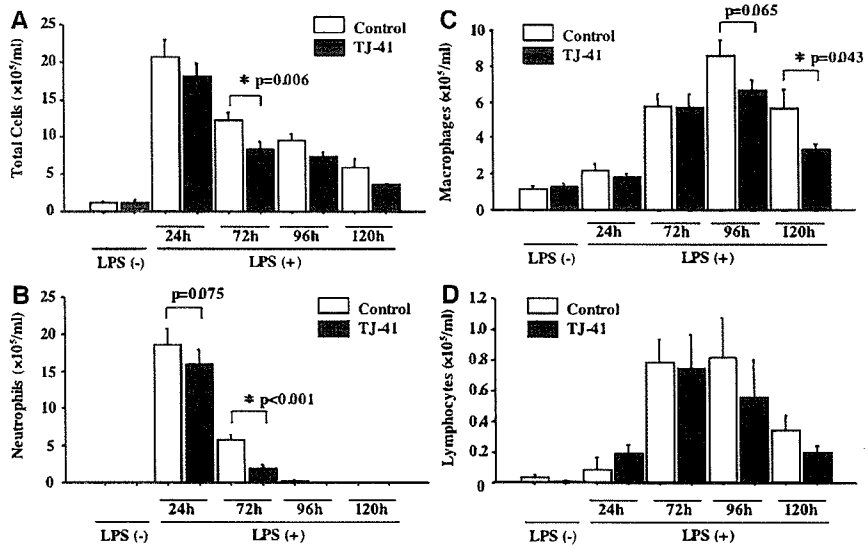

Fig. 2 Effects of TJ-41 on BALF cell analysis in a LPS-induced acute lung injury model. TJ-41 was administered for 8 weeks before LPS challenge and then was continued to 120 h after LPS challenge. BALF was collected and cell differentiation was determined before and at the indicated times after the intranasal administration of LPS, as described in Materials and Methods. Data are presented as the mean ± SEM (n = 5–10 in each group). *p < 0.05 compared with the control mice at each time point after LPS challenge

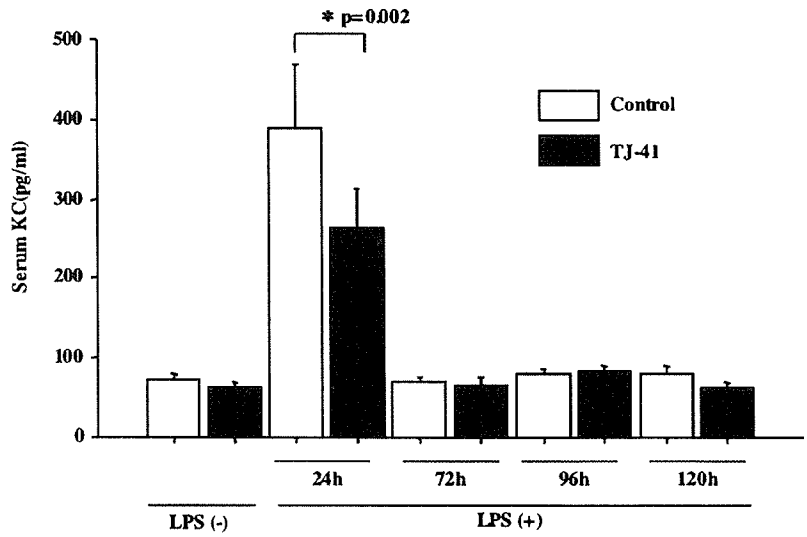

Fig. 3 Effects of TJ-41 on KC concentration in the serum in a LPS-induced acute lung injury model. TJ-41 was administered for 8 weeks before LPS challenge and then was continued to 120 h after LPS challenge. Serum was collected before and at the indicated times after the intranasal administration of LPS, as described in Materials and Methods. Data are presented as the mean ± SEM (n = 5–10 in each group). *p < 0.05 compared with control mice at each time point after LPS challenge

peaked at 24 h after LPS challenge and thereafter rapidly decreased (Fig. 3). This elevation was significantly attenuated by the preadministration of TJ-41 (p = 0.002; Control: 390.17 ± 79.37 pg/ml vs. TJ-41: 264.96 ± 47.02 pg/ml) (Fig. 3). On the other hand, the serum levels of GM-CSF in TJ-41-treated mice at 72 h after LPS challenge tended to be lower than those in untreated mice (p = 0.172; Control: 33.53 ± 7.64 pg/ml vs. TJ-41: 24.28 ± 9.06 pg/ml).

Effects of TJ-41 on Histopathologic Changes

After LPS challenge, edematous thickening of alveolar septa with the infiltration of neutrophils and macrophages and a few lymphocytes were observed (Fig. 4B-D). Neutrophils also infiltrated the alveolar space (Fig. 4B-D). At 24 h after LPS challenge, the alveolar thickening and cellular infiltration became most severe (Fig. 4B). After LPS challenge, alveolitis

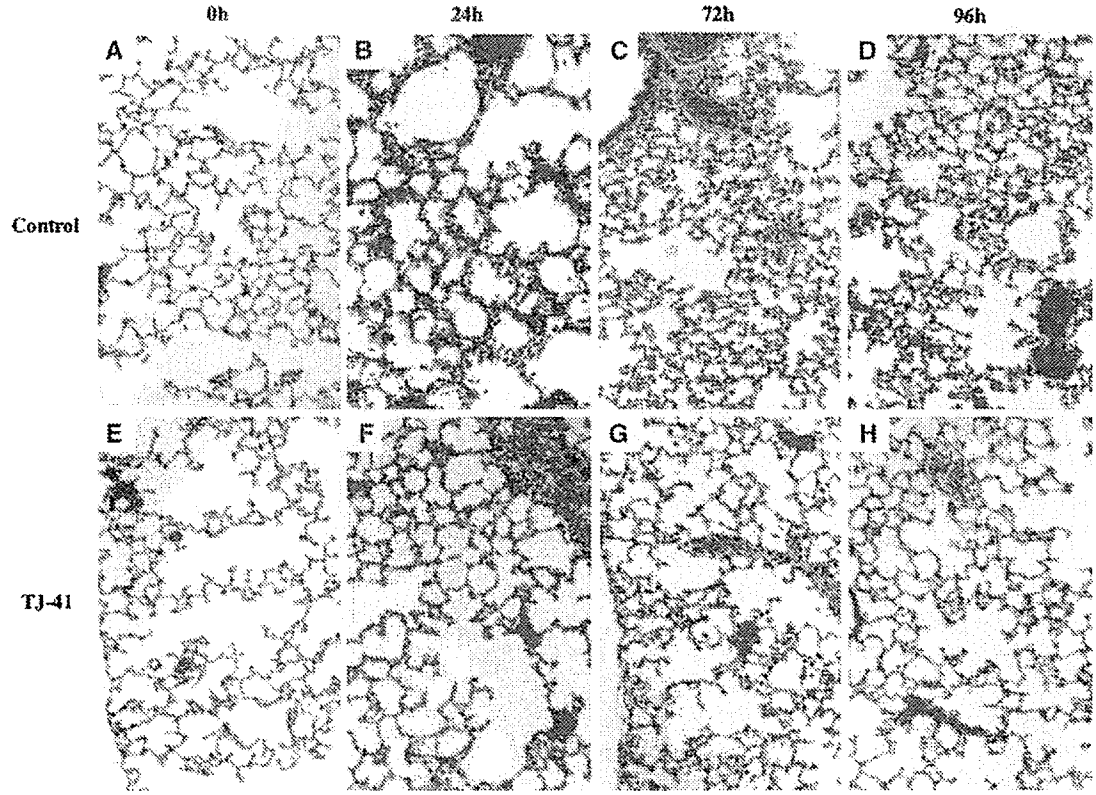

Fig. 4 Effects of TJ-41 on histopathologic changes in LPS-induced acute lung injury model. Representative pictures of lung tissue specimens from mice that received ordinary feed for 8 weeks (**A–D**) and those that received feed mixed with TJ-41 for 8 weeks (**E–H**). Histopathology was compared between the two groups at 0 h (**A, E**), 24 h (**B, F**), 72 h (**C, G**), and 96 h (**D, H**) after LPS challenge. No effect on the lung tissue findings was observed in mice that consumed feed mixed with TJ-41 for 8 weeks (**E**) compared with mice that consumed ordinary feed for 8 weeks (**A**). Alveolar thickening with the cellular infiltration of monocytes, neutrophils, and a few lymphocytes and neutrophil infiltration in the alveolar space are seen, particularly at 24 h after LPS challenge (**B, F**); however, these features were less severe in TJ-41-treated mice (**F**) [original × 100]

and neutrophil infiltration in the alveolar space was less severe in TJ-41-treated mice than in control mice (Fig. 4F-H).

Discussion

Although Ohtake et al. [10, 11] demonstrated that the oral administration of Sho-saiko-to ameliorated lung injury induced by intranasal LPS instillation in BALB/c mice, Ishizaki et al. [5] showed that Sho-saiko-to tended to induce acute pneumonitis by allergic-immunologic mechanisms in patients with chronic active hepatitis. Indeed, in some cases, Shosaiko-to leads to interstitial pneumonia, especially when used together with IFN, and some cases become serious. Therefore, we have tried to find other Japanese herbal medicines that have a protective effect on ALI/ARDS without such adverse effects. In this study we examined the preventive effects of Hochu-ekki-to on an LPS-induced ALI model. In BALF, the preadministration of TJ-41 caused a significant reduction in the total cells, neutrophils, and macrophages. The preadministration of TJ-41 significantly inhibited the increase in serum KC concentration at 24 h after LPS challenge. Furthermore, the histopathologic findings indicated that alveolitis was less severe in TJ-41-treated mice than in control mice. These findings suggest that the preadministration of TJ-41 could be expected to show an inhibitory effect on ALI/ARDS via the modulation of proinflammatory cytokines.

Clinical and experimental studies have provided circumstantial evidence of the occurrence of neutrophil-mediated injury in ALI/ARDS. Histologic studies of human lung specimens obtained early in the course of the disorder show marked accumulation of neutrophils [16]. Neutrophils predominated in pulmonary edema fluid and BALF obtained from the affected patients, and many animal models of ALI are neutrophil-dependent [16]. In the acute phase of ALI/ARDS, neutrophils are shown to adhere to the injured capillary endothelium and then migrate through the interstitium

into the air space, which is filled with protein-rich edema fluid. In the air space, alveolar macrophages secret cytokines, IL-1, IL-6, IL-8, and IL-10 and TNFα, all of which act locally to stimulate chemotaxis and activate neutrophils [16]. As shown in Figure 3, the serum levels of KC, which is a chemotaxin for neutrophils similar to human IL-8, in LPS-induced ALI mice receiving TJ-41 were significantly lower than those in control mice at 24 h after LPS challenge. These reductions may have contributed to the decreased neutrophil count in BALF of the LPS-induced ALI model treated with TJ-41 (Fig. 2B). It is of interest that KC production was affected by TJ-41, and a study of its suppression mechanism is now underway.

As shown in Figure 2C, the preadministration of TJ-41 caused a significant reduction in the macrophages in BALF of the LPS-induced ALI model. In one study of BALF from patients with ALI/ARDS, GM-CSF has been shown to increase [8]. GM-CSF is thus considered to play a role in the proliferation, differentiation, and survival of macrophages, while also regulating the expansion and maturation of their precursors [1]. The serum levels of GM-CSF in TJ-41-treated mice at 72 h after LPS challenge tend to be lower than those in untreated mice but the difference is not significant. The administration of TJ-41 decreased BALF concentrations of GM-CSF after influenza virus infection in mice [9]. We could not detect GM-CSF in the BALF by ELISA at any time after LPS challenge in our LPS-induced ALI model (data not shown). Further analysis is thus required to investigate the mechanism of the suppressive effect of TJ-41 on macrophage infiltration in the lungs of LPS-induced injury.

One possible explanation for the preventive effect of TJ-41 on ALI in this mouse model is its antioxidant effect. We expect that TJ-41 suppresses ROS production in an LPS-induced ALI model. We measured the amount of LPO in the BALF and serum, which was an indicator of oxidative stress [3]. The results of this study, however, demonstrated that the serum and BALF levels of LPO did not decrease in TJ-41-treated mice. These findings suggest that TJ-41 might have only a slight effect on ROS production in the lungs of the LPS-induced ALI model.

In summary, the results of this study proved that the preadministration of TJ-41 reduced LPS-induced ALI in BALB/c mice. The ameliorating effects of neutrophil infiltration on the lung seemed to be due to reduced KC production by TJ-41. These findings suggest that the preadministration of TJ-41 may therefore modulate the process of ALI/ARDS via regulating proinflammatory cytokine production. However, the precise mechanism by which TJ-41 modulates proinflammatory cytokine production remains obscure. Further studies are required to elucidate this issue.

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Mutsuo Yamaya

送信者： <bjp@bps.ac.uk>
宛先： <yamaya@geriat.med.tohoku.ac.jp>
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--- Full paper ---

Hochu-ekki-to inhibits rhinovirus infection in human tracheal epithelial cells

by

[1]Mutsuo Yamaya, [1]Takahiko Sasaki, [1]Hiroyasu Yasuda, [1]Daisuke Inoue, [1]Tomoko Suzuki, [1]Masanori Asada, [1]Motoki Yoshida, [1]Takashi Seki, [1]Kou Iwasaki, [2]Hidekazu Nishimura, & [1]Katsutoshi Nakayama

[1]Department of Geriatric and Respiratory Medicine, Tohoku University School of Medicine, Sendai, 980-8574, and [2]Virus Research Center, Clinical Research Division, Sendai National Hospital, Sendai 983-8520, Japan

Short title: Japanese herbal medicine and rhinovirus infection

Send correspondence to: Mutsuo Yamaya, M.D.
Department of Geriatric and Respiratory Medicine,
Tohoku University School of Medicine,
1-1 Seiryo-machi, Aoba-ku, Sendai, 980-8574, Japan
TEL: 81-22-717-7182
FAX: 81-22-717-7186
E-mail: yamaya@geriat.med.tohoku.ac.jp

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Abstract

Background and purpose: A traditional Japanese herbal medicine, hochu-ekki-to, has been used for the treatment of complaints of general fatigue caused by common colds. Hochu-ekki-to reduces the frequency of common cold in patients with chronic obstructive pulmonary disease. However, the inhibitory effects of hochu-ekki-to on the infection of rhinovirus (RV), the major cause of common colds, have not been studied.

Experimental approach: To examine the effects of hochu-ekki-to on RV infection in airways, human tracheal epithelial cells were infected with a major group rhinovirus-RV14.

Key results: RV14 infection increased virus titers, the content of cytokines in supernatants, and RV14 RNA in the cells. Hochu-ekki-to reduced supernatant virus titers, RV14 RNA in the cells, the susceptibility to RV infection, and supernatants cytokines concentrations including interleukin (IL)-1β, IL-6, IL-8 and tumor necrosis factor (TNF)-α after RV14 infection. Hochu-ekki-to reduced the mRNA expression of intercellular adhesion molecule (ICAM)-1, the receptor for RV14, supernatants concentrations of soluble form of ICAM-1, and the number and fluorescence intensity of acidic endosomes in the cells, from which RV RNA enters into the cytoplasm, at RV14 infection. Glycyrrhizin, one of biochemical compositions of hochu-ekki-to, reduced supernatant virus titers dose-dependently.

Conclusion and implications: These results suggest that hochu-ekki-to inhibits RV14 infection by reducing ICAM-1 and by blocking the RV RNA entry into the cytoplasm from the endosomes in airway epithelial cells. Glycyrrhizin may be partly associated with the inhibitory effects of hochu-ekki-to on RV14 infection. Hochu-ekki-to may modulate airway inflammation by reducing the production of cytokines in rhinovirus infection.

Key words: Hochu-ekki-to, common cold, intercellular adhesion molecule, endosome, rhinovirus

Abbreviations: CO_2, carbon dioxide; COPD, chronic obstructive pulmonary disease; DMSO, dimethylsulfoxide; EIA, enzyme linked immunoassay; ELISA, enzyme-linked immunosorbent assay; ICAM-1, intercellular adhesion molecule-1; LDH, lactate dehydrogenase; IFN, interferon; IL, interleukin; RV, rhinovirus; sICAM-1, soluble form of ICAM-1; TCID, tissue culture infective dose; TNF, tumor necrosis factor; USG, ultroser G..

Introduction

Rhinoviruses (RVs) are the major cause of the common cold and the most common acute infection illnesses in humans (Couch, 2001), and are also associated with acute exacerbations of bronchial asthma (Johnston *et al.*, 1995; Nicholson *et al.*, 1993) and chronic obstructive pulmonary disease (COPD) (Seemungal *et al.*, 2001; Sethi, 2004). Several mechanisms have been proposed, and the manifestations of RV-induced pathogenesis are suggested to be the result of virus-induced mediators of inflammation (Johnston, 2005; Sethi, 2004; Zhu *et al.*, 1996).

Hochu-ekki-to is a traditional Japanese herbal medicine which originated in China and is composed of 10 species of medical plants. It has been used for the treatment of complaints of general fatigue caused by common colds and of severe weakness. Sugiyama *et al.* (1997) administered hochu-ekki-to to 9 patients with COPD for 4 or 5 months, and found that the frequency of common cold in patients treated with hochu-ekki-to for 3 months was significantly lower than that in 17 patients without treatment of hochu-ekki-to. Based on these results, they concluded that hochu-ekki-to reduces the frequency of common cold in patients with COPD. Hochu-ekki-to has various immunoactive effects, including increased immunity in elderly persons (Kuroiwa *et al.*, 2004), mitogenic activity of lymphocytes (Iwama, *et al.*, 1986), and an augmentation of natural killer activity (Utsuyama *et al.*, 2001). However, the inhibitory effects of hochu-ekki-to on RV infection, the major cause of COPD exacerbations (Seemungal *et al.*, 2001; Sethi, 2004), are still uncertain.

Recent reports revealed that the major group of RVs enters the cytoplasm of infected cells after binding to its receptor intercellular adhesion molecule-1 (ICAM-1) (Casanovas & Springer, 1994; Greve *et al.*, 1989). The entry of RNA of a major group rhinovirus-RV14 into the cytoplasm of infected cells is suggested to be mediated by destabilization from receptor binding and by endosomal acidification (Casasnovas &

Springer, 1994). Macrolide antibiotics bafilomycin (Pérez & Carrasco, 1993; Suzuki *et al.*, 2001) and erythromycin (Suzuki *et al.*, 2002) inhibit the infection of the major group of RVs via the reduction of ICAM-1 expression and via the increase in endosomal pH. Glucocorticoid also inhibits RV14 infection via the reduction of ICAM-1 (Suzuki *et al.*, 2000). Hochu-ekki-to protects against murine cytomegalovirus infection (Hossain *et al.*, 1999) and *Listeria monocytogenes* infection (Yamaoka *et al.*, 1998). However, the effects of hochu-ekki-to on the RV infection have not been studied.

RV infection induces the production of cytokines including interleukin (IL)-1, IL-6 and IL-8 (Subauste *et al.*, 1995; Terajima *et al.*, 1997; Zhu *et al.*, 1996). These cytokines have proinflammatory effects (Akira *et al.*, 1990) and may be related to the pathogenesis of RV infections (Couch, 2001; Johnston, 2005). Hochu-ekki-to increases production of interferon (IFN)-γ in a murine model (Ishimitsu *et al.*, 2001). However, the effects of hochu-ekki-to on the cytokine production in airway epithelial cells by RV infection have not been studied.

Although the effects of hochu-ekki-to on the ICAM-1 expression and acidic endosomes are uncertain, we studied the effects of hochu-ekki-to on the RV infection in human airway epithelial cells. We also examined the effects of hochu-ekki-to on the production of ICAM-1 and cytokines, and on the endosomal pH to clarify the mechanisms responsible for the inhibition of RV infection.

Methods

Human tracheal epithelial cell culture

Isolation and culture of the human tracheal surface epithelial cells were performed as described previously (Suzuki *et al.*, 2001; 2002; Terajima *et al.*, 1997). The human tracheal surface epithelial cells were plated at 5×10^5 viable cells/ml in plastic tubes with round bottoms (16 mm diameter and 125 mm length, Becton Dickinson) coated with human placental collagen. Cells were immersed in 1 ml of a mixture of DMEM-Ham's F-12 (DF-12) medium (50/50, vol/vol) containing 2% ultroser G (USG) (BioSepra, Cergy-Saint-Christophe, France) and antibiotics (Suzuki *et al.*, 2001; 2002; Terajima *et al.*, 1997). The opening of the tubes was loosely covered with a screw cap to make air containing CO_2 move through the slit. Cells were immersed in 1 ml of medium and the tubes were laid and kept stationary in a humid incubator with a slant of ~ 5° (Suzuki *et al.*, 2001; 2002; Terajima *et al.*, 1997). Because of this position of the plastic tubes, the cells were attached and proliferated mainly on the inner surface of the lateral wall of the tubes, and the surface area of culture vessels of the plastic tubes covered by the cells became 11.4 ± 0.1 cm^2 (n=3). Cells in the tubes were cultured at 37°C in 5% CO_2-95% air.

Tracheas for cell cultures were obtained after death from 25 patients (age, 72 ± 3 yr; 9 female, 16 male) without complications with bronchial asthma or COPD. The causes of death included malignant tumor other than lung cancer (n=9), acute myocardial infarction (n=4), renal failure (n=3), congestive heart failure (n=2), cerebral bleeding (n=2), rupture of an aortic aneurysm (n=1), cerebral infarction (n=1), sepsis (n=1), mitral stenosis (n=1), and malignant lymphoma (n=1). Of 25 patients, 7 patients were ex-smokers, and 18 patients were non-smokers. This study was approved by the Tohoku University Ethics Committee.

Preparations and biochemical composition of hochu-ekki-to

Hocchu-ekki-to is composed of 10 species of medical plants, including *Astragali Radix, Atractylodis Lanceae Rhizoma, Ginseng Radix, Angelicae Radix, Bupleuri Radix, Zizyphi Fructus, Aurantii Nobilis Pericarpium, Glycyrrhizae Radix, Cimicifugae Rhizoma* and *Zingiberis Rhizoma.* Analysis of the biochemical composition of hochu-ekki-to with 3D HPLC revealed that the major components are hesperidin, glycyrrhizin and saikosaponin b2 (data from Tsumura Co., Tokyo, Japan), as described below. Furthermore, other compositions including in hochu-ekki-to were astragaloside IV, hinesol, ginsenoside, Z-ligustilide (data from Tsumura), although their contents were not measured.

Preparation of culture medium containing hochu-ekki-to

To prepare the culture medium containing hochu-ekki-to, the powder form of hochu-ekki-to was obtained from Tsumura Co., and was dissolved in dimethylsulfoxide (DMSO) (10 mg/ml) by vortexing for 1 min at room temperature. The solution was centrifuged at 1000 rpm for 5 min to remove any insoluble ingredients. The concentration of hesperidin, one of major components of hochu-ekki-to, has been used as an indicator of the concentration of hochu-ekki-to in supernatant. Analysis of the biochemical composition of hochu-ekki-to with 3D HPLC revealed that hochu-ekki-to (1g) contains 5.7 mg of hesperidin, 5.0 mg of glycyrrhizin and 0.1 mg of saikosaponin b2 (data from Tsumura Co.), suggesting that hochu-ekkito contains the same level of hesperidin and glycyrrhizin. On the other hand, the powder form of *Glycyrrhizae Radix*, liquorice roots, (1g) contains 25 mg of glycyrrhizin. The peak plasma concentration of glycyrrhizin was 228 ± 106 ng/ml (n =12) 13 h after ingestion of 0.5 g of the powder form of liquorice roots (data from Tsumura Co.). Therefore, after ingestion of 2.5 g hochu-ekki-to, a usual oral dose, the peak plasma concentration of glycyrrhizin was assumed to be 228 ng/ml. Furthermore, the plasma concentration of

glycyrrhizin was kept at 100 ng/ml after ingestion of 0.5 g of liquorice roots 24 h after (data from Tsumura Co.). Based on these data, the plasma concentration of hesperidin and glycyrrhizin was assumed to be 100 ng/ml. In the present study, the concentration of glycyrrhizin in the supernatants of hochu-ekki-to (20 mg) dissolved in DMSO (2 ml) was 43 ± 1 μg/ml (n=3). Furthermore, the concentration of hesperidin in the supernatants of hochu-ekki-to (20 mg) dissolved in DMSO (2 ml) was 56 ± 1 μg/ml (n=3). Therefore, cells were then exposed to diluted supernatants of hochu-ekki-to that contained 100 ng/ml of hesperidin.

Furthermore, the effects of hesperidin and glycyrrhizin on RV14 infection in human tracheal epithelial cells were also studied. The concentrations of hesperidin and glycyrrhizin were defined from these data. The supernatant containing hesperidin (Sigma) or glycyrrhizin (Wako, Japan) in DMSO (10mg/ml) was diluted in double distilled water and in DF-12 medium containing 2% USG and antibiotics.

Effects of hochu-ekki-to on viral infection

To examine the effects of hochu-ekki-to on the viral titers and the cytokine contents in supernatants, and the expression of ICAM-1 and RV14 RNA in the cells, the cultured human tracheal epithelial cells were treated with 100 ng/ml of hochu-ekki-to or vehicle (DMSO, 0.2 %) from 3 days before RV14 infection until the end of the experiments after RV14 infection (Suzuki *et al.*, 2001). The cells were then exposed to RV14 (10^5 $TCID_{50}$ units/ml) or vehicle (Eagle's minimum essential medium) for 60 min. The opening of tubes was sealed with rubber plugs and cells were cultured at 33°C with rolling in an incubator (HDR-6-T, Hirasawa, Tokyo, Japan) as previously described (Suzuki *et al.*, 2001; 2002; Terajima *et al.*, 1997).

In order to measure the time course of viral release during the first 24 h, we used three separate cultures from the same trachea. We collected the culture supernatants at either 1, 12, or 24 h after RV14 infection. Furthemore, to measure the viral titer during

1 to 3 days after RV14 infection, we used one culture from each trachea after collecting supernatants at 1 day (24 h) after RV14 infection. After collecting supernatants at 1 day after infection, the cells were rinsed with PBS and 1 ml of DF-12 medium containing 2% USG was replaced. Supernatants were also collected at 3 days after infection. Likewise, to measure the viral titer during 3 to 5 days after RV14 infection, after collecting supernatants at 3 days after infection, the cells were rinsed with PBS and 1 ml of the fresh medium was replaced. Supernatants were also collected at 5 days after RV14 infection. The cells were then rinsed with PBS and 1 ml of the fresh medium was replaced. Supernatants were also collected at 7 days after infection to measure the viral titer during 5 to 7 days after RV14 infection.

Viral stocks, and detection and titration of viruses

RV14 stocks were prepared from patients with common colds by infecting human embryonic fibroblast cells as described (Suzuki *et al.*, 2001; 2002; Terajima *et al.*, 1997). Detection and titration of RV14 were performed by observing the cytopathic effects of viruses on the fibroblast cells with methods as previously described (Suzuki *et al.*, 2001; 2002; Terajima *et al.*, 1997). The fibroblast cells were exposed to collected supernatants and the amount of specimen required to infect 50% of the fibroblast cells (tissue culture infective dose [$TCID_{50}$]) was determined. To demonstrate the time course of viral release, we expressed the rates of change in RV14 concentration in the supernatant. The rates were obtained by dividing the value of RV14 titer ($TCID_{50}$ units per ml) in supernatants by incubation time, and are expressed as $TCID_{50}$ units/ml/24h.

Qantification of rhinovirus RNA

To quantify the RV RNA and GAPDH mRNA expression in the cells after RV14 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 (Heid *et al.*, 1996; Holland *et al.*, 1991; Martell *et al.*, 1999). 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 standard curve was obtained between the fluorescence emission signals and C_T by means of 10-fold dilutions of the total RNA, extracted from 10^5 $TCID_{50}$ units/ml of RV14 in the supernatants of the human embryonic fibroblasts 7 days after infection with RV14 (10^4 $TCID_{50}$ units/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.

Study protocol

To examine the concentration-dependent effects of hochu-ekki-to on RV14 infection, cells were treated with hochu-ekki-to at concentrations ranging from 0.1 ng/ml to 500 ng/ml.

The effects of hochu-ekki-to on the susceptibility to RV14 infection were evaluated as previously described (Subauste *et al.*, 1995; Suzuki *et al.*, 2002) using epithelial cells pretreated with hochu-ekki-to (100 ng/ml, 3 days) or vehicle (DMSO, 0.2 %, 3 days). The epithelial cells were then exposed to serial 10-fold dilutions of RV14 or vehicle of RV14 (Eagle's minimum essential medium) for 1 h at 33°C. The presence of RV 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 RV used.

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 linked immunoassay (EIA).

Measurement of cytokine production

We measured IL-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α of culture supernatants by specific enzyme-linked immunosorbent assays (ELISAs) (Suzuki *et al.*, 2002; Terajima *et al.*, 1997). In order to examine the effects of hochu-ekki-to, supernatants were collected before and after RV14 infection. Cells were pretreated with hochu-ekki-to for 3 days before RV14 infection. 24 h before RV14 infection, cells were rinsed with PBS, and fresh DF-12 medium containing 2% USG was replaced. Supernatants were collected just before RV14 infection, and these supernatants were used as supernatants before RV14 infection for the measurement of cytokine release. After RV14 infection, supernatants were collected at 1, 3 and 5 days after RV14 infection. Cells were rinsed with PBS and fresh DF-12 medium containing 2% USG was replaced. Then supernatants were also collected at 7 days after RV14 infection. These supernatants were used as supernatants at 1 day, 3 days, 5 days and 7 days after RV14 infection for the measurement of cytokine release. To demonstrate the time course of cytokines release, we expressed the rates of change in cytokines concentration in the supernatant. The rates were obtained by dividing the value of cytokines concentration in supernatants by incubation time, and are expressed as pg/ml/24h.

Measurement of changes in acidic endosomes distribution

The fluorescence intensity of acidic endosomes in the cells was measured as previously described with a dye, LysoSensor DND-189 (Molecular Probes) (Suzuki *et al.*, 2001; 2002). The effects of hochu-ekki-to on the distribution of acidic endosomes were examined from 100 sec before to 300 sec after the treatment with hochu-ekki-to

(100 ng/ml) or vehicle (DMSO, 0.2 %). Fluorescence intensity of acidic endosomes was measured in 100 human tracheal epithelial cells, and mean value of fluorescence intensity was expressed as % of control value compared with the fluorescence intensity of the cells treated with vehicle of hochu-ekki-to (DMSO, 0.2 %).

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).

Statistical analysis

Results are expressed as means ± s.e. Statistical analysis was performed using two-way repeated measures of 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.

Results

Effects of hochu-ekki-to on rhinovirus infection in human tracheal epithelial cells

Exposing confluent human tracheal epithelial cell monolayers to RV14 (10^5 $TCID_{50}$ units/ml) consistently led to infection. No detectable virus was revealed at 1 h after infection. RV14 was detected in culture medium at 12 h, and the viral content progressively increased between 1 and 12 h after infection (Figure 1a). Evidence of continuous viral production was obtained by demonstrating that each of the viral titers of supernatants collected during either 12 to 24 h, 1 to 3 days, 3 to 5 days or 5 to 7 days after infection contained significant levels of RV14 (Figure 1a). The viral titer levels in supernatants increased significantly with time for the first 3 days ($P<0.05$ in each case by ANOVA). Treatment of the cells with hochu-ekki-to (100 ng/ml) significantly decreased the viral titers of RV14 in supernatants from 12 h after infection (Figure 1a). RV14 titer levels in culture supernatants of the cells from 7 ex-smokers did not differ from those from 18 non-smokers (data not shown). No virus was detected in supernatants after infection of ultraviolet (UV)-inactivated RV14 (Terajima *et al.*, 1997).

Further evidence of the inhibitory effects of hochu-ekki-to on RV14 replication in human tracheal epithelial cells was provided by real-time RT-PCR analysis. The amount of RV14 RNA in the cells increased with time until 120 h after RV14 infection (Figure 1b). Hochu-ekki-to (100 ng/ml) also reduced the amount of RV14 RNA in the cells (Figure 1b). No RV14 RNA was detected in the cells after infection of ultraviolet (UV)-inactivated RV14.

Hochu-ekki-to inhibited RV14 infection concentration-dependently and the maximum effect was obtained at 100 ng/ml and 500 ng/ml (Figure 2a).

To examine the effects of the biochemical components of hochu-ekki-to on RV14 infection, cells were pretreated with either glycyrrhizin or hesperidin with concentrations ranging from 0.1 ng/ml to 500 ng/ml. Glycyrrhizin reduced RV14 titers

of supernatants collected during 1 to 3 days concentration-dependently and the maximum effect was obtained at 100 ng/ml and 500 ng/ml (Figure 2b). The inhibitory effects of glycyrrhizin were consistent and stable even when the solution of glycyrrhizin was stored in a refrigerator for 48 h (data not shown). On the other hand, RV14 titers in the cells treated with 1 ng/ml of hochu-ekki-to were significantly lower than those in the cells treated with vehicle of hochu-ekki-to (DMSO, 0.2%) (Figure 2a). In contrast, RV14 titers in the cells treated with 1 ng/ml of glycyrrhizin did not differ from those in the cells treated with vehicle of glycyrrhizin (DMSO, 0.2%) (Figure 2b). Hesperidin did not reduce RV14 titers of supernatants collected during 1 to 3 days (Figure 2c).

To examine whether RV14 infection or hochu-ekki-to induced cytotoxic effects on the cultured cells and caused cell detachment from the tubes after the cells made a confluent sheet, we counted the cell numbers after RV14 infection and after the treatment with hochu-ekki-to. The cell numbers were constant in the confluent epithelial cells in the control medium, and the coefficient of variation was small (6.8%; n=15). Neither RV14 infection (10^5 $TCID_{50}$ units/ml; 5 days) nor hochu-ekki-to treatment (100 ng/ml; 5 days) had any effect on the cell numbers (data not shown). Cell viability, assessed by the exclusion of trypan blue (Terajima *et al.*, 1997), was consistently >96% in the hochu-ekki-to-treated culture. RV14 infection and hochu-ekki-to treatment (100 ng/ml) did not alter the amount of LDH in the supernatants. The amount of LDH in the supernatants was 31 ± 2 IU/l before RV14 infection, 33 ± 3 IU/l 3 days after RV14 infection (P>0.50, n=5), and 32 ± 2 IU/l after hochu-ekki-to treatment (100 ng/ml; 5 days) (P >0.50, n=5).

Likewise, glycyrrhizin (100 ng/ml) did not have an effect on the cell numbers (data not shown). Cell viability was consistently >96% in the glycyrrhizin-treated culture. The amount of LDH in the supernatants after glycyrrhizin treatment (100 ng/ml; 5 days) (32 ± 2 IU/l, n=5) did not differ from that before the treatment (31 ± 2 IU/l, n=5) (P >0.50).

Effects of hochu-ekki-to on susceptibility to type 14 rhinovirus infection

Treatment of the cells with hochu-ekki-to decreased the susceptibility of the cells to infection by RV14. The minimum dose of RV14 necessary to cause infection in the cells treated with hochu-ekki-to (100 ng/ml, 3 days) (3.1 ± 0.2 log $TCID_{50}$ units/ml, n=5, P<0.05) was significantly higher than that in the cells treated with vehicle of hochu-ekki-to (DMSO, 0.2 %) (2.1 ± 0.2 log $TCID_{50}$ units/ml) (n=5).

Effects of hochu-ekki-to on the expression of ICAM-1

To examine the effects of hochu-ekki-to on the expression of ICAM-1, the human tracheal epithelial cells were treated with hochu-ekki-to (100 ng/ml) or vehicle (DMSO, 0.2 %) for 3 days, and the mRNA was extracted and supernatants were collected. Hochu-ekki-to inhibited the baseline ICAM-1 mRNA expression in the cells before RV14 infection (Figure 3a). Hochu-ekki-to reduced the ICAM-1 mRNA expression by more than 50% compared with that of the cells treated with vehicle of hochu-ekki-to (DMSO, 0.2 %) (Figure 3a). Furthermore, concentrations of sICAM-1 in supernatants in the cells treated with hochu-ekki-to (100 ng/ml) was significantly lower than those in the cells treated with vehicle of hochu-ekki-to (Figure 3b).

Effects of hochu-ekki-to on cytokine production

Hochu-ekki-to (100 ng/ml) reduced the baseline secretion of IL-1β, IL-6 and IL-8 for 24 h before RV14 infection compared with that in the cells treated with vehicle of hochu-ekki-to (DMSO, 0.2 %) (Figure 4). Furthermore, the secretion of IL-6 and IL-8 increased 24 h after RV14 infection, and the secretion of IL-1β increased 3 days after RV14 infection. Hochu-ekki-to (100 ng/ml) also reduced the RV14 infection-induced secretion of IL-1β, IL-6 and IL-8 compared with that in the cells treated with vehicle of hochu-ekki-to (DMSO, 0.2 %) after RV14 infection (Figure 4).

TNF-α was not detectable in supernatants for 24 h before RV14 infection (Figure 4d). In contrast, the secretion of TNF-α increased 3 days after RV14 infection, and hochu-ekki-to reduced the RV14 infection-induced secretion of TNF-α compared with that in the cells treated with vehicle of hochu-ekki-to (DMSO, 0.2 %) 3 days after RV14 infection (Figure 4d). UV-inactivated RV14 did not increase the secretion of IL-1β, IL-6, IL-8 and TNF-α (Figure 4). Furthermore, secretion of IL-1β, IL-6, IL-8 and TNF-α in culture supernatants of the cells from 7 ex-smokers did not differ from those from 18 non-smokers (data not shown).

Effects of hochu-ekki-to on the acidification of endosomes

The effects of hochu-ekki-to on the changes in the distribution and the fluorescence intensity of acidic endosomes were examined from 100 sec before until 300 sec after the treatment with hochu-ekki-to (100 ng/ml) or vehicle (DMSO, 0.2 %). Acidic endosomes in human tracheal epithelial cells were stained green with LysoSensor DND-189 (Figure 5a). Green fluorescence from acidic endosomes was observed in a granular pattern in the cytoplasm (Figure 5a). Hochu-ekki-to (100 ng/ml) decreased the number and the fluorescence intensity of acidic endosomes with green fluorescence in the cells with time (Figure 5). The fluorescence intensity from acidic endosomes in the epithelial cells treated with hochu-ekki-to (100 ng/ml) for 300 sec was significantly reduced (Figure 5b and 5c).

Discussion

In the present study, we have shown that a traditional Japanese herbal medicine, hochu-ekki-to, reduced the viral titers in the supernatants and viral RNA of a major group rhinovirus-RV14, in cultured human tracheal epithelial cells. Pretreatment with hochu-ekki-to inhibited the expression of mRNA and protein of ICAM-1, the receptor for the major group of rhinviruses (RVs) (Greve *et al.*, 1989), before RV14 infection. The magnitude of inhibitory effects of hochu-ekki-to on ICAM-1 mRNA expression was similar to that of dexamethasone and erythromycin as previously described (Suzuki *et al.*, 2000, 2002). Because the minimum dose of RV14 necessary to cause infection in the cells treated with hochu-ekki-to was significantly higher than that in the cells treated with vehicle of hochu-ekki-to, hochu-ekki-to may inhibit RV14 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, hochu-ekki-to reduced the fluorescence intensity of acidic endosomes, from which RV RNA enters into the cytoplasm of the epithelial cells. The magnitude of inhibitory effects of hochu-ekki-to on the fluorescence intensity of acidic endosomes was similar to that of bafilomycin A_1 (Suzuki *et al.*, 2001) and erythromycin (Suzuki *et al.*, 2002). Hochu-ekki-to may also act by inhibiting RV14 RNA entry across acidic endosomes as demonstrated in HeLa cells and human tracheal epithelial cells treated with bafilomycin A_1 (Pérez & Carrasco, 1993; Suzuki *et al.*, 2001) and erythromycin (Suzuki *et al.*, 2002).

Glycyrrhizin, one of biochemical components of hochu-ekki-to, reduced RV14 titers in the supernatants concentration-dependently, while another component hesperidin, did not reduce RV14 titers. On the other hand, the magnitude of the inhibitory effect of glycyrrhizin was smaller than that of hochu-ekki-to. These findings suggest that that glycyrrhizin may be one of biochemical compositions of

hocchu-ekki-to which inhibits rhinovirus infection, although other components may also relate to the inhibitory effects of hochu-ekki-to on RV14 infection. Treatment of the cells with glycyrrhizin did not affect cell viability, and did not increase LDH concentration in the culture supernatants. Therefore, glycyrrhizin may partly relate to the inhibitory effects of hochu-ekki-to on RV14 infection.

RVs are the major cause of the common cold and the most common acute infection illness in humans (Couch, 2001). Furthermore, various viruses have been reported in the exacerbations in patients with COPD and bronchial asthma, including RV, influenza virus and respiratory syncytial virus (Johnston *et al.*, 1995; Nicholson *et al.*, 1993; Seemungal *et al.*, 2001; Sethi, 2004). Seemungal *et al.* reported that 64% of COPD exacerbations were associated with a cold before exacerbations (Seemungal *et al.*, 2004). Seventy-seven viruses were detected in 39% of COPD exacerbations, and 39 (58%) viruses were RV. RVs are also associated with the acute exacerbations of bronchial asthma (Johnston *et al.*, 1995; Nicholson *et al.*, 1993). Thus, RV may be a major pathogen of acute exacerbations of COPD and bronchial asthma.

Various mechanisms have been reported in the pathogenesis of exacerbations of COPD and bronchial asthma, including acute airway inflammation such as airway edema and eosinophil infiltration, acute airway hyperreactivity and mucus hypersecretion (Johnston, 2005; Sethi, 2004; Wedzicha, 2003). 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 observed in patients with bronchial asthma and COPD (Grunberg & Sterk, 1999; Riise *et al.*, 1994). Therefore, reduced ICAM-1 expression by hochu-ekki-to, in the present study, might also suggest the modulation of airway inflammation by hochu-ekki-to after RV infection.

Furthermore, neutrophilic and eosinophilc inflammation in the exacerbations of bronchial asthma and COPD are associated with a variety of mediators including IL-6

and IL-8, the production and secretion of which are stimulated by RV14 in airway epithelial cells as shown in the present and previous studies (Subauste *et al.*, 1995; Terajima *et al.*, 1997; Zhu *et al.*, 1996). Hochu-ekki-to reduces eosinophilia in ovoalbumin-sensitized mice through the reduced production of IL-4 (Ishimitsu *et al.*, 2001), suggesting that hochu-ekki-to may also modulate eosinophil-related inflammation in bronchial asthma. In the present study, RV14 infection increased the production of IL-1β, IL-6, IL-8 and TNF-α, and hochu-ekki-to also reduced RV14 infection-induced production of IL-1β, IL-6, IL-8 and TNF-α. Because hochu-ekki-to reduced viral titer of RV14 in supernatants, inhibiting effects of hochu-ekki-to on RV14 infection and on the cytokines production themselves might be associated with the reduced production of these proinflammatory cytokines in the cells treated with hochu-ekki-to after RV14 infection.

Hochu-ekki-to also reduced the baseline production of cytokines, including IL-1β, IL-6 and IL-8 before RV14 infection. The role of baseline production of these cytokines is uncertain. However, as we previously demonstrated, endogenously produced IL-1β is associated with the expression of ICAM-1 after RV infection in human tracheal epithelial cells (Terajima *et al.* 1997). In the present study, hochu-ekki-to reduced the RV titer levels and IL-1β secretion in culture supernatants of the cells. Pretreatment with hochu-ekki-to also inhibited the expression of mRNA and protein of ICAM-1. These findings suggest that production of ICAM-1 might be reduced by hochu-ekki-to partly through the reduction of IL-1β, and hochu-ekki-to might inhibit RV14 infection at least partly by reducing the production of its receptor, ICAM-1. However, the anti-viral effects and anti-inflammatory effects with baseline reductions of in IL-6 and IL-8 are still uncertain.

The endosomal pH is suggested to be regulated by vacuolar H^+-ATPases (Mellman *et al.*, 1986), and ion transport across the Na^+/H^+ antiporters (Marshansky & Vinay, 1996). Inhibitors of Na^+/H^+ antiporters 5-(N-ethyl-N-isopropyl)amiloride

(EIPA) and N"-[3-(Hydroxymethyl)-5-(1H-pyrrol-1-yl)benzoyl]guanidine methonesulfonate (FR168888) as well as a vacuolar H^+-ATPase inhibitor bafilomycin increase endosomal pH and inhibit RV14 infection in cultured human tracheal epithelial cells (Suzuki *et al.*, 2001). Although we have no data, increased endosomal pH by hochu-ekki-to in the present study may be associated with an inhibitory effect on vacuolar H^+-ATPases or Na^+/H^+ antiporters in the human tracheal epithelial cells.

Recent reports revealed that the major group of RVs enters the cytoplasm of infected cells after binding to its receptor ICAM-1 (Greve *et al.*, 1989). The entry of RNA of a major group rhinovirus-RV14 into the cytoplasm of infected cells is suggested to be mediated by the destabilization from receptor binding and by endosomal acidification (Casasnovas & Springer, 1994). The inhibitory effects of hochu-ekki-to on infection by RV14 and its effects on the endosomal pH in the present study are consistent with those of bafilomycin and erythromycin in previous studies (Pérez & Carrasco, 1993; Suzuki *et al.*, 2001; 2002). Furthermore, the inhibitory effects of hochu-ekki-to on ICAM-1 expression in airway epithelial cells might also be associated with inhibitory effects on RV14 infection, as previously reported on the inhibitory effects of dexamethasone, bafilomycin and erythromycin (Suzuki *et al.*, 2000; 2001; 2002).

Hochu-ekki-to reduced RV14 titer levels by about 1.5 logs of $TCID_{50}$. In contrast, hochu-ekki-to reduced the amount of RV14 RNA in the cells by 25%-50%, suggesting that the magnitude of inhibitory effects of hochu-ekki-to on RV14 RNA replication was smaller that on RV14 release into supernatants. The reason is uncertain. Hochu-ekki-to may also inhibit mechanisms after RV14 RNA replication other than attachment of RV14 to ICAM-1 and RV14 RNA entry through acidic endosomes, although we did not study on them.

Likewise, hochu-ekki-to inhibited ICAM-1 mRNA expression by 60%, sICAM-1 concentrations in supernatants by 45%, and fluorescent intensity from acidic endosomes

by 40% at the RV14 infection. In contrast, hochu-ekki-to inhibited RV14 RNA replication by 32% at 72 h and by 25% at 120 h, although hochu-ekki-to inhibited RV14 RNA replication by 55% at 24 h after RV14 infection. These findings suggest that all viral binding, internalization and release into the cytoplasm, and replication might be equally inhibited by hochu-ekki-to at the RV14 infection and during 24 h after RV14 infection. However, RV14 replication might not be strongly inhibited by hochu-ekki-to during 72 to 120 h after infection, although it is uncertain how hochu-ekki-to inhibits viral binding, internalization and release into the cytoplasm during 72 to 120 h after infection.

The RV14 titer levels in culture supernatants is low, but is consistent with the levels in throat swabs in patients with the symptoms of common cold as previously reported (Numazaki *et al.*, 1987). Zhu *et al.* (1996) demonstrated the increased symptom score of common cold after challenge of RV strain H with 800 $TCID_{50}$ and RV39 with 2,500 $TCID_{50}$. RV titer levels in their study were similar to those of RV14 in supernatants in this study, suggesting that minimal levels of RV infection and virus release may cause the symptoms of common cold. On the other hand, the rate of release of virus is constant from days 3 to 7. We have no data on the viral release after 7 days, and no data to show when it starts to come down. Because the time course of viral release after 7 days is also important on the pathogenesis of RV infection, further examinations are needed.

In summary, this is the first report that a traditional Japanese herbal medicine, hochu-ekki-to, inhibits infection by RV14 and decreases the susceptibility of cultured human tracheal epithelial cells to RV14 infection, probably through the inhibition of ICAM-1 expression and endosomal acidification. Hochu-ekki-to reduced baseline and rhinovirus infection-induced release of proinflammatory cytokines in supernatants including IL-1β, IL-6 and IL-8, and rhinovirus infection-induced release of TNF-α. Hochu-ekki-to may inhibit the infection of the major group of RVs, and modulate the

inflammatory responses in the airway epithelial cells after RV infection.

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