Levofloxacin inhibits rhinovirus infection in primary cultures of human tracheal epithelial cells

by

Mutsuo Yamaya,1 Hidekazu Nishimura,2 Yukimasa Hatachi,3 Hiroyasu Yasuda,4 Xue Deng,4 Takahiko Sasaki,5 Katsumi Mizuta,6 Hiroshi Kubo,1 Ryoichi Nagatomi7

1Department of Advanced Preventive Medicine for Infectious Disease, Tohoku University Graduate School of Medicine, 2Virus Research Center, Clinical Research Division, Sendai National Hospital, Sendai 983-8520, 3Department of Respiratory Medicine, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, 4Department of Innovation of New Biomedical Engineering Center, Tohoku University, 980-8574, 5Department of Respiratory Medicine, Tohoku University School of Medicine, 6Department of Microbiology, Yamagata Prefectural Institute of Public Health, Yamagata 990-0031, and 7Department of Medicine and Science in Sports and Exercise, Tohoku University School of Medicine, Sendai 980-8575, Japan

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Send correspondence to: Mutsuo Yamaya, MD, PhD
Professor and Chairman
Department of Advanced Preventive Medicine for Infectious Disease,
Tohoku University Graduate School of Medicine,
2-1 Seiryo-machi, Aoba-ku, Sendai, 980-8575, Japan
TEL: 81-22-717-7182
FAX: 81-22-717-7576
E-mail: myamaya@med.tohoku.ac.jp
Respiratory virus infections, including infection with rhinoviruses (RVs), relate to the exacerbation of chronic obstructive pulmonary disease (COPD). A new quinolone antibiotic, levofloxacin (LVFX), has been used to treat bacterial infections that cause COPD exacerbation as well as bacterial infections that are secondary to viral infection in COPD patients. However, the inhibitory effects of LVFX on RV infection and RV infection-induced airway inflammation have not been studied. We examined the effects of LVFX on type 14 rhinovirus (RV14, a major human RV) infection of human tracheal epithelial cells pretreated with LVFX. LVFX pretreatment reduced the RV14 titer and the level of cytokines in the supernatant, the amount of RV14 RNA in the cells after RV14 infection, and the cells’ susceptibility to RV14 infection. LVFX pretreatment decreased the mRNA level of intercellular adhesion molecule (ICAM)-1, a receptor for RV14, in the cells and the concentration of the soluble form of ICAM-1 in the supernatant before RV14 infection. LVFX pretreatment also decreased the number and the fluorescence intensity of the acidic endosomes from which RV14 RNA enters into the cytoplasm. LVFX pretreatment inhibited the activation of nuclear factor kappa-B proteins, including p50 and p65, in nuclear extracts. LVFX pretreatment did not reduce the titers of RV2 (a minor human RV) but reduced the titers of RV15 (a major human RV). These results suggest that LVFX inhibits major group rhinovirus infections in part by reducing ICAM-1 expression and the number of acidic endosomes. LVFX may also modulate airway inflammation in rhinoviral infections.

Key words: new quinolone; human tracheal epithelial cell; rhinovirus; intercellular adhesion molecule; acidic endosome; pro-inflammatory cytokine
Rhinoviruses (RVs) are the main cause of the common cold, and they are responsible for the most common acute infectious illness in humans (41). In addition, RVs are associated with the exacerbation of inflammatory chronic pulmonary diseases, such as chronic obstructive pulmonary disease (COPD) (30). New quinolone antibiotics, such as levofloxacin (LVFX), have clinical benefits in the treatment of COPD exacerbation, including a longer infection-free period and a reduction in hospitalizations after treatment compared with treatment with other antibiotics (7, 28, 34). Several reasons have been suggested for the clinical effects of quinolone antibiotics, including a high serum concentration of the drug that far exceeds the minimal inhibitory concentration (MIC) (10), a broader antibiotic spectrum (4), and anti-inflammatory properties (40). However, the inhibitory effects of new quinolone antibiotics on RV infections and on RV infection-induced airway inflammation have not been studied.

Several mechanisms for the RV-induced exacerbation of COPD have been proposed, including virus-induced mucus hypersecretion, airway inflammation (30), and smooth muscle contraction. RV infection induces the production of cytokines and monokines, including interleukin (IL)-1, IL-6, and IL-8 (33, 48). These cytokines and monokines have pro-inflammatory effects (1), and they may also be involved in the pathogenesis of RV infections and RV infection-induced exacerbation of COPD. LVFX pretreatment reduces lipopolysaccharide (LPS)-induced IL-1β production in a murine macrophage-like cell line (AW264.7 cells) (14) and reduces IL-6 and IL-8 production in a human lung epithelial cell line (40). However, the inhibitory effects of new quinolone antibiotics on RV infection-induced airway inflammation have not been well studied.

Type 14 rhinovirus (RV14, a major human RV) enters the cytoplasm of infected
cells after binding to the receptor known as intercellular adhesion molecule (ICAM)-1 (6, 11). The entry of the RNA from this group of RVs into the cytoplasm of infected cells is suggested to be mediated by destabilization of cell membrane due to ICAM-1 binding. Furthermore, the entry of the RNA into the cytoplasm is mediated by endosomal acidification when the virions enter the cell via endosomes before they enter the cytoplasm (6). Glucocorticoids (37), the macrolide antibiotics bafilomycin (25, 35) and erythromycin (36), the proton pump inhibitor lansoprazole (29), and the β2 agonist procaterol (43) inhibit RV infection by reducing ICAM-1 expression or increasing endosomal pH. One of the new quinolone antibiotics, ciprofloxacin, inhibits the expression of ICAM-1 by monocytes (19). However, the inhibitory effects of new quinolone antibiotics on the RV infection of human airway epithelial cells are still unclear.

We studied the effects of LVFX on the RV infection of primary cultures of human airway epithelial cells. We also examined the effects of LVFX on ICAM-1 production and on endosomal pH to clarify the mechanisms responsible for the inhibition of RV infection.

**MATERIALS AND METHODS**

**Human tracheal epithelial cell culture**

Human tracheal surface epithelial cells were isolated and cultured as described previously (43). The cells were plated at 5 x 10⁵ viable cells/ml in plastic tubes with round bottoms (16 mm in diameter and 125 mm in length; Becton Dickinson, Franklin Lakes, NJ, USA) that were coated with human placental collagen. The plastic tubes were fixed in an inclined stainless-steel tube rack (30 cm wide, 10 cm high and 10 cm deep, TE-HER TUBE RACK INCLINABLE® RF-6; Hirasawa Works Co. Ltd., Tokyo,
Japan), which was placed in a humid incubator. The tubes were kept stationary, and
the cells were immersed in 1 ml of Dulbecco’s modified Eagle's medium
(DMEM–Ham’s F-12 medium (50/50 vol/vol) containing 2% Ultroser G (USG; Pall
BioSepra, Cergy-Saint-Christophe, France). The cells were then cultured at 37°C in
5% CO2–95% air in an incubator.

To enhance RV14 release from the cells, the tubes containing the cultured cells were
subjected to rolling (41, 43). As described below, the tubes were rolled during
epithelial cell culture (43) to study the effects of LVFX on the release of RV14 and
cytokines. We also assessed RV14 RNA replication after RV14 infection and cellular
susceptibility to RV14 infection. In contrast, acidic endosomes could be observed in
the cells living on coverslips in stationary Petri dishes. Therefore, the cells used for
the measurement of acidic endosomes were cultured under stationary conditions.
Furthermore, to study the effects of LVFX on nuclear factor-kappa B (NF-κB)
activation prior to RV14 infection, cells were cultured in tubes under stationary
conditions (43).

To examine the effects of LVFX on the adhesion and growth of human tracheal
epithelial cells, the cells were placed and cultured in tubes in medium containing 2%
USG supplemented with LVFX (3 μg/ml) or vehicle (water). The concentration of
LVFX was chosen according to the reasons described below (Treatment with LVFX).
Cells were counted 24 h after plating and when the cells made confluent sheets 5-7 days
after plating. We measured the time required for the cells to generate confluent sheets.
We also measured the concentration of lactate dehydrogenase (LDH) in the medium 3
days after the treatment of confluent cell sheets with LVFX (3 μg/ml).

The tracheas from which cells were derived for culturing were obtained after death
from 33 patients (age 71 ± 3 y; 13 female, 20 male). None of the patients had been
diagnosed with bronchial asthma, whereas three of the patients had COPD. None of
the patients were being treated with LVFX at the time of death. Of the 33 patients, 13
were ex-smokers and 20 had never smoked. This study was approved by the Tohoku University Ethics Committee.

Culture of human embryonic fibroblast cells

Human embryonic fibroblast cells (HFL-III cells, Riken Bio Resource Center Cell Bank, Cell No: RCB0523; Tsukuba, Japan) were cultured in flasks (25-cm² surface area; Becton Dickinson) and then plated in plastic dishes (96-well, Becton Dickinson) or in plastic tubes with round bottoms. The cells were then cultured at 37°C in 5% CO₂–95% air (43).

Viral stocks

Stocks of RV14 (a major group RV) and RV2 (a minor group RV) (6, 11, 12) were prepared from a patient with a common cold by infecting human embryonic fibroblast cells as previously described (22, 34). Stocks of RV15 (a major group RV) (39) were also prepared as previously described (18). We used RV stocks that had been passaged 3-5 times.

Detection and titration of viruses

RVs (RV2, RV14 or RV15) in the supernatant (cell-culture medium) were detected and titrated using the endpoint methods (8) by infecting replicate confluent human embryonic fibroblast cells in plastic 96-well plates (Becton Dickinson) with serial 10-fold dilutions of virus-containing supernatants, as previously described (43). The presence of the typical cytopathic effects of RVs was monitored in all replicate cell cultures for 7 days (168 h) (43). On the basis of these data, the TCID₅₀ (tissue culture infective dose) was calculated as previously described (22). The rates of RV release into the supernatant are expressed as TCID₅₀ units/ml/24 h (43).
Quantification of rhinoviral RNA

To quantify the RV14 RNA and ribosomal RNA (18S, rRNA) expression in the human tracheal epithelial cells after RV14 infection, a two-step real-time quantitative reverse transcription (RT)-PCR using the TaqMan technique (Roche Molecular Diagnostic Systems) was performed using the TaqMan® Gene Expression Master Mix (Applied Biosystems, Bedford, CA, USA) and methods described by Nolan et al. (21), as previously reported (43).

In the first step, cDNA was reverse transcribed from RV14 RNA using the QuantiTect Reverse Transcription Kit (Qiagen) and the RV reverse primer 5’-CGGACACCCAAAGTAGTCGGT-3’.

In the second step, real-time PCR was performed using this cDNA and the TaqMan® Gene Expression Master Mix. The cDNA sample (2 μl) was mixed with the TaqMan Gene Expression Master Mix (10 μl), a forward primer (5’-GCACCTTCTGGTTTCCCCAGGAGC-3’; 0.5 μl), a reverse primer (5’-CGGACACCCAAAGTAGTCGGT-3’; 0.5 μl), the Taqman RV14 probe (5’-[FAM]CCTTTAACCGTTATCCGCCA[TAMRA]-3’; 0.5 μl), and RNAase-free water (6.5 μl).

To quantify the rRNA, the conversion of rRNA to cDNA and real-time PCR were performed using the same two-step process described above. To obtain quantitative data, the minimum number of PCR cycles required to detect the fluorescent signal was defined as the cycle threshold (Ct) of RV14 RNA and rRNA from the cells, and quantitative data were obtained as described previously (43).

Viral infection of the epithelial cells

Infection of the human tracheal epithelial cells with RVs (RV2, RV14 or RV15) or vehicle [Minimum Essential Medium (MEM) plus 2% ultra-low γ-globulin calf serum] was performed using previously described methods (34, 43). A stock solution of RVs (100 μl in each tube, 1.0 x 10⁴ TCID₅₀ units/100μl, 5.0 x 10⁻² TCID₅₀ units/cell) was
added to the human tracheal epithelial cells in the tubes (34, 43) except where other
virus doses are indicated. After a 1-h incubation at 33°C, RV14 stocks were aspirated,
the cells were rinsed with phosphate-buffered saline (PBS) and then fed with fresh
medium and cultured at 33°C with rolling.

Treatment with LVFX

The mean peak concentration of LVFX in the alveolar epithelial lining fluid has
been reported to be 3.4 μg/ml at 1 h after oral ingestion of 500 mg LVFX (47).
Therefore, to examine the effects of LVFX, cultured human tracheal epithelial cells
were treated with either LVFX (3 μg/ml = 8.1 x 10⁻⁶M, supplied from Daiichi-Sankyo,
Co., Ltd, Tokyo, Japan) or vehicle (0.1% double distilled water). The cells were
treated with LVFX or vehicle beginning three days (72 h) before infection with RVs
until the end of the experimental period after RV infection (except when other
concentrations or times are listed) as described previously in the reports using other
agents (36, 43).

To examine the effects of LVFX on the RV14 infection when the cells were treated
with LVFX just after RV14 infection, the cells were treated with vehicle (water) before
infection with RV14. After a 1-h exposure to RV14, the cells were rinsed with PBS
and then fed with fresh medium supplemented with LVFX (3 μg/ml, 8.1 x 10⁻⁶M) and
cultured at 33°C with rolling.

Likewise, to examine the time-dependent effects of LVFX on RV14 titers, the cells
were pretreated with LVFX (3 μg/ml) for time periods ranging from 0 to 3 days (72 h)
before RV14 infection.

To examine the concentration-dependent effects of LVFX on RV14 infection, cells
were treated with LVFX at concentrations ranging from 0.01 μg/ml to 10 μg/ml.
To examine the effects of LVFX on ICAM-1 mRNA expression in the cells and the
concentration of the soluble form of ICAM-1 (sICAM-1) in the supernatant, the cells
were pretreated with LVFX (3 μg/ml = 8.1 x 10^{-6}M) or vehicle (water) for 3 days (72 h) before RV14 infection. The supernatants were collected, and RNA was extracted from cells just before RV14 infection.

Collection of supernatants for measurements

In the cells cultured in the tubes, we measured the time course of viral release using previously described methods (43). To measure the release of RVs (RV2, RV14 or RV15) during the first 24 h, we used three separate cultures from each trachea. We collected the supernatants at 1 h, 12 h, or 24 h after RV infection. We also collected supernatants at 3 days (72 h), 5 days (120 h), and 7 days (168 h) after infection. At 1 day (24 h), 3 days (72 h), and 5 days (120 h) after infection, supernatants were collected, cells were rinsed with PBS, the medium was replaced with fresh medium, and the cell culture was continued.

Likewise, to examine the effects of LVFX on the secretion of IL-1β, IL-6, and IL-8, supernatants were collected immediately before infection and then again 1 day (24 h), 3 days (72 h), and 5 days (120 h) after RV14 infection.

Effects of LVFX on susceptibility to rhinovirus infection

The effects of LVFX on the cellular susceptibility to RV14 infection were evaluated as previously described (43). Epithelial cells were pretreated with LVFX (3 μg/ml) or vehicle for 3 days (72 h) before infection. The epithelial cells were exposed to serial 10-fold dilutions of RV14 at doses ranging from 10^1 to 10^5 TCID_{50} units/ml in media containing LVFX or vehicle for 1 h at 33°C. After exposure to RV14, fresh medium with no LVFX was added. The cells in the tubes were then cultured at 33°C with rolling.

We collected the supernatants 1 day (24 h) and 3 days (72 h) after RV14 infection and measured the RV14 titers in the supernatants with the human embryonic fibroblast
cell assay described above to assess whether infection occurred at each dose (10^1, 10^2, 10^3, 10^4, and 10^5 TCID_{50} units/ml) of RV14 (43).

Measurement of ICAM-1 expression
The level of ICAM-1 mRNA was examined using two-step real-time RT-PCR analysis using the methods described above (Quantification of rhinoviral RNA), with a forward primer designed for ICAM-1 (43). The concentration of sICAM-1 in the supernatants was measured with an enzyme immunoassay (EIA) (43).

Measurement of changes in acidic endosomes
The distribution and the fluorescence intensity of acidic endosomes in the cells were measured, as previously described, with the LysoSensor DND-189 dye (Molecular Probes, Eugene, OR, USA) (35). Live-cell imaging was performed by observing cells on coverslips in Petri dishes with a fluorescence microscope (OLYMPUS IX70; OLYMPUS Co. Ltd., Tokyo, Japan). The fluorescence intensity was calculated using a fluorescence image analyzer system (Lumina Vision®; Mitani Co. Ltd., Fukui, Japan) equipped with a fluorescence microscope. The fluorescence intensity of the acidic endosomes was measured in 100 human tracheal epithelial cells, and the mean value of the fluorescence intensity was expressed as the percentage of the control value compared with the fluorescence intensity of the cells before any treatment.

We studied the effects of a long period of treatment with LVFX (3 μg/ml, 72 h) on acidic endosomes because the cells were pretreated with LVFX or vehicle (water) for 3 days (72 h) before RV14 infection.

Measurement of cytokine production
We measured IL-1β, IL-6, and IL-8 levels in supernatants with specific enzyme-linked immunosorbent assays (ELISAs) (43) in duplicate human tracheal...
epithelial cells in plastic tubes at all time points mentioned above. The concentrations of IL-1β, IL-6, and IL-8 in the supernatants of the cells were measured before and after infection with RV14 or exposure to culture medium for RV14 stocks (MEM supplemented with 2% ultra-low γ-globulin calf serum) in the presence of LVFX. We also measured the concentrations of IL-1β, IL-6, and IL-8 in the supernatants of the cells that were exposed to ultraviolet-inactivated RV14 or vehicle of RV14 stocks.

NF-κB assay

Nuclear extracts from human tracheal epithelial cells were prepared using a TransFactor extraction kit (BD Bioscience/CLONTECH, Mountain View, CA, USA). The presence of p50, p65, and c-Rel subunits was assayed using a TransFactor Family Colorimetric Kit-NF-κB (BD Bioscience/CLONTECH) according to the manufacturer’s instructions, as previously described (43). The results were expressed in terms of optical density (OD), which provides the quantitative levels of the NF-κB subunits (43).

The cells were treated with LVFX (3 μg/ml) or vehicle for 3 days. To adjust the data for cell numbers, we counted the number of cells that were cultured in the tubes. We found that there were only small differences in cell numbers in each tube from the same tracheae (data not shown). We also counted cells to monitor the cell number in the NF-κB experiment using five different tracheae. We found that the cell number was between 1.9 x 10^6 and 2.3 x 10^6 per tube in five different tracheae. To adjust the data for cell numbers, we calculated the OD according to 1.9 x 10^6 cells.

Statistical analysis

The results are expressed as the mean ± SEM. Statistical analysis was performed using a one-way analysis of variance (ANOVA). Subsequent post-hoc analysis was performed using Bonferroni’s method. For all analyses, values of P<0.05 were assumed to be significant. The term “n” refers to the number of donors (tracheae).
from which cultured epithelial cells were obtained.
RESULTS

Effects of LVFX on rhinoviral infection in human tracheal epithelial cells

Exposing confluent human tracheal epithelial cell monolayers to RV14 (5.0 x 10^{-2} TCID_{50} units/cell) consistently led to infection. No virus was detected at 1 h after infection; however, RV14 was detected in the supernatants (culture medium) at 12 h, and the viral content progressively increased between 1 and 12 h after infection (Fig. 1A). Evidence of continuous viral production was obtained by demonstrating that each of the supernatants collected from 12 h to 24 h (1 day), 1 day (24 h) to 3 days (72 h), 3 days (72 h) to 5 days (120 h), or 5 days (120 h) to 7 days (168 h) after infection contained significant levels of RV14 (Fig. 1A). The viral titers in supernatants increased significantly with time for the first 3 days (72 h) (\(P<0.05\) by ANOVA).

Furthermore, in the tracheal cells from subjects whose cells were infected with RV14, the supernatants collected from 1 (24 h) to 3 days (72 h) after infection contained consistent levels of RV14 (4.49 ± 0.15 log TCID_{50} units/ml/24 h, n=33).

Pretreatment of the cells with LVFX (3 \(\mu\)g/ml = 8.1 x 10^{-6}M) resulted in significantly lower viral titers of RV14 in the supernatants from 12 h after infection (Fig. 1A) and reduced RV14 titers in the supernatants in a concentration-dependent manner (Fig. 1B). Pretreatment of the cells with LVFX reduced viral titers of RV14 in supernatants at concentrations of 0.5 \(\mu\)g/ml or greater (Fig. 1B).

The inhibitory effects of LVFX on the RV14 titers were time-dependent. The maximum inhibitory effect was obtained when the cells were pretreated with LVFX for 3 days (72 h) (Fig. 1C). Significant inhibitory effects were observed even when the cells were treated with LVFX (3 \(\mu\)g/ml) just after RV14 infection (Fig. 1C).

Pretreatment of the cells with LVFX (3 \(\mu\)g/ml) did not reduce the viral titers of RV2 in supernatants (Fig. 1D). In contrast, pretreatment of the cells with LVFX (3 \(\mu\)g/ml) reduced viral titers of RV15 in the supernatants (Fig. 1D).
RV14 titers in the supernatants collected from the cells of the 13 ex-smokers from 1 day (24 h) to 3 days (72 h) after infection did not differ from those of the 20 patients who had never smoked (4.49 ± 0.13 log TCID$_{50}$ units/ml/24 h vs. 4.51 ± 0.15 log TCID$_{50}$ units/ml/24 h, respectively, $P>0.02$). Likewise, the RV14 titers in the supernatants from the three patients with COPD did not differ from those of the 30 patients without COPD (data not shown). No virus was detected in the supernatants after infection with ultraviolet (UV)-inactivated RV14 (data not shown).

To measure the effects of LVFX on cell attachment, the human tracheal epithelial cells were placed in tubes containing the medium supplemented with LVFX (3 μg/ml) or vehicle (water). The number of cells that attached to the culture vessels was then counted 24 h later. The number of the cells treated with LVFX was the same as the number of the cells treated with the vehicle (1.51 ± 0.1 x 10$^5$ in LVFX vs. 1.49 ± 0.1 x 10$^5$ in vehicle, n=3) after initially placing 5 x 10$^5$ cells in each tube. Pretreatment with LVFX (3 μg/ml) for 3 days (72 h) did not change cell viability (99 ± 1% in LVFX vs. 98 ± 1% in vehicle, n=5, $P>0.50$), as assessed by the trypan blue exclusion assay.

Furthermore, until 7 days (168 h) after the start of cell culture, LVFX- and vehicle-treated cells made confluent cell sheets in the same amount of time following their placement in tubes (data not shown). The cell numbers in the confluent sheets cultured in the medium supplemented with LVFX (3 μg/ml) did not differ from cell counts in the unsupplemented medium (2.1 ± 0.3 x 10$^6$ of cells/tube in LVFX vs. 2.2 ± 0.3 x 10$^6$ of cells/tube in vehicle, n=5, $P>0.50$). Pretreatment with LVFX (3 μg/ml) for 3 days (72 h) did not alter the lactate dehydrogenase (LDH) concentration (29 ± 2 IU/l/24 h in LVFX vs. 31 ± 3 IU/l/24 h IU/ml in vehicle, n=5, $P>0.50$) in the supernatants collected 3 days (72 h) after LVFX treatment.

**Effects of LVFX on viral RNA replication**

Further evidence of the inhibitory effects of LVFX on RV14 RNA replication in
human tracheal epithelial cells was provided by real-time RT-PCR analysis. The RNA extraction was performed at 1 day (24 h), 3 days (72 h) and 5 days (120 h) after RV14 infection. RV14 RNA was consistently observed in the cells from 1 day (24 h) after infection onwards, and the levels increased with time after infection (Fig. 2). In preliminary experiments, the maximum level of RV14 RNA replication was observed at 3 days (72 h) after infection (data at 120 h not shown), whereas RV14 RNA was not observed in the cells prior to infection (data not shown). Pretreatment with LVFX (3 μg/ml) resulted in a lower level of RV14 RNA at 1 day (24 h) and 3 days (72 h) after infection (Fig. 2). Detectable amounts of RV14 RNA were not observed in the cells after infection with ultraviolet-inactivated RV14 (Fig. 2).

Effects of LVFX on susceptibility to rhinovirus infection

Pretreatment of the cells with LVFX (3 μg/ml) decreased the susceptibility of the cells to infection by RV14. When viral release was measured in supernatants collected 3 days (72 h) after RV14 infection, the minimum dose of RV14 necessary to cause infection of the cells treated with LVFX (3.2 ± 0.3 log TCID$_{50}$ units/ml, n=5) was significantly higher than that for the cells treated with the vehicle alone (2.1 ± 0.2 log TCID$_{50}$ units/ml, n=5, P<0.05).

Effects of LVFX on the expression of ICAM-1

LVFX (3 μg/ml, 72 h) reduced baseline ICAM-1 mRNA expression in the cells by approximately 50% compared with the levels in the cells treated with the vehicle alone prior to RV14 infection (Fig. 3A). Furthermore, the concentrations of sICAM-1 in the supernatants from cells treated with LVFX (3 μg/ml) were significantly lower than those from the cells treated with the vehicle alone prior to RV14 infection (Fig. 3B).

Effects of LVFX on the acidification of endosomes
Acidic endosomes in human tracheal epithelial cells were stained green with LysoSensor DND-189 (Fig. 4A-4C) as described previously (43). Treatment with the vehicle for 3 days (72 h) did not change the number of fluorescent acidic endosomes in the cells (Fig. 4B) or the fluorescence intensity of the acidic endosomes compared with the cells prior to any treatment (Fig. 4D). In contrast, treatment with LVFX (3 μg/ml, 72 h) reduced the number of acidic endosomes showing green fluorescence (Fig. 4C) and also reduced the fluorescence intensity of the acidic endosomes compared with measurements of cells treated with vehicle and compared to cells prior to any treatment (Fig. 4D).

**Effects of LVFX on cytokine production**

LVFX (3 μg/ml) pretreatment reduced the baseline secretion of IL-1β, IL-6, and IL-8 for 24 h before RV14 infection compared with the secretion levels of cells treated with vehicle alone (Fig. 5). RV14 infection increased the secretion of IL-1β, IL-6, and IL-8. The maximum secretion was observed at 1 day (24 h) after RV14 infection for IL-6 and IL-8 and peaked at 3 days (72 h) after infection for IL-1β. LVFX (3 μg/ml) also reduced the RV14 infection-induced secretion of IL-1β, IL-6, and IL-8 compared with the secretion levels in the cells treated with vehicle alone (Fig. 5). Exposure to culture medium used for RV14 stocks (vehicle of RV14 stock; MEM supplemented with 2% ultra-low γ-globulin calf serum) and infection with ultraviolet-inactivated RV14 did not change the concentrations of IL-1β, IL-6, and IL-8 in the supernatants (Fig. 5).

The secretion levels of IL-1β, IL-6, and IL-8 in the supernatants of the cells from the four ex-smokers did not differ from the levels secreted by cells from the four patients who had never smoked (data not shown). Likewise, the secretion of IL-1β, IL-6, and IL-8 in the supernatants of the cells from three patients with COPD did not differ from the levels secreted by cells from the 30 patients without COPD (data not shown).
Effects on NF-kappa B

LVFX (3 μg/ml, 72 h) treatment significantly reduced the levels of the p50, p65, and c-Rel subunits of NF-κB in the nuclear extracts of cells cultured under stationary conditions prior to RV14 infection compared with the NF-κB levels in the cells treated with vehicle alone (Figs. 6A-6C).
DISCUSSION

In the present study, we have shown that the new quinolone antibiotic levofloxacin (LVFX) reduced the titers of a major group rhinovirus (RV), RV14, in supernatants and reduced the RNA replication of the virus in primary cultures of human tracheal epithelial cells. Pretreatment with LVFX reduced the mRNA and protein expression levels of ICAM-1, the receptor for RV14 (6, 11), prior to RV14 infection. The minimum dose of RV14 necessary to cause infection of the cells treated with LVFX was significantly higher than that for the cells treated with the vehicle alone. Pretreatment of the cells with LVFX also reduced the titers of RV15, one of the major group RVs (39), but did not reduce the titers of RV2, a minor group RV (12). These findings suggest that LVFX might inhibit infection by major group RVs, including RV14 and RV15, in part through reducing the production of its receptor, ICAM-1.

Major human RVs, including RV14 and RV15, enter the cytoplasm of infected cells after binding to the receptor known as intercellular adhesion molecule (ICAM)-1 (6, 11, 39). The entry of the RNA from this group of RVs into the cytoplasm of infected cells is suggested to be mediated by destabilization of cell membrane due to ICAM-1 binding. Furthermore, the entry of the RNA into the cytoplasm is mediated by endosomal acidification, which occurs when the virions enter the cell via endosomes before they enter the cytoplasm (6). In contrast, a minor group RV, RV2, enters the cytoplasm of infected cells after binding to its receptor, a low density lipoprotein (LDL) receptor (12). The entry of RV2 is also mediated by endosomal acidification in the cells (26). Therefore, the inhibition of ICAM-1 expression and the reduction in the number of acidic endosomes might mean that fewer whole virions enter the cytoplasm. However, in this study, LVFX significantly reduced ICAM-1 expression, whereas the magnitude of the reduction of the fluorescence intensity from acidic endosomes was small. Therefore, the reduction of ICAM-1 expression might mainly contribute to the
inhibition of infection by major group RVs, including RV14 and RV15, induced by LVFX in this study. Although we did not examine the effects of LVFX on the expression of the LDL receptor, the small effects of LVFX on acidic endosomes might be associated with the finding in this study that LVFX did not inhibit infection by RV2.

In the present study, LVFX reduced ICAM-1 expression in the primary cultures of human tracheal epithelial cells. The reduction of ICAM-1 expression resulting from treatment with ciprofloxacin has previously been reported by Mori et al. in monocytes (19). The inhibitory effects of LVFX on ICAM-1 expression by human tracheal epithelial cells might be associated with the inhibitory effects of LVFX on RV14 infection. This phenomenon has previously been reported in terms of the inhibitory effects of various agents, including dexamethasone, erythromycin, the proton pump inhibitor lansoprazole, and the β2 agonist procaterol (29, 36, 37, 43).

Human embryonic fibroblast cells did not show any morphological changes that demonstrate the presence of RV14 when the supernatants collected 1 h after infection were added to fibroblasts. In contrast, the supernatants collected 12 h after infection produced morphological changes in the fibroblasts, indicating the presence of RVs (8, 22). These findings suggest that the supernatants collected 12 h after infection contained significant amounts of RV14 virions that had been newly produced after infection, as reported previously (43).

Furthermore, in the tracheal cells from all subjects infected with RV14, the supernatant fluids collected from 1 day (24 h) to 3 days (72 h) after infection contained significant levels of RV14. These findings suggest that the human tracheal epithelial cells from all subjects were consistently infected with RV14.

Lachowicz-Scroggins et al. demonstrated with immunohistochemistry that RV16 was detected in some, but not all, of the cultured cells after infection with 10^5 TCID_{50} units/ml of RV16 (15), which was the concentration of RV14 used in this study. These data suggest the existence of cells in the cell sheets that were not infected during their
exposure to RV16 stocks but could be instead infected with RV16 that was replicated and released from adjacent cells. As we described in the Materials and Methods section, after exposure to RV14, RV14 stocks were aspirated and removed from the cells, the cells were rinsed with PBS, and fresh medium supplemented with LVFX or vehicle (water) was added to the tubes. Likewise, to measure the time course of RV14 titers, supernatants were collected at 1 day (24 h), 3 days (72 h), and 5 days (120 h) after infection. Cells in the tubes were rinsed with PBS, fresh medium supplemented with LVFX or vehicle was added, and the cell culture was continued. Therefore, supernatants might contain RV14 that was released by a second round of replication during the 7 days of the experiments in addition to that released after the exposure to RV14 stocks. Because the cells were treated with LVFX after RV14 infection in this study, LVFX might also inhibit a second round of replication of RV14. Furthermore, RV14 virions released into the supernatants were sufficient to cause infection of the cells because RV14 titers in the supernatants were higher than those to cause infection in this study. Therefore, these findings suggest that the RV14 present in the supernatants might also include RV14 virions that were released by a second round of replication in this study.

The mean peak concentration of LVFX in the alveolar epithelial lining fluid has been reported to be 3.4 μg/ml at 1 h after oral ingestion of 500 mg LVFX (47). Pretreatment of the cells with LVFX reduced the viral titers of RV14 in the supernatants at concentrations of 0.5 μg/ml or greater. The inhibitory effects of LVFX on the RV14 titers were time-dependent, whereas significant inhibitory effects were observed even when the cells were treated with LVFX (3 μg/ml) immediately after RV14 infection. These findings suggest the clinically relevant possibility that LVFX may inhibit RV infection when patients receive LVFX after RV infection.

In the present study, treatment with LVFX reduced various cell functions, including ICAM-1 expression, endosomal acidification, the production of cytokines, and NF-κB.
expression. However, treatment of the cells with LVFX did not change cell attachment, the growth and viability of the cells, or LDH concentrations in supernatants. These findings were consistent with previous reports that ofloxacin, a fluoroquinolone, does not affect the number of cultured primary human renal proximal tubular epithelial cells (31). Shimoda et al. demonstrated that LVFX does not exacerbate retinal degeneration induced by phototoxicity in mouse eyes (32). Thus, LVFX might not affect cell functions such as attachment and growth and there was not any apparent cytotoxicity by LVFX at the concentrations used.

As we reported previously, RV14 infection increased ICAM-1 expression in the cultured human tracheal epithelial cells (29, 36, 38). We also previously demonstrated that anti-IL-1β antibodies inhibited RV14-induced ICAM-1 expression (29, 38), and treatment with IL-1β (200 pg/ml) increased ICAM-1 expression in human tracheal epithelial cells in an immunohistochemical analysis (38). In the present study, RV14 infection increased the concentration of IL-1β (200 pg/ml) in supernatants. Regarding the effects of ICAM-1 on inflammation, ICAM-1 plays a vital role in the recruitment and migration of immune effector cells to sites of local inflammation in patients with COPD (27). These findings suggest that RV14 infection-induced cytokine levels may be sufficient to modulate inflammation. The inhibitory effects of LVFX on ICAM-1 shown in this study may also be associated with the inhibition of airway inflammation and the subsequent exacerbation of COPD after RV infection (30) and with the clinical benefits in the treatment of COPD exacerbation (29).

RVs are associated with the exacerbation of COPD (30). Neutrophilic inflammation, which takes place during the exacerbation of COPD, has been suggested to be associated with a variety of mediators, including IL-6, after RV infection (30). Ciprofloxacin reduces the induction of tumor necrosis factor in rabbit alveolar macrophages in response to a lipopolysaccharide (LPS) derived from P. aeruginosa (23). These data suggest that new quinolones may modulate the inflammatory response.
response seen in infections caused by Gram-negative bacilli. In the present study, LVFX reduced the RV14 infection-induced production of IL-1β, IL-6, and IL-8. The inhibitory effects of LVFX on IL-1β production are consistent with previous findings in LPS-stimulated peripheral blood mononuclear cells (46), although IL-8 production was affected little by LVFX. Trovafloxacin reduces the production of IL-6 and IL-1β by human monocytes in response to LPS stimulation (13). LVFX reduces LPS-induced IL-1β production in a murine macrophage-like cell line (14) and reduces IL-6 and IL-8 production in human bronchial epithelial cell lines (40). Similar to the inhibitory effects of glucocorticoids (37) and the β2 agonist procaterol (43), LVFX may also modulate the airway inflammation induced by RV14 infections.

Endosomal pH is suggested to be regulated by the vacuolar H+-ATPases (17) and ion transport across Na+/H+ exchangers (16, 20). The vacuolar H+-ATPase inhibitor bafilomycin and the Na+/H+ exchanger inhibitors 5-(N-ethyl-N-isopropyl) amiloride (EIPA) and N''-[3-(Hydroxymethyl)-5-(1H-pyrrol-1-yl) benzoyl] guanidine methanesulfonate (FR168888) have been shown to increase endosomal pH and inhibit the RV14 infection of cultured human tracheal epithelial cells (35). In the present study, LVFX increased the endosomal pH; however, it is unknown if LVFX inhibits vacuolar H+-ATPases or Na+/H+ exchangers. The physiological functions, other than the anti-microbial effects, of new quinolone antibiotics, including LVFX, have not been well studied in airway epithelial cells. However, H+-ATPases are functional in the bacterium Streptococcus pneumoniae and are associated with the mechanisms of quinolone resistance (9). These findings suggest the possibility that LVFX may have an effect on H+-ATPases in airway epithelial cells.

NF-κB activation increases the expression of the ICAM-1 gene and genes encoding various pro-inflammatory cytokines (24, 48). In the present study, LVFX reduced the expression of ICAM-1 prior to RV14 infection and reduced the secretion of pro-inflammatory cytokines in supernatants before and after RV14 infection.
Moxifloxacin has been shown to decrease NF-κB activation in a human bronchial epithelial cell line (5). The reduction in the expression of ICAM-1 and the secretion of interleukins by human tracheal epithelial cells in response to LVFX may be associated with a decrease in NF-κB activity.

We previously reported that macrolide antibiotics and the mucolytic agent L-carbocisteine inhibit respiratory syncytial virus (RSV) infection by reducing its receptors, activated RhoA, isoform A of the Ras-homologus (Rho) family, and/or ICAM-1 (2, 3). We also reported that a macrolide antibiotic, clarithromycin, and L-carbocisteine inhibit seasonal influenza virus infection by reducing the expression of its receptor, SAα2,6Gal, and reducing the number of acidic endosomes in human tracheal epithelial cells (44, 45). In this study, we demonstrated that LVFX reduces ICAM-1 expression and slightly but significantly reduces the number of acidic endosomes. These findings suggest the possibility that LVFX may also inhibit infection by other viruses such as RSV and influenza virus. Recent reports have demonstrated that the fluoroquinolones LVFX and ofloxacin inhibit human polyomavirus BK (BKV) replication in primary human kidney cells (31). Further experiments are needed to study this possibility.

In summary, this is the first report showing that the new quinolone antibiotic LVFX reduces the release of RV14 into human tracheal epithelial cell supernatants, reduces the replication of RV14 RNA in these cells, and decreases the susceptibility of these cells to RV14 infection. These effects may occur, in part, through a reduction in the expression of ICAM-1, the receptor for RV14, and a reduction in the number of acidic endosomes, through which RV14 RNA enters the cytoplasm. Pretreating cells with LVFX reduced the baseline and RV14 infection-induced release of IL-1β, IL-6, and IL-8 into the supernatant. LVFX may therefore inhibit infection by RV14 and modulate inflammatory responses in the airways after RV infection.
REFERENCES


FIGURE LEGENDS

Fig. 1. (A) The time course of viral release into the supernatants of human tracheal epithelial cells obtained at different times after exposure to 5.0 × 10^{-2} TCID_{50} units/cell of RV14 in the presence of LVFX (3 μg/ml) (closed circles) or vehicle (0.1% double distilled water) (open circles). The epithelial cells isolated from each donor were treated with LVFX or vehicle beginning 3 days before infection until the end of the experimental period. To examine whether the supernatants contained a significant amount of RV14, human embryonic fibroblast cells were observed for evidence of cytopathic changes for 7 days (168 h) after exposing the fibroblasts to the supernatants. The rates of change in RV14 concentration in the supernatants are expressed as TCID_{50} units/ml/24 h. The results are reported as the mean ± SEM from five different tracheae (two ex-smokers and three non-smokers). Significant differences compared to treatment with the vehicle alone are indicated by *(P<0.05) and ***(P<0.01).

(B) Concentration-dependent effects of LVFX on viral release into supernatants collected between 1 day (24 h) and 3 days (72 h) after infection. The cells were treated with LVFX (closed circles) or vehicle (Control; open circle) beginning 3 days (72 h) before RV14 infection until the end of the experimental period. The epithelial cells isolated from each donor were treated with LVFX or vehicle. To examine whether the supernatants contained a significant amount of RV14, human embryonic fibroblasts were observed for evidence of cytopathic changes for 7 days (168 h) after exposing the fibroblasts to the supernatants. The rates of change in the RV14 concentration in the supernatants are expressed as TCID_{50} units/ml/24 h. The results are reported as the mean ± SEM from 3 (0.5 μg/ml of LVFX), 5 (0.01, 0.1, 1, 3, and 10 μg/ml...
(C) RV14 titers in the supernatants of the cells pretreated with LVFX (3 μg/ml) for times ranging from 0 (just after) to 3 days (72 h) and the RV14 titers in the supernatants of the cells treated with a vehicle (water) for 3 days (72 h). The results are reported as the mean ± SEM from 3 different tracheae (two ex-smokers and one non-smoker). Significant differences compared to treatment with the vehicle alone are indicated by *(P<0.05) and **(P<0.01).

(D) Effects of LVFX (3 μg/ml) on the titers of RV2 and RV15 in the supernatants collected between 1 day (24 h) and 3 days (72 h) after infection. The cells were treated with LVFX or vehicle (water) beginning 3 days (72 h) before RV infection until the end of the experimental period. The epithelial cells isolated from each donor were treated with LVFX or vehicle. The RV2 and RV15 titers in the supernatants are expressed as TCID₅₀ units/ml/24 h. The results are reported as the mean ± SEM from 3 different tracheae. Significant differences compared to treatment with the vehicle alone (vehicle) are indicated by *(P<0.05). LVFX did not reduce RV2 titers.

Fig. 2. Replication of viral RNA in human tracheal epithelial cells at 1 day (24 h) or 3 days (72 h) after infection with RV14 in the presence of LVFX (3 μg/ml) (RV + LVFX) or vehicle (RV) or after infection with ultraviolet-inactivated RV14 in the presence of vehicle of LVFX (UV-RV), as detected by real-time quantitative RT-PCR. The epithelial cells isolated from each donor were treated with LVFX or vehicle. The results are expressed as the relative amount of RNA expression
Fig. 3. (A) The expression of ICAM-1 mRNA before RV14 infection in human tracheal epithelial cells treated with LVFX (3 μg/ml, 72 h, LVFX) or vehicle (Control), detected by real-time quantitative RT-PCR. The epithelial cells isolated from each donor were treated with LVFX or vehicle. ICAM-1 mRNA levels were normalized to the constitutive expression of ribosomal RNA (rRNA). The expression of ICAM-1 mRNA in the cells treated with vehicle was set to 1.0. The results are reported as the mean ± SEM from five different tracheae (two ex-smokers and three non-smokers). Significant differences compared to treatment with the vehicle alone are indicated by *(P<0.05).

(B) Concentration of sICAM-1 in supernatants before RV14 infection of human tracheal epithelial cells treated with LVFX (3 μg/ml, 72 h, LVFX) or vehicle (Control) as detected by enzyme immunoassay. sICAM-1 concentrations in the supernatants are expressed in ng/ml. The results are reported as the mean ± SEM from five different tracheae (two ex-smokers and three non-smokers). Significant differences compared to control values are indicated by *(P<0.05).

Fig. 4. (A-C) Changes in the distribution of acidic endosomes (green fluorescence) in human tracheal epithelial cells before treatment with LVFX (A) and 3 days (72 h) after treatment with LVFX (3 μg/ml) (C) or vehicle (B). Data are...
Fig. 5. (A-C) Altered time course for cytokine release into the supernatants of human tracheal epithelial cells before (time 0) and after infection with RV14 in the presence of LVFX (3 μg/ml) or vehicle of LVFX (water). The epithelial cells isolated from each donor were treated with LVFX or vehicle. The concentrations of cytokines in the supernatants are expressed in pg/ml. The results are reported as the mean ± SEM from eight different tracheae (four ex-smokers and four non-smokers in RV14 infection). Significant differences compared to values before RV14 infection (time 0) in the presence of vehicle are indicated by *(P<0.05) and ***(P<0.01). Significant differences compared to RV14 infection alone (RV + vehicle) at each time point after infection are indicated by +(P<0.05) and +++(P<0.01). Exposure to culture medium used for RV14 stocks (vehicle) and infection with ultraviolet-inactivated RV14 (UV-RV) did not change the concentrations of cytokines in the supernatants; the results are reported as the mean ± SEM from 3 different tracheae (two ex-smokers and one non-smoker).

Fig. 6. Amount of p50 (A), p65 (B), and c-Rel (C) in the nuclear extracts of human
tracheal epithelial cells treated with LVFX (3 μg/ml) or vehicle (Control) for 3 days (72 h) prior to RV14 infection. The results are expressed as the optical density (OD) and are reported as the mean ± SEM from five different tracheae (two ex-smokers and three non-smokers). Significant differences compared to control values (Control) prior to RV14 infection are indicated by *(P<0.05).
Levofloxacin Inhibits Rhinovirus Infection in Primary Cultures of Human Tracheal Epithelial Cells

Mutsuo Yamaya, Hidekazu Nishimura, Yukimasa Hatachi, Hiroyasu Yasuda, Xue Deng, Takahiko Sasaki, Katsumi Mizuta, Hiroshi Kubo, and Ryoichi Nagatomi

Department of Advanced Preventive Medicine for Infectious Disease, Tohoku University Graduate School of Medicine, Sendai, Virus Research Center, Clinical Research Division, Sendai National Hospital, Sendai, Department of Respiratory Medicine, Graduate School of Medicine, Kyoto University, Kyoto, Department of Innovation of New Biomedical Engineering Center, Tohoku University, Sendai, Department of Respiratory Medicine, Tohoku University School of Medicine, Sendai, Department of Microbiology, Yamagata Prefectural Institute of Public Health, Yamagata, and Department of Medicine and Science in Sports and Exercise, Tohoku University School of Medicine, Sendai, Japan

Volume 56, no. 8, p. 4052—4061, 2012. Page 4058, Figure 4: panel A in the published paper is incorrect and should appear as shown below.

FIG 4 (A to C) Changes in the distribution of acidic endosomes (green fluorescence) in human tracheal epithelial cells before treatment with LVFX (A) and 3 days (72 h) after treatment with LVFX (3 μg/ml) (C) or vehicle (B). Data are representative of data from five different experiments (two ex-smokers and three nonsmokers). Bar = 100 μm. (D) Fluorescence intensity of acidic endosomes 3 days (72 h) after treatment with LVFX (3 μg/ml) or vehicle and before treatment (Before). The results are reported as the fluorescence intensity divided by the fluorescence intensity before treatment (expressed as a percentage) and are reported as the means ± SEM from five samples (two ex-smokers and three nonsmokers). Significant differences compared to values before treatment are indicated (*, P < 0.05).