhibitory effects of dexamethasone on ICAM-1 expression might be partly mediated by a reduction in IL-1β synthesis.

We observed that dexamethasone inhibited baseline and RV infection-induced production of proinflammatory cytokines and ICAM-1. The expression of genes for many cytokines and ICAM-1 induced by RV infection are suggested to be mediated by the activation of

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nuclear factor-κB (3, 20, 35). Glucocorticoids activate their receptors that may then bind to activated nuclear factor-κB and prevent it from binding to κB sites on genes that have a role in inflammatory processes (3). Therefore, in the present study, dexamethasone might reduce cytokine production after RV infection by inhibiting RV infection via the reduction of its receptor, ICAM-1. It is also possible that dexamethasone might further inhibit the production of cytokines and ICAM-1 induced by RV infection via its inhibitory effects on the production of cytokines and ICAM-1 (3, 15, 23).

In the present study, dexamethasone treatment did not change the baseline electrical resistance and short-circuit current but inhibited increases in short-circuit current induced by isoproterenol and bradykinin. These findings are consistent with previous studies (16, 25) showing that glucocorticoid does not affect baseline $Na^+$ absorption and $Cl^-$ secretion but inhibits fluid and mucus secretion in response to mediators in airway epithelial cells.

Pretreatment with UV irradiation not only affected RV replication but also completely abolished cytokine production, including IL-1β, in the present study. UV irradiation blocks RV replication but not the capability to enter the cells (11). In a previous report by Terajima et al. (31), monoclonal antibodies to ICAM-1 completely blocked RV14 infection when human tracheal epithelial cells were exposed to RV14 for 15 min. We observed that monoclonal antibodies to ICAM-1 completely blocked IL-1β induction when cells were exposed to RV14 for 15 min (data not shown). However, when the epithelial cells were exposed to RV14 for 60 min, the inhibitory effects of monoclonal antibodies to ICAM-1 on RV14 titer and IL-1β production in the culture medium were significant but partial (31). Our results that UV irradiation abolished RV14 infection-induced cytokine production is consistent with a previous report (29) that UV irradiation reduced RV14-induced IL-8 production by 80% in the respiratory epithelial cell line BEAS-2B. Therefore, replication as well as entry of RV14 may be needed for induction of IL-1β production in human tracheal epithelial cells, although the precise mechanism of IL-1β induction after RV14 infection is still uncertain.

A randomized controlled trial of glucocorticoid prophylaxis against experimental RV infection in healthy subjects shows that the trend toward less of an increase in nasal obstruction, middle ear pressure, mucus weight, and nasal mucus kinin and albumin concentrations during the first 2 days after viral inoculation in the steroid group was temporally related to the simultaneous administration of oral prednisone and intranasal dexamethasone (7). However, there was no difference in the rate of infection or the level of viral shedding between patients receiving glucocorticoids or a placebo (7). A further study is therefore needed to clarify an antiviral action of glucocorticoids in RV infection in vivo.

In the present study, dexamethasone reduced the viral titers of RV14 but not of RV2 in the cell lysates and culture supernatants of human tracheal epithelial cells. Hydrocortisone also reduced the viral titers of RV14 but not of RV2 in culture supernatants and cell lysates that did not contain significant levels of hydrocortisone. We found that neither dexamethasone nor hydrocortisone affects RV titration using cytopathic effects on human embryonic fibroblasts. Furthermore, dexamethasone reduced RV14 RNA content in the cells. These findings suggest that glucocorticoid may inhibit infection of RV14 but not of RV2 in human tracheal epithelial cells.

In summary, dexamethasone may decrease the susceptibility of RV14 infection to cultured human tracheal epithelial cells via inhibition of ICAM-1 induction. Dexamethasone-induced inhibition of cytokine production and ICAM-1 induction may modulate airway inflammation after RV infection.

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