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The proton pump inhibitor lansoprazole inhibits rhinovirus infection in cultured human tracheal epithelial cells

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Abstract

To examine the effects of lansoprazole, a proton pump inhibitor, on rhinovirus infection in airways, human tracheal epithelial cells were infected with a major subgroup of rhinoviruses, type 14 rhinovirus. Rhinovirus increased the mRNA expression of intercellular adhesion molecule-1 (ICAM-1) in the cells, the major rhinovirus receptor, and the content of the soluble form of ICAM-1 (sICAM-1) and cytokines in supernatants. Lansoprazole reduced supernatant titers and RNA of rhinovirus, the susceptibility to rhinovirus infection, the ICAM-1 mRNA production, the number and fluorescence intensity of acidic endosomes in the cells, and supernatants sICAM-1 and cytokine concentrations including interleukin-1β. Antibody to interleukin-1β reduced baseline and rhinovirus-induced ICAM-1 production. These results suggest that lansoprazole inhibits rhinovirus infection by reducing ICAM-1 via partly endogenous production of interleukin-1β, and by blocking the rhinovirus RNA entry into the endosomes. Lansoprazole may modulate airway inflammation by reducing the production of cytokines and ICAM-1 in rhinovirus infection.

Keywords: Asthma; Common cold; ICAM-1 (intercellular adhesion molecule-1); Proton pump; Lansoprazole

1. Introduction

Prospective studies have shown an association between asthma attacks and infection by various viruses including rhinoviruses, influenza viruses, and respiratory syncytial viruses (Johnston et al., 1995; Nicholson et al., 1993). Studies using polymerase chain reaction (PCR)-based diagnostics have demonstrated that rhinoviruses are responsible for 80–85% and 45% of the asthma flares in 9- to 11-year-old children and adults, respectively (Johnston et al., 1995; Nicholson et al., 1993).

Recent reports revealed that the major rhinovirus enters the cytoplasm of infected cells after binding to its receptor intercellular adhesion molecule-1 (ICAM-1) (Greve et al., 1989; Casasnovas and Springer, 1994). The entry of RNA of a major rhinovirus, type 14 rhinovirus, into the cytoplasm of infected cells is suggested to be mediated by the destabilization from receptor binding and by endosomal acidification (Casasnovas and Springer, 1994). Macrolide antibiotics bafilomycin (Bowman et al., 1988; Pérez and Carrasco, 1993; Suzuki et al., 2001) and erythromycin (Suzuki et al., 2002) inhibit major rhinovirus infection via the reduction of ICAM-1 expression (Suzuki et al., 2001, 2002) and via the increase in endosomal pH (Bowman et al., 1988; Pérez and Carrasco, 1993; Suzuki et al., 2001, 2002). On the other hand, in addition to the inhibitory effects on H^+–K^+ ATPase in gastric parietal cells, proton pump inhibitors, lansoprazole and omeprazole, inhibit various cell functions including the production of ICAM-1 (Ohara and Arakawa, 1999; Watanabe et al., 2001). Ohara and Arakawa (1999) demonstrated in an immunohistochemical study that the number of peripheral blood monocytes expressing

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ICAM-1 is significantly decreased after lansoprazole treatment. Furthermore, Watanabe et al. (2001) demonstrated that omeprazole inhibits ICAM-1 expression in gastric mucosa after injection of interleukin-1β. Omeprazole also increases pH in the renal cell vacuoles (Sabolic et al., 1994). These findings suggest the possibility that lansoprazole may also inhibit type 14 rhinovirus infection. However, the effects of lansoprazole on type 14 rhinovirus infection have not been investigated.

Omeprazole also inhibits cytokine production in the epithelial cells (Kountouras et al., 2000). Rhinovirus infection induces the production of cytokines including interleukin-1 (Subauste et al., 1995; Terajima et al., 1997). These cytokines have proinflammatory effects (Akira et al., 1990) and may be related to the pathogenesis of rhinovirus infections. Endogenous interleukin-1β regulates the ICAM-1 expression after rhinovirus infection (Terajima et al., 1997). However, the effects of lansoprazole on the cytokine production by rhinovirus infection have not been studied.

We therefore examined the effects of lansoprazole on the production of ICAM-1 and cytokines, and on the endosomal pH to clarify the mechanisms responsible for the inhibition of rhinovirus infection.

2. Methods

2.1. Viral stocks

RV14 stocks were prepared from patients with common colds by infecting human embryonic fibroblast cells as described (Numazaki et al., 1987; Terajima et al., 1997; Suzuki et al., 2000), and identified with a microneutralization test using an antibody for type 14 rhinovirus (Gwaltney, 1966).

2.2. Detection and titration of viruses

Detection and titration of rhinoviruses were performed by observing the cytopathic effects of viruses on human embryonic fibroblast cells with methods as previously described (Numazaki et al., 1987; Terajima et al., 1997; Suzuki et al., 2000), and the amount of specimen required to infect 50% of the human embryonic fibroblast cells (tissue culture infective dose [$TCID_{50}$]) was determined.

2.3. Detection and quantification of rhinovirus RNA

Detection and quantification of rhinovirus RNA in the epithelial cells were performed by reverse transcription (RT)-PCR (Terajima et al., 1997; Suzuki et al., 2000) as previously described.

Furthermore, to quantify the rhinovirus RNA and GAPDH mRNA expression in the human tracheal epithelial cells after rhinovirus infection, real-time quantitative RT-PCR using the Taqman technique (Roche Molecular Diagnostic Systems) was performed as previously described (Martell et al., 1999, Suzuki et al., 2002). Taqman technology exploits the 5′ –3′ nucleolytic activity of AmpliTaq DNA polymerase (Holland et al., 1991; Heid et al., 1996). In principle, the method uses a dual-labeled fluorogenic hybridization probe, a Taqman probe that specifically anneals the template between the PCR primers. The probe contains a fluorescent reporter (6-carboxyfluorescein [FAM]) at the 5′ end and a fluorescent quencher (6-carboxytetramethylrhodamine [TAMRA]) at the 3′ end. According to the progression of PCR, the Taqman probe is degraded and releases the reporter, resulting in an increase in fluorescence emission. The use of a sequence detector (ABI PRISM 7700; Applied Biosystems, CA) allows measurement of the amplified product in direct proportion to the continuous increase in fluorescence emission during PCR. In the present experiment, the reaction mixture for RT-PCR was prepared in a single buffer system without the addition of reagents or changing of the tubes between the RT reaction and PCR (Martell et al., 1999). Briefly, 100 ng of RNA dissolved in 10 μl of water from each aliquot of human tracheal epithelial cells was denatured at 90 °C for 90 s. Each RNA sample (100 ng/10 μl of water) was mixed in 40 μl of buffer containing the following reagents for the one-step RT-PCR reaction: 50 mM KCl, 10 mM Tris–HCl (pH 8.3), 0.01 mM EDTA, 60 nM Passive Reference 1 (Applied Biosystems), 5 mM $MgCl_2$, 100 nM forward primer (5′ -GCACTTCTGTTTCCCC-3′), 100 nM reverse primer (5′ -CGGACACCCAAAGTAG-3′), 0.3 mM deoxynucleoside triphosphate (Boehringer), 0.4 U/μl RNase inhibitor (Promega), 0.4 U/μl Moloney murine leukemia virus RT (Perkin Elmer), 0.0025 U/μl *Taq* Gold Polymerase (Perkin Elmer), and 100 nM Taqman probe. Taqman probe type 14 rhinovirus [5′ -(FAM) CGAGGTATAGGCTGTACCCACTGCCAAAA (TAMRA)-3′] was designed for type 14 rhinovirus. We used the program PrimerExpress (Applied Biosystems) to design the probe and primers according to the guidelines for the best performance of the PCR. The fragment of rhinovirus RNA extracted from the human tracheal epithelial cells before or at either 24 or 72 h after infection by type 14 rhinovirus infection was reverse transcribed into cDNA (30 min at 48 °C) and amplified by PCR for 40 cycles (15 s at 95 °C and 1 min at 60 °C). Whole reactions of the RT-PCR and detection of the fluorescence emission signal for every PCR cycle were performed at the same time in a single tube in a sequence detector (ABI 7700). The minimum PCR cycle to detect the fluorescent signal was defined as the cycle threshold (Cτ), which is predictive of the quantity of an input target fragment (Heid et al., 1996). The standard curve was obtained between the fluorescence emission signals and Cτ by means of 10-fold dilutions of the total RNA, extracted from 10^5 $TCID_{50}$ U/ml of type 14 rhinovirus in the supernatants of the human

embryonic fibroblasts 7 days after infection with type 14 rhinovirus (10^4 $TCID_{50}$ U/ml). Real-time quantitative RT-PCR for GAPDH was also performed using the same PCR products. The expression of rhinovirus RNA was normalized to the constitutive expression of GAPDH mRNA.

2.4. Human tracheal epithelial cell culture

Tracheas for cell culture were obtained after death from 70 patients (age, 63±2 years; 33 females, 37 males). Isolation and culture of the human tracheal surface epithelial cells were performed as described previously (Yamaya et al., 1992; Terajima et al., 1997). The human tracheal epithelial cells were cultured at 37 °C before the rhinovirus infection. We routinely ruled out contamination with *Mycoplasma pneumoniae* and *Chlamydia pneumoniae* in the supernatants of the cultured human tracheal epithelial cells using PCR techniques (Gaydos et al., 1992; Skakni et al., 1992).

2.5. Measurement of LDH concentration

The amount of lactate dehydrogenase (LDH) in the culture supernatants was measured with the method as described by Amador et al. (1963).

2.6. Effects of lansoprazole on viral infection

To examine the effects of lansoprazole on rhinovirus infection, the cells treated with lansoprazole (10 μM, 3 days) (Sabolic et al., 1994) were exposed to type 14 rhinovirus (10^5 $TCID_{50}$ U/ml) or vehicle (Eagle's minimum essential medium) for 60 min and cultured at 33 °C with rolling as previously described (Terajima et al., 1997) because significant rhinovirus titers in the supernatants could not be detected when the cells were cultured at 37 °C. The viral content in the supernatant is expressed as $TCID_{50}$ U/ml.

Furthermore, to examine the effects of lansoprazole on the viral titers, cytokine production, and expression of ICAM-1, the cultured human tracheal epithelial cells were treated with 10 μM lansoprazole or vehicle (0.1% ethanol) (Sabolic et al., 1994) from 3 days before rhinovirus infection until the end of the experiments after rhinovirus infection (Suzuki et al., 2002).

The effects of lansoprazole on the susceptibility to rhinovirus infection were evaluated as previously described (Subauste et al., 1995; Suzuki et al., 2002) using epithelial cells pretreated with lansoprazole (10 μM, 3 days) or vehicle (0.1% ethanol, 3 days). The epithelial cells were then exposed to serial 10-fold dilutions of type 14 rhinovirus or vehicle of type 14 rhinovirus (Eagle's minimum essential medium) for 1 h at 33 °C. The presence of rhinovirus in the supernatants collected for 1–3 days after infection was determined with the human embryonic fibroblast cell assay described above to assess whether infection occurred at each dose of rhinovirus used.

2.7. Measurement of ICAM-1 expression

The mRNA of ICAM-1 was examined with real-time RT-PCR analysis as previously described (Suzuki et al., 2002). Furthermore, concentrations of a soluble form of ICAM-1 (sICAM-1) in culture supernatants were measured with enzyme immunoassay (EIA).

To determine the role of endogenous interleukin-1β in the expression of ICAM-1 mRNA in human tracheal epithelial cells and in the sICAM-1 concentrations in culture supernatants of the epithelial cells, confluent cells were preincubated using a monoclonal mouse anti-human interleukin-1β (10 μg/ml; Genzyme) or vehicle of a monoclonal mouse anti-human interleukin-1β (phosphate-buffered saline) for 3 days after rhinovirus infection as previously described (Terajima et al., 1997).

2.8. Measurement of cytokine production

We measured interleukin-1β, interleukin-6, interleukin-8, and tumor necrosis factor (TNF)-α of culture supernatants by specific enzyme-linked immunosorbent assays (ELISAs) before and at 1, 3, and 5 days after infection with RV14 as previously described (Terajima et al., 1997; Suzuki et al., 2002).

2.9. Measurement of changes in acidic endosomes distribution

The distribution of acidic endosomes in the cells was measured as previously described with a dye, LysoSensor DND-189 (Molecular Probes) (Gu et al., 1997; Suzuki et al., 2001, 2002). The effects of lansoprazole on the distribution of acidic endosomes were examined from 100 s before to 300 s after the treatment with lansoprazole (10 μM) or vehicle of lansoprazole (0.1% ethanol). Furthermore, we studied the effects of long periods of lansoprazole treatment (10 μM, 3 days and 7 days) on the fluorescence intensity of acidic endosomes. Fluorescence intensity of acidic endosomes was measured in 100 human tracheal epithelial cells, and mean value of fluorescence intensity was expressed as a percent of control value compared with the fluorescence intensity of the cells treated with vehicle of lansoprazole (0.1% ethanol).

2.10. Statistical analysis

Results are expressed as mean±S.E. Statistical analysis was performed using two-way repeated-measures analysis of variance (ANOVA). Subsequent post-hoc analysis was made using Bonferroni's method. For all analyses, values of $P<0.05$ were assumed to be significant. *n* refers to the

number of donors (tracheae) from which cultured epithelial cells were used.

3. Results

3.1. Effects of lansoprazole on rhinovirus infection of human tracheal epithelial cells

Exposing confluent human tracheal epithelial cell monolayers to type 14 rhinovirus (10^5 $TCID_{50}$ U/ml) consistently led to infection. Furthermore, lansoprazole inhibited type 14 rhinovirus infection concentration-dependently and the maximum effect was obtained at 10 μM (Fig. 1). To examine whether type 14 rhinovirus infection or lansoprazole induced cytotoxic effects on the cultured cells and caused cell detachment (Winther et al., 1990) from the tubes after the cells made a confluent sheet, we counted the cell numbers after type 14 rhinovirus infection and after the treatment with lansoprazole. The cell numbers were constant in the confluent epithelial cells in the control medium, and the coefficient of variation was small (7.5%; n=29). Neither type 14 rhinovirus infection (10^5 $TCID_{50}$ U/ml; 5 days) nor lansoprazole treatment (10 μM; 5 days) had any effect on the cell numbers. Cell numbers after type 14 rhinovirus infection ($2.1\pm0.2\times10^6$, P>0.50, n=7) were not significantly different from those after sham infection ($2.0\pm0.1\times10^6$, n=7). Likewise, cell numbers after lansoprazole treatment ($2.0\pm0.1\times10^6$, P>0.50, n=7) were not significantly different from those after treatment with the vehicle alone ($2.0\pm0.1\times10^6$, n=7). Cell viability, assessed by the exclusion of trypan blue (Terajima et al., 1997), was consistently >96% in the rhinovirus-infected culture and the lansoprazole-treated culture. Type 14 rhinovirus infection and lansoprazole treatment (10 μM) did not alter the amount of lactate dehydrogenase (LDH) in the supernatants. The amount of LDH in the supernatants was 28±2 IU/l before type 14 rhinovirus infection, 29±2 IU/l 5 days after type 14 rhinovirus infection (P>0.50, n=7), and 30±3 IU/l after lansoprazole treatment (10 μM; 5 days) (P>0.20, n=7).

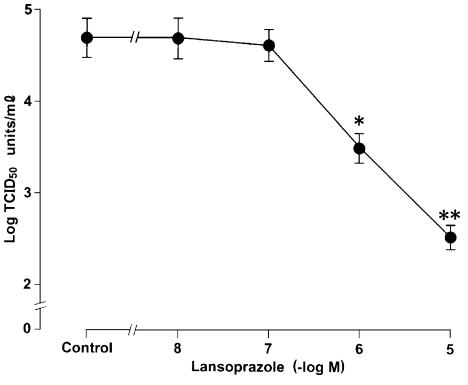

Fig. 1. Concentration–response effects of lansoprazole on the viral titers in supernatants collected during 1–3 days after infection. The cells were treated with lansoprazole or vehicle (Control; 0.1% ethanol) from 3 days before type 14 rhinovirus infection until the end of the experiments after type 14 rhinovirus infection. Viral content in the supernatant is expressed as $TCID_{50}$ units per milliliter. Results are mean±S.E. from seven different tracheae. Significant differences from vehicle alone (control) are indicated by *P<0.05 and **P<0.01.

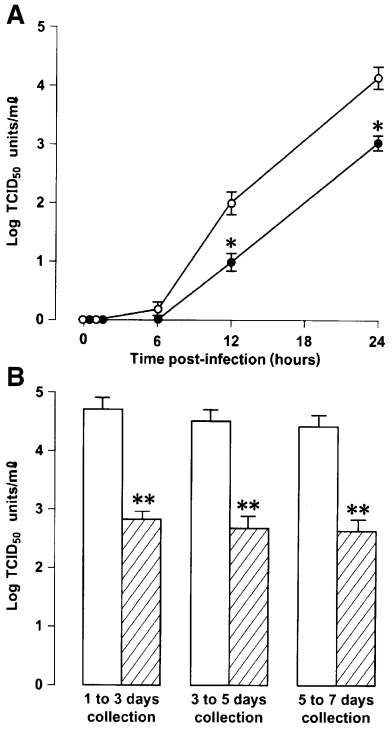

Fig. 2. Viral titers in supernatants of human tracheal epithelial cells obtained at different times after exposure to 10^5 $TCID_{50}$ U/ml of type 14 rhinovirus (A and B) in the presence (closed circles and hatched bars) of lansoprazole (10 μM) or vehicle of lansoprazole (0.1% ethanol) (open circles and open bars). Viral titers in supernatants collected sequentially during the first 24 h after infection (A). Viral titers in supernatants collected during 1–3 days, 3–5 days, and 5–7 days after infection (B). The cells were treated with lansoprazole or vehicle (0.1% ethanol) from 3 days before type 14 rhinovirus infection until the end of the experiments after type 14 rhinovirus infection. Viral content in the supernatant is expressed as $TCID_{50}$ units per milliliter. Results are mean±S.E. (A) or mean+S.E. (B) from seven different tracheae. Significant differences from viral infection alone are indicated by *P<0.05 and **P<0.01.

To measure the time course of viral release during the first 24 h, we used four separate cultures from the same trachea, and calculated the results from seven different tracheae. We collected the culture supernatants at either 1, 6, 12, or 24 h after type 14 rhinovirus infection. No detectable virus was revealed at 1 h after infection. Type 14 rhinovirus was detected in culture medium 6 h after infection, and the viral content progressively increased between 6 and 24 h after infection (Fig. 2A). To examine the effects of lansoprazole on the viral titers, the cells were treated with 10 μM lansoprazole (Sabolic et al., 1994) or vehicle (0.1% ethanol) from 3 days before rhinovirus infection until the end of the experiments after rhinovirus infection. Evidence of continuous viral production was obtained by demonstrating that the viral titers of supernatants collected during 1–3 days, 3–5 days, and 5–7 days after infection each contained

significant levels of type 14 rhinovirus (Fig. 2A and B). The viral titer levels in supernatants increased significantly with time for the first 24 h ($P<0.05$ in each case by ANOVA), then remained constant thereafter. Type 14 rhinovirus infection of the epithelial cells was constant and the coefficient of variation of the viral titers in the supernatants during 1–3 days was small (8.3%; $n=56$). Treatment of the cells with lansoprazole significantly decreased the viral titers of type 14 rhinovirus in supernatants from 12 h after infection (Fig. 2A and B). Omeprazole (10 μM) also reduced the viral titers of type 14 rhinovirus in supernatants (2.8±0.2 log $TCID_{50}$ U/ml, during 1–3 days, $P<0.01$, $n=5$) compared with those of type 14 rhinovirus infection alone (4.7±0.3 log $TCID_{50}$ U/ml, during 1–3 days, $n=5$). To examine whether lansoprazole is toxic to RV, we incubated type 14 rhinovirus with lansoprazole (10 μM) or vehicle (0.1% ethanol) for 24 h at 33 °C, and washed the type 14 rhinovirus by ultracentrifugation with sucrose gradient (Korant et al., 1972). The virus titers in the supernatants of the cells infected with type 14 rhinovirus (10^5 $TCID_{50}$ U/ml, 60 min) after treatment with lansoprazole (3.1±0.3 log $TCID_{50}$ U/ml, 24 h after infection, $P>0.50$, $n=5$) were not different from those infected with type 14 rhinovirus (10^5 $TCID_{50}$ U/ml, 60 min) after treatment with the vehicle alone (3.1±0.3 log $TCID_{50}$ U/ml, 24 h after infection, $n=5$).

Therefore, the infection load was kept constant between experimental and control samples. We confirmed the presence of beating cilia on the epithelial cells using an inverted microscope (MIT-2; Olympus, Tokyo, Japan) from the beginning of the cell culture to the end of the experiments as reported previously (Suzuki et al., 2000, 2002), and a dome formation when the cells made confluent cell sheets on days 5–7 of culture as described by Widdicombe et al. (1987).

3.2. Effects of lansoprazole on viral RNA by PCR

Further evidence of the inhibitory effects of lansoprazole on infection by type 14 rhinovirus and viral replication in human tracheal epithelial cells was provided by PCR analysis. The RNA extraction was performed at 72 h after type 14 rhinovirus infection. Lansoprazole (10 μM) decreased the intensity of the product band of type 14 rhinovirus from 24 and 72 h after infection (Fig. 3A and B). Omeprazole (10 μM) also decreased the intensity of the product band of type 14 rhinovirus from 72 h after infection (Fig. 3B).

3.3. Effects of lansoprazole on susceptibility to type 14 rhinovirus infection

To examine the effects of lansoprazole on the susceptibility to infection by type 14 rhinovirus, the human tracheal epithelial cells were treated with lansoprazole (10 μM) from 3 days before infection with type 14 rhinovirus until just before infection with type 14 rhinovirus. The cells were then

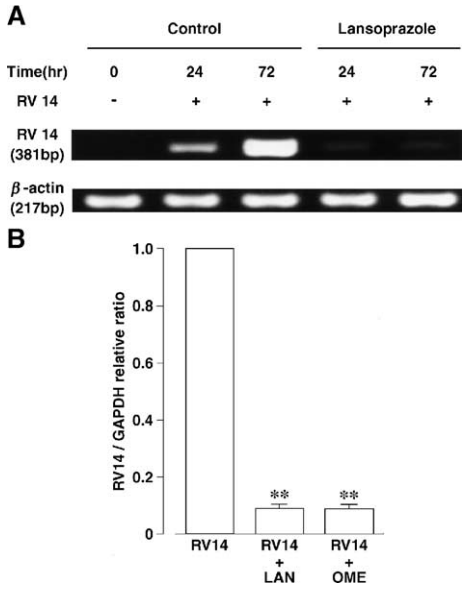

Fig. 3. (A) Replication of rhinovirus RNA from human tracheal epithelial cells before and at 24 and 72 h after infection by type 14 rhinovirus in the presence of lansoprazole (10 μM) or vehicle alone (Control, 0.1% ethanol) as detected by RT-PCR. Data are representative of three different experiments. (B) Replication of rhinovirus RNA from human tracheal epithelial cells at 72 h after infection by type 14 rhinovirus in the presence of lansoprazole (10 μM) (RV14+LAN), omeprazole (10 μM) (RV14+OME), or vehicle alone (0.1% ethanol) (RV14) as detected by real-time quantitative RT-PCR. Results are expressed as relative amounts of type 14 rhinovirus RNA expression (ratio) compared with those of cells treated with vehicle alone (0.1% ethanol) (RV14), and represent mean+S.E. from seven different tracheae. Significant differences from treatment with vehicle alone (RV14) are indicated by $^{**}P<0.01$. To examine the effects of lansoprazole and omeprazole on viral RNA in the cells, the cells were treated with lansoprazole, omeprazole, or vehicle alone (0.1% ethanol) from 3 days before type 14 rhinovirus infection until the RNA extraction at 72 h after type 14 rhinovirus infection.

exposed to serial 10-fold dilutions of type 14 rhinovirus for 1 h at 33 °C. The presence of type 14 rhinovirus in the supernatants collected for 1–3 days after infection was determined with the human embryonic fibroblast cell assay described above to assess whether infection occurred at each dose of rhinovirus used. Treatment of the cells with lansoprazole decreased the susceptibility of the cells to infection by type 14 rhinovirus. The minimum dose of type 14 rhinovirus necessary to cause infection in the cells treated with lansoprazole (10 μM, 3 days) (3.2±0.1 log TCID50 U/ml, $n=7$, $P<0.05$) was significantly higher than that in the cells treated with vehicle of lansoprazole (0.1% ethanol) (2.1±0.1 log $TCID_{50}$ U/ml ($n=7$).

3.4. Effects of lansoprazole on the expression of ICAM-1

To examine the effects of lansoprazole on the expression of ICAM-1, the human tracheal epithelial cells were treated with lansoprazole (10 μM) or vehicle (0.1% ethanol) from 3 days before rhinovirus infection until the assay of the expression after rhinovirus infection. The mRNA was extracted at 72 h after rhinovirus infection or sham

infection. Type 14 rhinovirus infection increased ICAM-1 mRNA in the absence of lansoprazole. Lansoprazole inhibited the increases in ICAM-1 mRNA induced by type 14 rhinovirus infection as well as the baseline ICAM-1 mRNA expression in the cells (Fig. 4A). Lansoprazole reduced the ICAM-1 mRNA expression by more than 60% compared with that of the cells treated with vehicle of lansoprazole (0.1% ethanol) (Fig. 4A). Likewise, type 14 rhinovirus infection increased sICAM-1 concentrations in supernatants in the absence of lansoprazole (Fig. 4B). Lansoprazole inhibited the increases in sICAM-1 induced by type 14 rhinovirus infection as well as the baseline sICAM-1 concentrations in supernatants (Fig. 4B).

Furthermore, we studied the effects of antibody to interleukin-1β on the ICAM-1 expression because endogenous interleukin-1β regulates ICAM-1 expression in human tracheal epithelial cells (Terajima et al., 1997). Antibody to interleukin-1β (10 μg/ml; Genzyme) significantly reduced baseline and type 14 rhinovirus infection-induced ICAM-1 expression (Fig. 4C) and sICAM-1 concentrations in supernatants (Fig. 4D). On the other hand, the magnitude of the inhibitory effects of lansoprazole on ICAM-1 mRNA expression and sICAM-1 concentration in supernatants was significantly larger than those of antibody to interleukin-1β ($P<0.05$). An isotype-matched mouse IgG_1 control monoclonal antibody (10 μg/ml; Chemicon International) did not reduce the baseline and type 14 rhinovirus infection-induced ICAM-1 mRNA expression (1.1±0.1 ICAM-1/GAPDH relative ratio in the absence of type 14 rhinovirus infection, $n=5$; and 2.6±0.3 ICAM-1/GAPDH relative ratio in the presence of type 14 rhinovirus infection, $n=5$), and did not reduce sICAM-1 concentration in supernatants (206±12 ng/ml in the absence of type 14 rhinovirus infection, $n=5$; and 311±15 ng/ml in the presence of type 14 rhinovirus infection, $n=5$) ($P>0.20$).

3.5. Effects of lansoprazole on cytokine production

To examine the effects of lansoprazole on cytokine production after rhinovirus infection, the human tracheal epithelial cells were treated with lansoprazole (10 μM) or vehicle (0.1% ethanol) from 3 days before rhinovirus infection until the collection of the supernatants after rhinovirus infection (Fig. 5). The secretion of interleukin-1β, interleukin-6, interleukin-8, and TNF-α all increased in response to type 14 rhinovirus. In contrast, ultraviolet-irradiated type 14 rhinovirus did not increase interleukin-1β, interleukin-6, interleukin-8, and TNF-α (data not shown). The maximal production of interleukin-1β and TNF-α was observed at 3 days after type 14 rhinovirus infection, and that of interleukin-6 and interleukin-8 was observed at day 1 after type 14 rhinovirus infection. Furthermore, lansoprazole inhibited the baseline and rhinovirus infection-induced production of all of these cytokines (Fig. 5). Lansoprazole inhibited both basal and rhinovirus-induced increases in interleukin levels (interleukin-1β, interleukin-6, and inter-

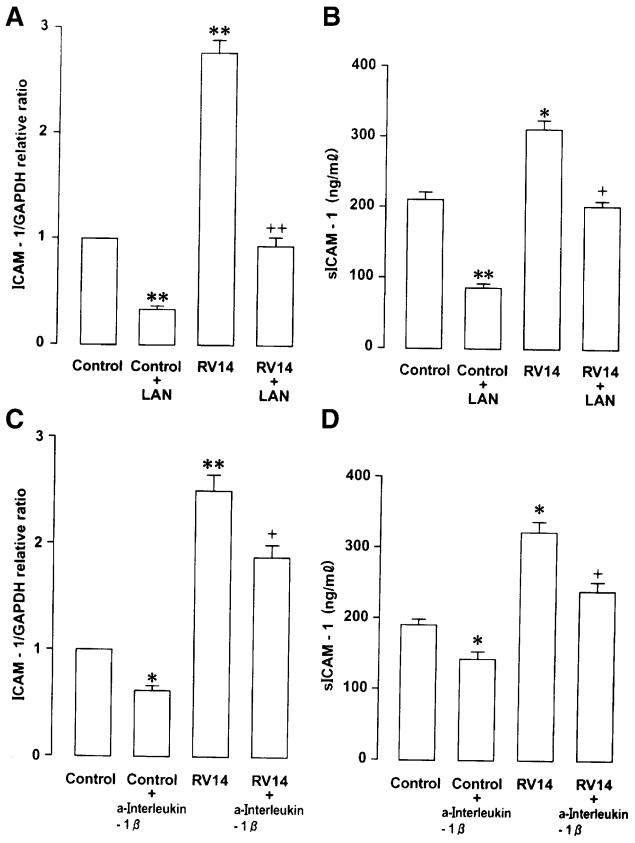

Fig. 4. (A) Effects of lansoprazole (LAN, 10 μM) or vehicle of lansoprazole (Control; 0.1% ethanol) on the expression of ICAM-1 mRNA in human tracheal epithelial cells 3 days after infection of type 14 rhinovirus or sham (Control; Eagle's minimum essential medium) detected by real-time quantitative RT-PCR. ICAM-1 mRNA was normalized to the constitutive expression of GAPDH mRNA. Results are mean+S.E. from 14 different tracheae. Significant differences from corresponding control values are indicated by $^{**}P<0.01$. Significant differences from type 14 rhinovirus infection are indicated by $^{++}P<0.01$. (B) Effects of lansoprazole (LAN, 10 μM) or vehicle of lansoprazole (Control; 0.1% ethanol) on the sICAM-1 concentrations in supernatants of human tracheal epithelial cells during 1–3 days after type 14 rhinovirus or sham (Control; Eagle's minimum essential medium) infection. Results are mean+S.E. from 14 different tracheae. Significant differences from corresponding control values are indicated by $^{*}P<0.05$ and $^{**}P<0.01$. Significant differences from type 14 rhinovirus infection are indicated by $^{+}P<0.05$. (C) Effects of antibody to interleukin-1β (a-interleukin-1β, 10 μg/ml) or vehicle of interleukin-1β (Control; phosphate-buffered saline) on the expression of ICAM-1 mRNA in human tracheal epithelial cells 3 days after type 14 rhinovirus or sham (Control; Eagle's minimum essential medium) infection detected by real-time quantitative RT-PCR. ICAM-1 mRNA was normalized to the constitutive expression of GAPDH mRNA. Results are mean+S.E. from five different tracheae. Significant differences from corresponding control values are indicated by $^{*}P<0.05$ and $^{**}P<0.01$. Significant differences from RV14 infection are indicated by $^{+}P<0.05$. (D) Effects of antibody to interleukin-1β (a-interleukin-1β, 10 μg/ml) or vehicle of antibody to interleukin-1β (Control; phosphate-buffered saline) on the sICAM-1 concentrations in supernatants of human tracheal epithelial cells during 1–3 days after type 14 rhinovirus or sham (Control; Eagle's minimum essential medium) infection. Results are mean+S.E. from 14 different tracheae. Significant differences from corresponding control values are indicated by $^{*}P<0.05$. Significant differences from type 14 rhinovirus infection are indicated by $^{+}P<0.05$.

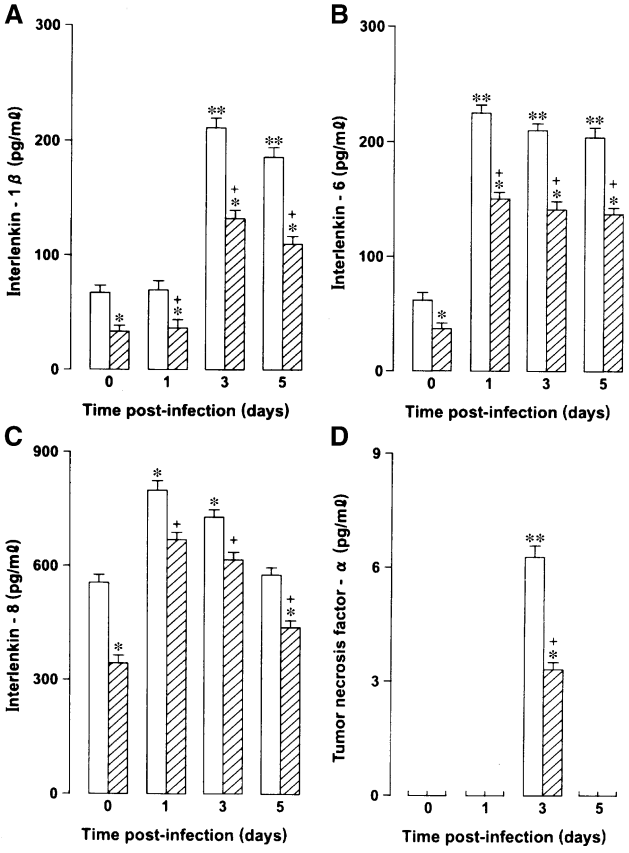

Fig. 5. (A–D) Time course of release of cytokines into supernatants of human tracheal epithelial cells after infection of type 14 rhinovirus in the presence of lansoprazole (10 μM) (hatched bars) or vehicle of lansoprazole (0.1% ethanol) (open bars). Results are mean+S.E. from seven different tracheae. Significant differences from values before type 14 rhinovirus infection (time 0) in the presence of vehicle of lansoprazole (0.1% ethanol) are indicated by $*P<0.05$ and $**P<0.01$. Significant differences from corresponding values of type 14 rhinovirus alone are indicated by $^{+}P<0.05$.

leukin-8) by 40–60%. Basal levels of TNF-α were below detection; however lansoprazole decreased rhinovirus-induced increases in TNF-α by approximately 50%. Of the cytokines measured, interferon (IFN)-α, IFN-β, and IFN-γ in the supernatants were under the limit of detection of the assay, and type 14 rhinovirus infection did not alter interleukin-1α production (11±1 pg/ml 3 days after type 14 rhinovirus infection versus 12±2 pg/ml 3 days after sham infection, $P>0.20$, $n=7$).

3.6. Effects of lansoprazole on the acidification of endosomes

The effects of lansoprazole on the changes in the distribution of acidic endosomes were examined from 100 s before until 300 s after the treatment with lansoprazole (10 μM) or vehicle (0.1% ethanol). Acidic endosomes in human tracheal epithelial cells were stained green with LysoSensor DND-189. Green fluorescence from acidic endosomes was observed in a granular pattern in the cytoplasm (Fig. 6A). Lansoprazole decreased the number and the fluorescence intensity of acidic endosomes with green fluorescence in the cells with time (Fig. 6B). The fluorescence intensity from acidic endosomes in the epithelial cells treated with lansoprazole for 300 s was significantly reduced (Fig. 6C). Furthermore, the fluorescence intensity from acidic endosomes in the epithelial cells treated with lansoprazole for 3 days and 7 days was also studied. The fluorescence intensity from acidic endosomes in the cells treated with lansoprazole for 3 days and 7 days was reduced to less than 10% of the intensity in the cells treated with vehicle of lansoprazole (0.1% ethanol) (Fig. 6C).

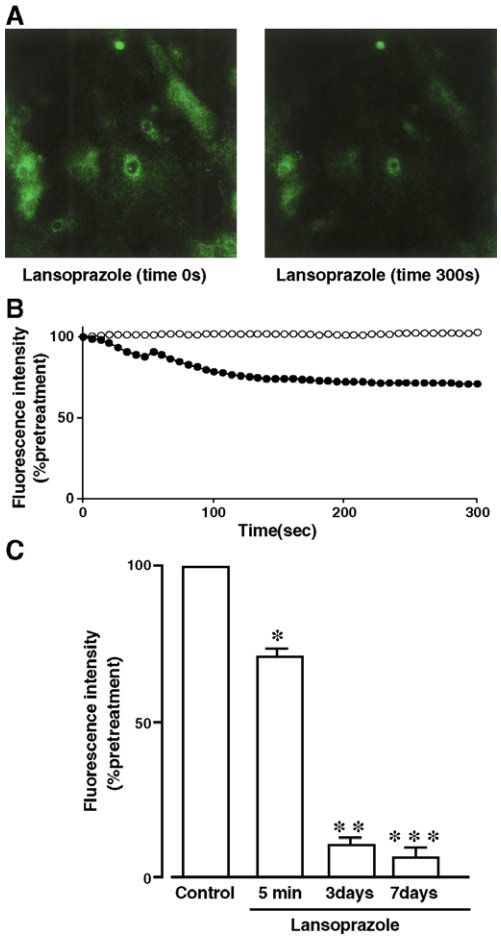

Fig. 6. (A) Changes in the distribution of acidic endosomes with green fluorescence in the human tracheal epithelial cells before (time 0 s) and 300 s (time 300 s) after treatment with lansoprazole (10 μM). Data are representative of three different experiments. (B) Time course changes in the intensity of green fluorescence from acidic endosomes in human tracheal epithelial cells after treatment with either lansoprazole (10 μM, closed circles) or vehicle of lansoprazole (0.1% ethanol) (control, open circles). Inhibitors were administrated at time 0. Acidic endosomes in human tracheal epithelial cells were stained with green with a dye, LysoSensor DND-189. Data are representative of three different experiments. (C) The fluorescence intensity of acidic endosomes 300 s, 3 days, and 7 days after the addition of lansoprazole (10 μM) or vehicle of lansoprazole (control, 0.1% ethanol). Results mean+S.E. from seven different tracheae. Significant differences from vehicle (control) are indicated by $*P<0.05$, $**P<0.01$, and $***P<0.001$.

4. Discussion

In the present study, we have shown that a proton pump inhibitor, lansoprazole, reduced the viral titers in the supernatants and viral RNA of a major subgroup of rhinoviruses, type 14 rhinovirus, in cultured human tracheal epithelial cells. Pretreatment with lansoprazole inhibited the expression of mRNA of ICAM-1, the receptor for the major rhinoviruses (Greve et al., 1989). The magnitude of inhibitory effects of lansoprazole on ICAM-1 mRNA expression was similar to that of dexamethasone and erythromycin as previously described (Suzuki et al., 2000, 2002). Contents of the soluble form of ICAM-1 (sICAM-1) in culture supernatants were also reduced by lansoprazole. Because the minimum dose of type 14 rhinovirus necessary to cause infection in the cells treated with lansoprazole was significantly higher than that in the cells treated with vehicle of lansoprazole, lansoprazole may inhibit type 14 rhinovirus infection at least partly by reducing the production of its receptor, ICAM-1, as observed in human tracheal epithelial cells treated with dexamethasone (Suzuki et al., 2000) and erythromycin (Suzuki et al., 2002). Furthermore, lansoprazole reduced the number and fluorescence intensity of acidic endosomes in cultured human tracheal epithelial cells. The magnitude of inhibitory effects of lansoprazole on fluorescence intensity of acidic endosomes was similar to that of bafilomycin A_1 (Suzuki et al., 2001) and erythromycin (Suzuki et al., 2002). In the epithelial cells treated with lansoprazole for 3 days, the fluorescence intensity reduced to less than 10% of that of the cells treated with vehicle of lansoprazole (0.1% ethanol). Omeprazole, another proton pump inhibitor, also reduced supernatant titers and RNA of type 14 rhinovirus. Since lansoprazole was not toxic to type 14 rhinovirus, lansoprazole may act by inhibiting type 14 rhinovirus RNA entry across acidic endosomes as demonstrated in HeLa cells and human tracheal epithelial cells treated with bafilomycin A_1 (Pérez and Carrasco, 1993; Suzuki et al., 2001) and erythromycin (Suzuki et al., 2002).

The epithelial cells in human airways express ICAM-1 on their surface, which is the site of attachment for 90% of the approximately 100 rhinovirus serotypes (Greve et al., 1989; Stanway, 1994). 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 (Terajima et al., 1997; Papi and Johnston, 1999) have shown that rhinovirus infection upregulates ICAM-1 expression on the airway epithelial cells, an effect that would facilitate viral cell attachment and entry. The increases in the expression of protein and mRNA of ICAM-1 on human tracheal epithelial cells induced by type 14 rhinovirus infection in the present study are in accord with those in previous studies (Terajima et al., 1997; Papi and Johnston, 1999).

In the present study, we observed that infection of type 14 rhinovirus increased the production of interleukin-1β, interleukin-6, interleukin-8, and TNF-α in the cultured human tracheal epithelial cells, and that lansoprazole reduced the baseline and rhinovirus infection-induced increases in cytokine production. These cytokines induce the growth and differentiation of T and B lymphocytes, production of other cytokines, prostaglandin E_2 synthesis, and degranulation from neutrophils (Akira et al., 1990). Furthermore, these cytokines are known to mediate a wide variety of proinflammatory and immunoregulatory effects (Akira et al., 1990), and are suggested to play an important role in the pathogenesis of rhinovirus infections. Therefore, lansoprazole may inhibit the airway inflammation as well as rhinovirus infection.

In addition to lansoprazole, an antibody to interleukin-1β also reduced the baseline and type 14 rhinovirus infection-induced ICAM-1 mRNA expression and sICAM-1 concentrations in supernatants. On the other hand, the magnitude of inhibitory effects of lansoprazole on the baseline and type 14 rhinovirus infection-induced ICAM-1 mRNA expression and sICAM-1 concentrations was significantly larger than that of antibody to interleukin-1β. Lansoprazole also reduced baseline and type 14 rhinovirus infection-induced interleukin-1β release in supernatants. Therefore, lansoprazole might reduce baseline and type 14 rhinovirus infection-induced ICAM-1 production partly via endogenous interleukin-1β production and via the other mechanisms. Further studies are needed to clarify the mechanisms.

The endosomal pH is suggested to be regulated by vacuolar H^+-ATPases (Mellman et al., 1986). Furthermore, endosomal pH is also regulated by ion transport across the Na^+/H^+ antiporters (Marshansky and Vinay, 1996; Nass and Rao, 1998). Inhibitors of Na^+/H^+antiporters 5-(*N*-ethyl-*N*-isopropyl)amiloride (EIPA) and *N*″-[3-(hydroxymethyl)-5-(1*H*-pyrrol-1-yl)benzoyl]guanidine methonesulfonate (FR168888) as well as a vacuolar H^+-ATPase inhibitor bafilomycin increase endosomal pH and inhibit type 14 rhinovirus infection in cultured human tracheal epithelial cells (Suzuki et al., 2001). In the present study, lansoprazole increased the endosomal pH, and the effect of lansoprazole on acidic endosomes was stable for at least 7 days. Although we have no data to demonstrate whether lansoprazole inhibits vacuolar H^+-ATPases or Na^+/H^+ antiporters, another proton pump inhibitor, omeprazole, inhibits vacuolar H^+-ATPases in rat renal cortical and medullary endosomes (Sabolic et al., 1994), suggesting the possibility that lansoprazole may have an inhibitory effect on vacuolar H^+-ATPases in airway epithelial cells.

Recent reports have revealed the mechanisms of rhinovirus entry into the cytoplasm of infected cells (Pérez and Carrasco, 1993; Casasnovas and Springer, 1994; Prchla et al., 1994; Bayer et al., 1998, 1999). Type 14 rhinovirus forms rhinovirus-soluble ICAM-1 complexes and these complexes can release viral RNA (Casasnovas and Springer, 1994). Furthermore, type 14 rhinovirus releases RNA after exposure to an acidic pH (Casasnovas and Springer, 1994), and infection of HeLa cells and human tracheal epithelial

cells by type 14 rhinovirus is inhibited by bafilomycin (Pérez and Carrasco, 1993; Suzuki et al., 2001). Therefore, the entry of type 14 rhinovirus RNA into the cytoplasm of the infected cells appears to be mediated by the destabilization by receptor binding, by endosomal acidification, or both (Casasnovas and Springer, 1994). The inhibitory effects of lansoprazole on infection by type 14 rhinovirus and its effects on the endosomal pH in the present study are consistent with those of bafilomycin and erythromycin in previous studies (Pérez and Carrasco, 1993; Prchla et al., 1994; Suzuki et al., 2001, 2002).

In summary, this is the first report that the proton pump inhibitors, lansoprazole and omeprazole, inhibit infection by type 14 rhinovirus and decrease the susceptibility of cultured human tracheal epithelial cells to type 14 rhinovirus infection, probably through the inhibition of ICAM-1 expression and endosomal acidification. Lansoprazole reduced baseline and rhinovirus infection-induced release of proinflammatory cytokines in supernatants including interleukin-1β. An antibody to interleukin-1β reduced baseline and rhinovirus infection-induced ICAM-1 expression and sICAM-1 concentrations. On the other hand, the magnitude of inhibitory effects of lansoprazole on the baseline and type 14 rhinovirus infection-induced ICAM-1 mRNA expression and sICAM-1 concentrations was significantly larger than that of antibody to interleukin-1β. Therefore, lansoprazole might reduce baseline and type 14 rhinovirus infection-induced ICAM-1 production partly via endogenous interleukin-1β production and via the other mechanisms. Proton pump inhibitors, including lansoprazole, may inhibit the infection of major subgroups of rhinoviruses, and modulate the inflammatory responses in the airway epithelial cells after rhinovirus infection. However, clinical applications to prevent rhinovirus infection and treat the airway inflammation caused by rhinovirus infection are still uncertain. Further studies are needed to clarify the clinical usefulness of lansoprazole and omeprazole for rhinovirus infection.

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