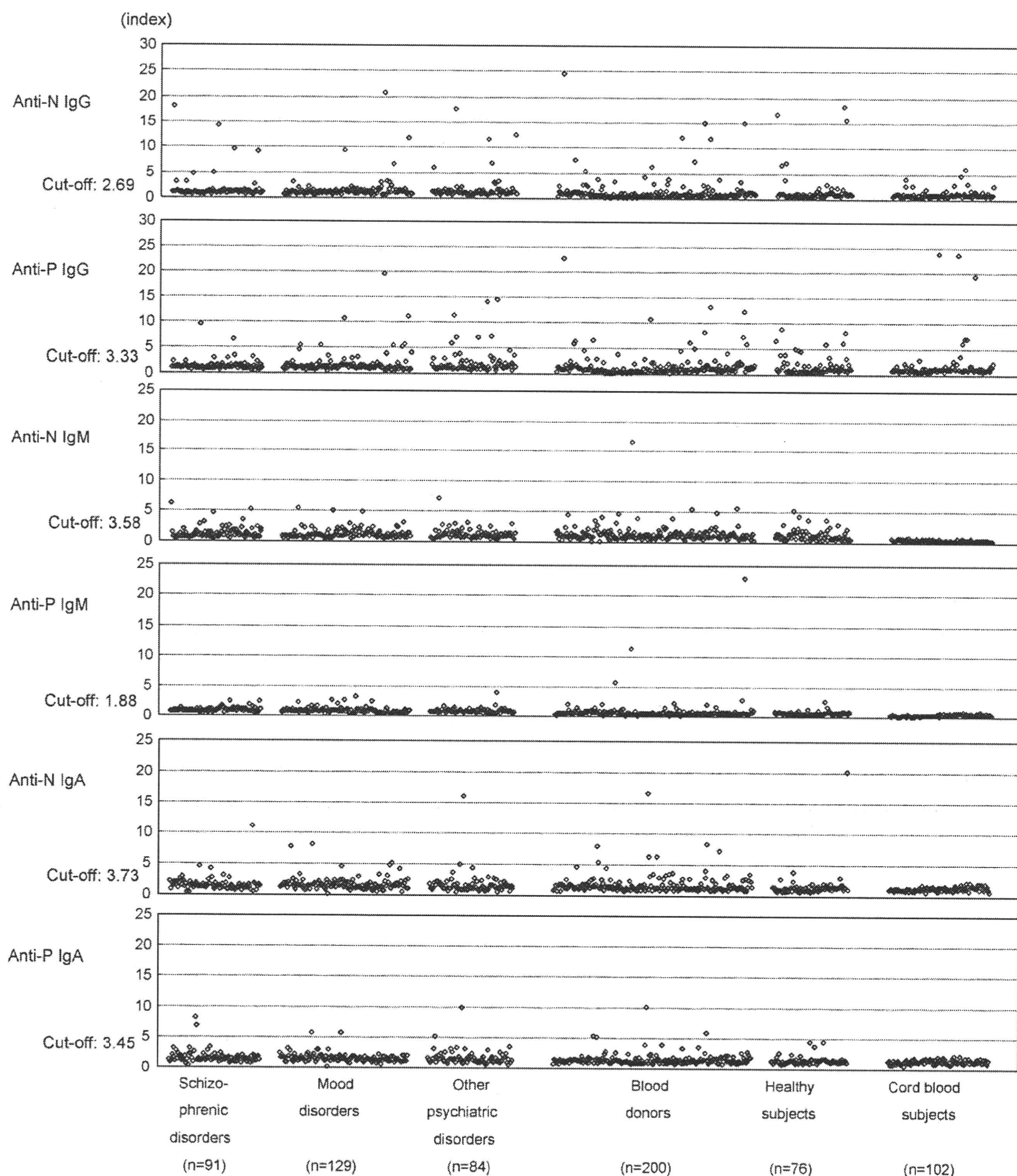


**Fig. 1.** Results of all the subjects for the anti-BDV antibodies.

Seven specific IgM from five patients were re-examined 12–78 months after the first sampling, among which six specific IgM were shown to be persistently positive (Fig. 2).

The results of the avidity examination are shown in Fig. 3. The monoclonal IgG of mouse against BDV-N and the polyclonal rabbit serum against BDV-P showed high avidity incubated in 5 M urea, with the titer persisting at 90% and 75%, respectively. The titer of the human anti-BDV-N and -P IgG decreased to 30–92% and 7–81% in 3 M urea, and 7–61% and 4–23% in 5 M urea, respectively. Most of the specific IgM and IgA showed lower avidity than the specific IgG except a few subjects.

## 4. Discussion

Contrary to most of the other studies, including our previous one,[6] the seroprevalence in control subjects was not signifi-

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**Table 3**
Subjects having two or more anti-BDV antibodies

| Sample no. | Sex | Age | Diagnosis | Anti-N TgG; cut-off point: 2.69 | Anti-P TgG; cut-off point: 3.33 | Anti-N TgM; cut-off point: 3.58 | Anti-P TgM; cut-off point: 1.88 | Anti-N TgA; cut-off point: 3.73 | Anti-P TgA; cut-off point: 3.45 |
|---|---|---|---|---|---|---|---|---|---|
| Samples having four antibodies | | | | | | | | | |
| D58 | | | | 11.93 | 13.04 | (−) | (−) | 8.29 | 6.07 |
| 164 | F | 62 | BPII | 9.40 | 10.71 | (−) | (−) | 4.68 | 5.85 |
| D184 | | | | 6.18 | 10.50 | (−) | (−) | 16.74 | 10.23 |
| Samples having three antibodies | | | | | | | | | |
| D89 | | | | 14.92 | 12.28 | (−) | 2.78 | (−) | (−) |
| 32 | F | 28 | BP I, with psychotic features | (−) | 4.49 | 5.54 | 2.34 | (−) | (−) |
| Samples having two antibodies of the same isotype | | | | | | | | | |
| D104 | | | | 24.58 | 22.69 | (−) | (−) | (−) | (−) |
| 281 | F | 51 | BP I, with psychotic features | 20.84 | 19.69 | (−) | (−) | (−) | (−) |
| H70 | | | | 18.28 | 6.09 | (−) | (−) | (−) | (−) |
| 299 | F | 54 | Panic disorder | 17.77 | 11.27 | (−) | (−) | (−) | (−) |
| H1 | | | | 16.90 | 6.65 | (−) | (−) | (−) | (−) |
| H72 | | | | 15.69 | 8.23 | (−) | (−) | (−) | (−) |
| D52 | | | | 15.02 | 8.19 | (−) | (−) | (−) | (−) |
| 280 | F | 62 | Somatoform disorder | 12.61 | 3.60 | (−) | (−) | (−) | (−) |
| D31 | | | | 12.09 | 4.46 | (−) | (−) | (−) | (−) |
| 134 | M | 34 | Dysthymic disorder; alcohol abuse | 11.79 | 11.02 | (−) | (−) | (−) | (−) |
| 263 | M | 63 | Amnestic disorder; epilepsy | 11.70 | 14.02 | (−) | (−) | (−) | (−) |
| 199 | F | 43 | Sch, paranoid type | 9.54 | 6.53 | (−) | (−) | (−) | (−) |
| D114 | | | | 7.70 | 5.86 | (−) | (−) | (−) | (−) |
| D43 | | | | 7.47 | 4.90 | (−) | (−) | (−) | (−) |
| H18 | | | | 7.12 | 3.82 | (−) | (−) | (−) | (−) |
| 275 | M | 45 | Limbic encephalitis | 7.01 | 3.40 | (−) | (−) | (−) | (−) |
| 307 | M | 44 | Dysthymic disorder | 6.82 | 5.46 | (−) | (−) | (−) | (−) |
| H14 | | | | 6.71 | 3.80 | (−) | (−) | (−) | (−) |
| CB73 | | | | 6.08 | 6.95 | (−) | (−) | (−) | (−) |
| D123 | | | | 5.29 | 4.56 | (−) | (−) | (−) | (−) |
| CB69 | | | | 4.60 | 3.64 | (−) | (−) | (−) | (−) |
| 322 | F | 60 | Posterior cortical atropy | 3.43 | 14.47 | (−) | (−) | (−) | (−) |
| CB75 | | | | 3.07 | 6.95 | (−) | (−) | (−) | (−) |
| H55 | | | | 2.87 | 5.88 | (−) | (−) | (−) | (−) |
| 286 | F | 68 | MDD, recurrent | 3.38 | 3.85 | (−) | (−) | (−) | (−) |
| D168 | | | | (−) | (−) | 16.48 | 11.38 | (−) | (−) |
| 140 | F | 54 | MDD, single episode | (−) | (−) | 5.01 | 2.72 | (−) | (−) |
| D11 | | | | (−) | (−) | 4.55 | 2.00 | (−) | (−) |
| D140 | | | | (−) | (−) | 4.12 | 2.14 | (−) | (−) |
| H56 | | | | (−) | (−) | 3.73 | 2.60 | (−) | (−) |
| 246 | M | 41 | Somatoform disorder | (−) | (−) | (−) | (−) | 16.08 | 9.95 |
| 85 | F | 30 | MDD, recurrent | (−) | (−) | (−) | (−) | 8.13 | 5.87 |
| D138 | | | | (−) | (−) | (−) | (−) | 7.91 | 5.29 |
| Samples having two antibodies of the different isotypes | | | | | | | | | |
| D90 | | | | (−) | 5.96 | (−) | 22.93 | (−) | (−) |
| D26 | | | | (−) | (−) | (−) | 2.24 | 3.76 | (−) |
| 117 | F | 61 | Sch, paranoid type | 4.91 | (−) | (−) | (−) | 4.30 | (−) |
| 317 | F | 59 | Dysthymic disorder | (−) | 5.11 | (−) | (−) | 4.20 | (−) |
| D135 | | | | 3.72 | (−) | (−) | (−) | (−) | 5.44 |
| D199 | | | | 2.88 | (−) | (−) | (−) | (−) | 3.98 |

Only the index values of the positive results are shown. D: blood donors, H: healthy subjects, CB: cord bloods, with no roman letters: psychiatric patients. BPI: bipolar I disorder, BPII: bipolar II disorder, Sch: shizophrenia, MDD: major depressive disorder.

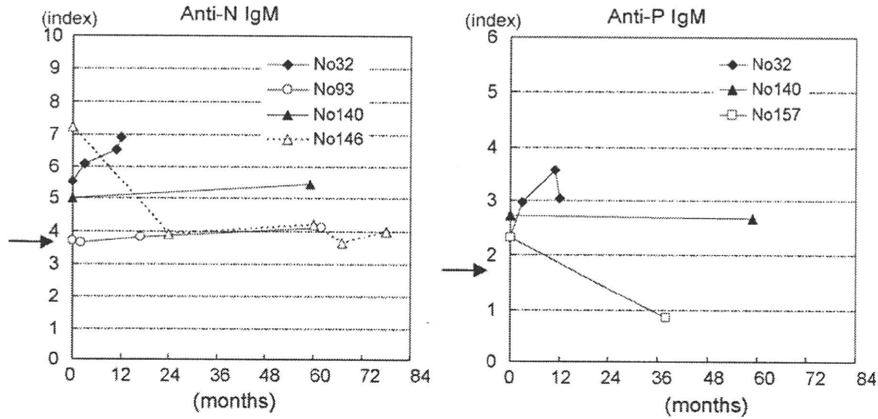


**Fig. 2.** Results of the patients showing positive anti-BDV IgM results and sampled two or more times. Arrows indicate the cut-off points: 3.58 and 1.88 for anti-BDV-N and -P IgM, respectively.

cantly different from that in psychiatric patients. As the control subjects in this study were sampled consecutively, they should reflect the general Japanese population. The samples of the healthy subjects of our previous study were not used in this study. They had been carefully selected from healthy people, and screened for the absence of six autoantibodies including anti-nuclear, anti-DNA, anti-mitochondrial, anti-thyroid microsomal, and anti-thyroglobulin antibodies and rheumatoid factor. Whether subclinical autoimmunity and/or some other factor might be related to the seropositivity remains to be clarified. These results lead us to the following speculations: (1) the general Japanese population might have been exposed to BDV or a related virus with a considerable incidence; (2) in most cases, it is not pathogenic; and (3) the specific antibody does not necessarily indicate its relation to their present symptoms. Age analysis revealed higher seroprevalence in elders, which is consistent with the previous report,[7] suggesting the life-long opportunity of exposure to BDV or a related virus. Why the schizophrenic patients had a tendency of having less anti-BDV-P IgG and why the age difference was found only in anti-BDV-N IgG should be explored in future.

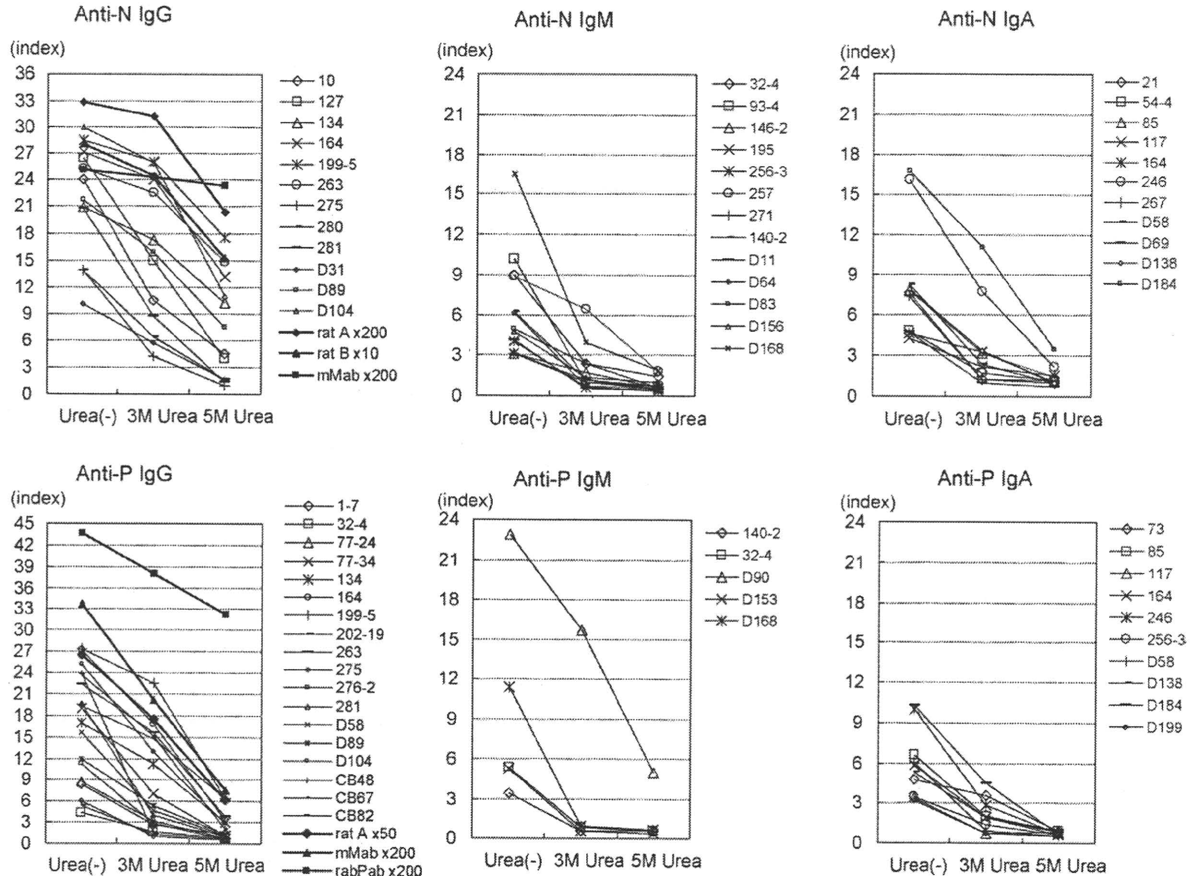


**Fig. 3.** The avidity of the anti-BDV antibodies. For IgG, results of non-human samples were included. Rats A and B: sera from BDV infected rats, mMab: mouse monoclonal antibody, rabPab: serum from immunized rabbit by BDV-P antigen.

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The specific IgM and IgA against BDV-N and -P were successfully detected using radioligand assay. This method is highly sensitive and specific because it uses antigens without deformity in liquid phase and can detect conformational antibodies. The specific IgM and IgA were not found in cord blood subjects, which is compatible because IgM and IgA do not pass through placenta. Excluding cord blood subjects, 137 out of 580 subjects had at least one of the six antibodies, 51 of which did not have IgG but had IgM and/or IgA. Testing of IgM and IgA, therefore, increased the sensitivity from 86/580 (14.8%) to 137/580 (23.6%). Forty-four out of all 682 subjects (6.5%) had two or more specific antibodies, and most of them had antibodies of the same isotype against two BDV antigens, suggesting that these antibodies had been produced by exposure to BDV or a related virus rather than simultaneously cross-reacted to two BDV antigens.

Allmang et al.[3] found that in most positive sera by IFA, the titers decreased to less than those of 16-fold dilution when incubated in 3 M urea. In our study, the titers persisted at 50% or more in 3 M urea in the majority of subjects having high titers. The difference might be due to the preserved conformational structure of the antigens in radioligand assay. However, the titers of most sera reduced to less than 50% in 5 M urea, which suggested lower avidity than normal matured IgG, because matured antibodies would persist in 6 or 7 M urea.[8,9] Anti-BDV-N IgG had higher avidity than anti-BDV-P IgG. The flexible part of N-terminal of BDV-N[10] is reported to be the most preferred site of epitope,[3] and it is suspected that the flexibility might be the cause of the higher avidity.

The specific IgM is ordinarily produced during a few months at the first infection or at the active phase of reactivation or re-infection. However, six specific IgM against BDV did not disappear for 12–78 months, whereas only one specific IgM turned negative after 38 months. Yamaguchi et al. (50th Meeting of the Japanese Society for Virology, 2002) had found a year-long delay of class-switching in horse BDV infection. Our study also revealed the persistence of the specific IgM in humans, which may indicate continuous active infection or disturbed immune response.

Low avidity of the specific IgG might suggest a cross-reaction of unspecific antibodies. Combined with the persistence of the specific IgM, however, it could be hypothesized that the immune response against BDV might be disturbed. This hypothesis could explain the mystery of human anti-BDV antibodies; i.e. low titer, persistent low avidity, and discordance of results among different methods and laboratories. Rubin et al.[11] reported that BDV infection to the stromal cells of the thymus in BDV inoculated rats was related to the absence or amelioration of the cell-mediated encephalitis, which might suggest some role of the thymus in the disturbed immune response.

IgA against BDV antigens was also detected, among which three subjects had high titer of IgG and IgA against both antigens, suggesting mucosal involvement of BDV infection. In experimentally infected animals, BDV has been detected in nasal secretion, saliva, urine and stool.[12]

Our findings supported the possible exposure to BDV or a related virus in humans. Further studies are necessary to clarify the role of BDV in humans. In order to examine the role of BDV in human diseases, other diagnostic methods are needed.

## Acknowledgements

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ORIGINAL ARTICLE

# Erythromycin attenuates MUC5AC synthesis and secretion in cultured human tracheal cells infected with RV14

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**Erythromycin attenuates MUC5AC synthesis and secretion in cultured human tracheal cells infected with RV14**
INOUE D, KUBO H, SASAKI T, YASUDA H, NUMASAKI M, SASAKI H, YAMAYA M. *Respirology* 2008; **13**: 215–220

**Background and objective:** The common cold is a major cause of asthma exacerbation and chronic obstructive lung disease. Rhinovirus is reported to be responsible for more than 50% of cases of the common cold. In a previous study, we reported that rhinovirus infection of cultured airway cells induced MUC5AC mucin overproduction and hypersecretion by activating the p44/42 mitogen-activated protein kinase (p44/42 MAPK) pathway. The aim of this study was to examine the effect of erythromycin on RV14-induced airway mucin overproduction and hypersecretion.
**Methods:** RV14-infected human tracheal epithelial cells were treated with erythromycin.
**Results:** Erythromycin blocked RV14-induced MUC5AC protein overproduction and hypersecretion, and also blocked RV14-induced p44/42 MAPK activation in the cells.
**Conclusions:** Erythromycin may attenuate RV14-induced MUC5AC overproduction and hypersecretion by blocking the p44/42 MAPK pathway or its upstream regulators.

**Key words:** asthma, erythromycin, mucin, p44/42 MAPK, rhinovirus.

## INTRODUCTION

Mucus hypersecretion leads to exacerbation of asthma and chronic obstructive lung disease by causing severe airway narrowing.[1,2] In normal airways, a small amount of mucus is secreted. Mucus is transported to the mouth by the beating of cilia and is subsequently swallowed, thus removing particles trapped in the airways. This airway clearance is impaired when excessive secretion occurs, leading to airflow obstruction.

The main component of mucus is mucin. MUC5AC and MUC5B are reported to constitute 95–98% of secreted mucin in airways. Mucus with a high concentration of MUC5AC or MUC5B has a high viscosity and is likely to cause airway narrowing.[2–4] Although little is known about the functional differences between the two mucin proteins, the ratio of MUC5AC to MUC5B is very low in COPD patients compared with asthma patients, and this may cause pathological differences between the two diseases.[2–4]

Macrolide antibiotics are reported to inhibit airway mucus hypersecretion induced by several stimuli. Azithromycin, a macrolide antibiotic, inhibits *Pseudomonas aeruginosa*-induced MUC5AC production in NCI-H292 airway cells by inhibiting p44/42 MAP kinase.[5] Erythromycin (EM) suppresses LPS-induced mucus hypersecretion in the airways of ovalbumin sensitized rats.[6] Clarithromicyn has also been reported to suppress airway mucin hypersecretion and p44/42 MAPK activation in a murine diffuse pan bronchiolitis model.[7] Although common viral airway infections resulting in mucus hypersecretion are the most frequent cause of exacerbations of obstructive lung disease, it is not known whether macrolide antibiotics inhibit mucus secretion in viral infections.

In a previous study, we showed that rhinovirus (RV), which is reported to cause more than 50% of common colds, induced mucin hypersecretion in cultured tracheal epithelial cells via the p44/42 MAPK pathway.[8]

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In the present study, it was hypothesized that macrolide antibiotics may attenuate RV-induced mucin hypersecretion. Therefore, the question of whether EM can attenuate RV-induced mucin secretion and the role of p44/42 MAPK was investigated.

# METHODS

## Human tracheal epithelial cells

Tracheas for cell culture were obtained from 21 patients (mean age 63.3 ± 10.5 years; 10 female), 3–6 h after death under a protocol approved by the Tohoku University Ethics Committee. None of the patients had a respiratory illness, including pneumonia, bronchial asthma and COPD. The causes of death were acute myocardial infarction ($n = 6$), congestive heart failure ($n = 5$), malignant tumour other than lung cancer without lung metastasis ($n = 4$), cerebral bleeding ($n = 2$), cerebral infarction ($n = 2$) and unidentified arrhythmia resulting in sudden death without any other abnormality ($n = 2$). Tracheal epithelial cells were isolated and cultured in medium containing Dulbecco's modified Eagle's medium (DMEM; Gibco-BRL Life Technologies, Palo Alto, CA, USA), Ham's F-12 medium (Gibco), fetal calf serum (FCS; Gibco), penicillin/streptomycin (Sigma Chemical Co., St Louis, MO, USA), gentamicin (Sigma), amphotericin B (Sigma), and Ultroser G serum substitute (USG; BioSepra, Cergy-Saint-Christophe, France) as described previously.[9,10] The cells were plated at $5 \times 10^5$ viable cells/mL ($2 \times 10^5$ cells/cm$^2$) in plastic round-bottom tubes (125 × 16 mm, Becton Dickinson, Franklin Lake, NJ, USA) coated with human placental collagen, and cultured in DMEM-Ham's F-12 containing 2% USG.

## RV14 virus

RV14 was isolated in our laboratory from patients with common colds.[9–11] Stocks of RV14 were generated as described previously.[8] Briefly, RV14 was cultured in human embryonic fibroblast cells in glass tubes. The cells were incubated for several days until cytopathological effects were obvious, after which the cultures were filtered, frozen at −80°C, thawed and sonicated. The virus-containing fluid was stored in aliquots at −80°C. The viral content of stock solutions was determined using the human embryonic fibroblast cell assay described below.

## Detection and titration of virus

RV14 virus was detected by exposing confluent human embryonic fibroblast cells in glass tubes to 10-fold serial dilutions of virus-containing Eagle's minimum essential medium (Gibco) supplemented with 2% ultra-low IgG fetal calf serum (Gibco). After 7 days, the cytopathological effects of viruses on human embryonic fibroblast cells were observed.[9–11] The amount of virus required to infect 50% of human embryonic fibroblast cells (TCID50) was determined.

## Detection of RV14 RNA by RT-PCR

RV14 RNA was detected by RT-PCR[12] and quantified by real-time RT-PCR using the TaqMan method (Roche Molecular Diagnostic Systems, Basel, Switzerland) as previously described.[13] A forward primer (5′-GCACTTCTGTTTCCCC-3′), a reverse primer (5′-CGGACACCCAAAGTAG-3′), and a TaqMan probe (5′-[FAM] CGAGGTATAGGCTGTACCCACTGCCAAAA [TAMRA]-3′) were specifically designed to detect RV14 cDNA.

## Viral infection of human tracheal epithelial cells in the presence of erythromycin

Cells were pre-incubated with medium containing 10 μM EM for 3 h before infection.[13] Incubation with EM for up to 72 h did not affect growth of cultured tracheal epithelial cells, as measured by cell counting (Calcein-AM kit; Dojindo, Kumamoto, Japan) (data not shown).[14] Cells were infected with RV14 by incubation with 100 μL of medium containing 10 μM EM and RV14 at a titre of $10^5$ TCID50, for 1 h at room temperature. The medium was then replaced with 1 mL of medium containing 10 μM EM and the cells were cultured at 33°C in a rolling incubator (HDR-6-T; Hirasawa, Tokyo, Japan).[9,10]

## Real-time quantitative RT-PCR assessment of MUC5AC mRNA expression

Real-time quantitative RT-PCR using the TaqMan technology was performed as previously described.[8,15] Total cellular RNA from human tracheal epithelial cells was isolated with RNAzol B (Tel-Test Inc., Friendswood, TX, USA) according to the manufacturer's instructions. Total RNA was reverse transcribed to cDNA and amplified by PCR for 40 cycles (15 s at 95°C, 1 min at 60°C).[8] MUC5AC forward primer (5′-CAGCCACGTCCCCTTCAATA-3′), reverse primer (5′-ACCGCATTTGGGCATCC-3′), and TaqMan probe (FAM-5′-CCACCTCCGAGCCCGTCACTGAG-3′-TAMRA) were used according to the manufacturer's protocol (Applied Biosystems, Foster City, CA, USA). RT-PCR was performed using a ABI PRISM 7700 instrument (Applied Biosystems) with β-actin (Applied Biosystems) as a control. Levels of mRNA expression were calculated by the comparative threshold cycle (Ct) method.[16] At least five independent experiments were performed for each mRNA.

## Measurement of MUC5AC protein

To examine the effects of RV14 on mucin production and secretion from cultured cells, a specific ELISA for MUC5AC was performed, as described previously.[8,17]

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Total mucin was measured by the same procedure using 17Q2 antibody (5 μg/mL, MMS 662R, BabCO, Richmond, CA, USA).[18,19]

### Immunoblotting for activated p44/42 MAP kinase

Immunoblotting for activated p44/42 MAP kinase was performed as previously described,[8,20] with some modifications. After 75 min of RV14 infection and EM treatment, cells were lysed (20 mM sodium phosphate, pH 7.8, 150 mM NaCl, 5 mM EDTA, 50 mM HEPES, 1% Triton X-100, 50 mM NaF, 1 mM sodium orthovanadate, 5 mM PMSF, 10 mg/mL each of leupeptin and aprotinin) and incubated for 30 min at 4°C. After centrifugation, supernatants were suspended in SDS sample buffer and boiled for 5 min. Proteins were separated by SDS-PAGE on a 4–15% acrylamide gel and transferred to nitrocellulose membranes, which were incubated with 5% fat-free skim milk for 1 h, then incubated overnight with antiphospho-p44/42 MAPK mAb (2 mg/mL) or total p44/42 MAPK mAb (2 mg/mL) (PhosphoPlus p44/42 MAP kinase antibody kit; Cell Signaling Technology, Beverly, MA, USA). Bound antibodies were visualized by the avidin-biotin-alkaline phosphatase complex method (ABC kit; Vector Laboratories, Burlingame, CA, USA).

### Statistical analysis

Data are presented as a mean ± SEM and initial statistical analyses were performed by analyses of variance (ANOVA) across all groups, except for the data for MUC5AC protein, which was not normally distributed. When overall significance was identified by ANOVA, the data were subjected to Bonferroni adjustment to identify which groups were significantly different. Statistical analysis of the data for MUC5AC protein was performed using the Kruskal–Wallis non-parametric test. When overall significance was identified, the Steel test was performed to identify which groups were significantly different. Statistical significance was defined as $P < 0.05$.

## RESULTS

### Pre-treatment of human tracheal epithelial cells with EM for less than 3 h does not inhibit RV14 infection

In a previous report, we showed that RV14 infection is decreased by EM pretreatment for longer than 24 h.[13] To determine the time at which EM does not decrease RV14 infection in cultured human tracheal epithelial cells, the amount of RV14 was measured in cells pretreated with EM for 1, 2, 3, 8, 12 and 24 h. Cells were then infected with RV14 in medium that contained EM, followed by an 8-h incubation in medium containing EM only. Next, the number of RV14 RNA copies in the cell (Fig. 1a) and the titre of RV14 in the supernatant (Fig. 1b) were measured. When cells were infected with RV14 following an EM pre-incubation shorter than 4 h, neither the number of RV14 RNA copies in the cells, nor the RV14 viral titre in the cell supernatant decreased, while an EM pre-incubation time of longer than 12 h significantly decreased both the number of RV14 RNA copies and the RV14 viral titres (Fig. 1). An EM pre-incubation time of 8 h did not decrease the RV14 titre but did decrease the number of RV14 RNA copies (Fig. 1).

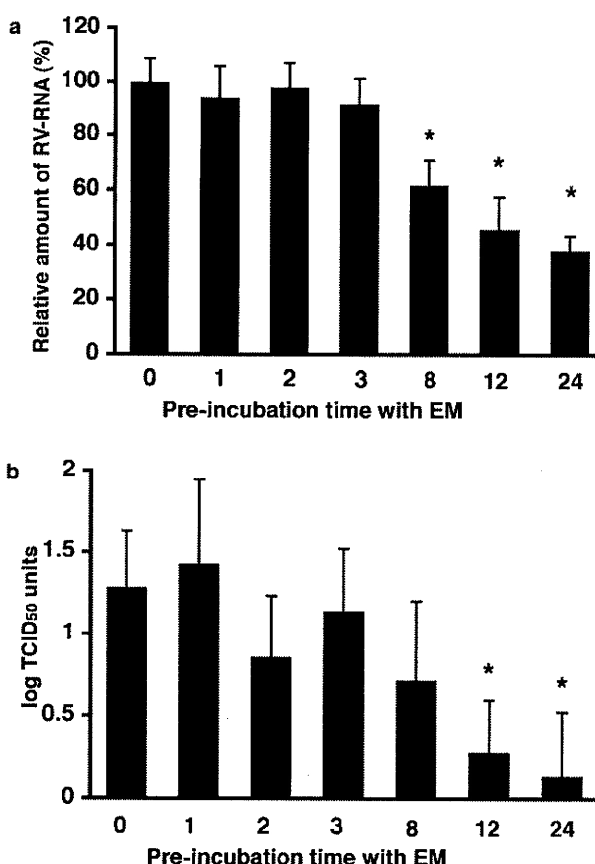


**Figure 1** Viral RNA copies (a) and RV14 titre in supernatants (b) of RV14-infected human tracheal epithelial cells collected 8 h after exposure to RV14. Viral RNA results are expressed as relative RNA expression (%) compared with maximum RV14 RNA after 24 h of erythromycin (a). The titre of RV14 at infection was $10^5$ $TCID_{50}$ (b). The cells were pre-incubated with 10 μM EM for 0, 1, 2, 3, 8, 12 or 24 h before infection. Data are mean ± SEM of separate experiments with cells from seven different donors. *Significant difference compared with viral infection alone, $P < 0.01$. EM, erythromycin.

### Inhibitory effect of EM on RV14-induced MUC5AC overproduction and hypersecretion

To determine if EM could inhibit RV14-induced MUC5AC upregulation in RV14 infected airway

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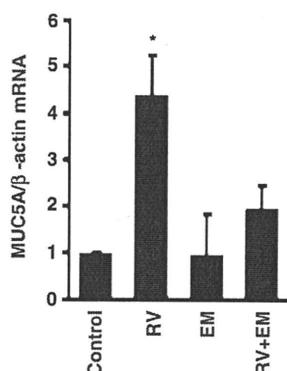


**Figure 2** The expression of MUC5AC in cultured human tracheal epithelial cells, measured after 3 h of pretreatment with erythromycin (EM) and 4 h of RV14 infection (RV) by quantitative real time RT-PCR. Expression of mRNA was normalized to the constitutive expression of β-actin mRNA. Results are mean ± SEM from five samples. *Significantly different from control values, $P < 0.05$.

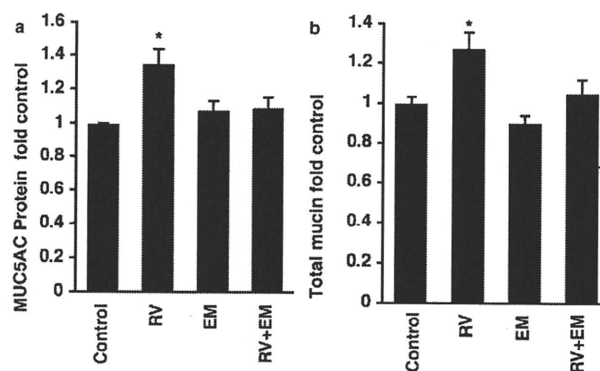


**Figure 3** Concentrations of MUC5AC protein (a) and total mucin (b) in supernatants of cultured human tracheal epithelial cells measured by ELISA after 3 h of pretreatment with erythromycin (EM) followed by 4 h of RV14 infection (RV). 45M1 and 17Q2 antibodies were used to label MUC5AC and total mucin, respectively. 17Q2 antibody labels not only MUC5AC but also MUC5B, which is a major constituent of airway mucus. Results are mean ± SEM from five samples. *Significantly different from control values, $P < 0.05$.

epithelial cells,[8] cells were infected with RV14 after pre-incubation with EM for 3 h. After a 4-h incubation with medium containing only EM, the levels of MUC5AC mRNA, MUC5AC protein in the cells, and MUC5AC protein secreted were measured. The 3-h pre-incubation with EM significantly attenuated RV14-induced MUC5AC mRNA expression in human tracheal epithelial cells (Fig. 2) without attenuating the viral titre or the number of virus copies (Fig. 1).

A 3-h pre-incubation with EM significantly attenuated RV14-induced MUC5AC secretion in supernatants (Fig. 3a), and also attenuated RV14-induced increase of MUC5AC protein in cell lysates (Fig. 4a). The 3-h pre-incubation with EM attenuated RV14-induced increase of total mucin in the cell supernatants (Fig. 3b). Pre-incubation with EM also attenuated RV14-induced increase of total mucin in cell lysates (Fig. 4b). EM treatment alone did not change the amount of MUC5AC or total mucin in cell supernatants or lysates (Figs 3,4).

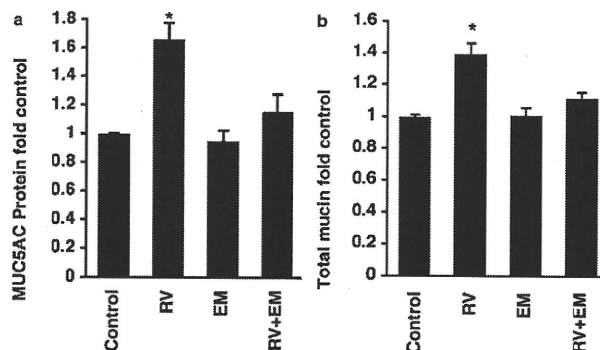


**Figure 4** Concentrations of MUC5AC protein (a) and total mucin (b) in cell lysates of cultured human tracheal epithelial cells measured by ELISA after 3 h of pretreatment with erythromycin (EM) and 4 h of RV14 infection (RV). Cell lysates were prepared by homogenization in an appropriate buffer. Results are mean ± SEM from five samples. *Significantly different from control values, $P < 0.05$.

### Suppression of RV14-induced p44/42 MAPK activation by erythromycin in cultured human tracheal epithelial cells

Treatment with EM inhibited the activation of p44/42 MAPK induced by RV14 infection in tracheal epithelial cells (Fig. 5). EM did not appear to alter the activation of p44/42 MAPK in non-infected cells.

## DISCUSSION

Erythromycin attenuated RV14-induced MUC5AC production and secretion in cultured human tracheal epithelial cells. MUC5AC mRNA expression was also attenuated by EM treatment, suggesting that EM affects pretranscriptional mechanisms. Furthermore, EM attenuated RV14-induced p44/42 MAPK activation. As we previously reported that the p44/42 MAPK pathway was important in RV14-induced mucin production and secretion,[8] attenuation of p44/42 MAPK by EM may play an important role in the attenuation of MUC5AC production and secretion in tracheal epithelial cells. This hypothesis seems consistent with earlier studies, which reported that macrolides may attenuate MUC5AC production by inhibiting p44/42 MAPK activation by certain stimuli.[5,7] One of these reports showed that, in cell lines infected with *Pseudomonas aeruginosa*, MUC5AC production was attenuated by azithromycin,[5] while another study showed that, in an animal model of diffuse pan-bronchiolitis, both p44/42 MAPK activation and MUC5AC production were attenuated by

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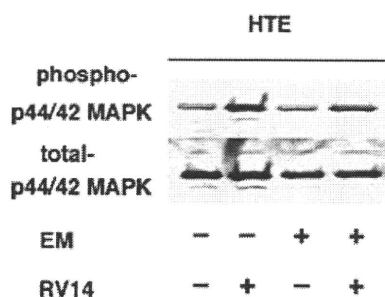


**Figure 5** Tyrosine phosphorylation of p44/42 MAPK in human tracheal epithelial cells was evaluated by Western blot analysis. Phosphorylated p44/42 MAPK and total p44/42 MAPK were evaluated after 3 h of pretreatment with erythromycin (EM) followed by 75 min of infection with RV14 or vehicle. Data are representative of three separate experiments.

clarithromycin.[7] However, another report indicated that the macrolide, roxithromycin, reduced MUC5AC production by a mechanism other than p44/42 MAPK.[21] Another study showed that clarithromycin, on the contrary, activates p44/42 MAPK after 2 h.[22] Possible explanations for the discrepancy among these reports is that different macrolides may have different effects,[22] and that different stimuli may induce MUC5AC production through different pathways. As macrolides are frequently used clinically, further studies are needed to clarify the effect of each drug, and which specific pathway is involved.

Given the fact that EM did not appear to inhibit activation of p44/42 MAPK in non-infected cells, it is possible that the effect of EM is specific to RV14-induced p44/42 MAPK activation. The question of whether macrolides affect p44/42 MAPK activity in non-infected cells was not tested in a previous study[5] that reported a similar macrolide effect to that observed in the present study. One previous study showed that in non-infected cells, clarithromycin first inactivated p44/42 MAPK for the first 30 min and then activated p44/42 MAPK a few hours later,[22] which is a different time-course for the effect of macrolides on p44/42 MAPK activation compared with that observed in the present study. This discrepancy may have been due to the use of a different macrolide.

A 3-h treatment with EM attenuated RV14-induced mucin secretion in cultured human tracheal epithelial cells, suggesting that even after a short duration of treatment, EM may be effective. In our previous study, pre-incubation with EM for 24–72 h reduced RV14 infection through downregulation of ICAM-1, the RV14 binding site on the airway epithelial cell.[13] There we concluded that EM might be useful for prophylaxis of COPD exacerbations, which are characterized by mucus hypersecretion. In the present study, pre-incubation with EM for 3 h inhibited RV14-induced mucus production but did not affect the RV14 titre in infected cells. These results suggest that EM may also be useful when administered at the time patients are first infected with the common cold virus, as many cells remain uninfected at that stage. The present results suggest that 3 h after administration to patients, EM will prevent mucin hypersecretion by newly infected cells. In addition, after 24 h EM will also prevent further infection with RV14.

We believe that attenuation of RV14-induced mucin production by EM is not a consequence of a decrease in cell number induced by EM. There is a report that clarithromycin delays cell growth after 4 days,[23] but under the conditions of the present study EM did not affect cell numbers. However, it was confirmed that after 5 days there was a decrease in cell number.

The present study showed that secretion of MUC5AC by airway epithelial cells was not increased by RV14 infection. However, the amount of MUC5AC stored in the cells was increased significantly by infection. In addition, it is known that viral infections induce inflammatory cytokines, many of which are known to cause MUC5AC secretion.[14] The combination of these two effects may contribute to hyper-secretion of MUC5AC that could potentially be fatal.

The need to pre-incubate the cells with EM for 3 h or for EM to be present throughout the whole RV14 infection process is a limitation of the present study. Although the EM pre-incubation did not inhibit RV14 infection, the question of whether RV14 infected the cells in a normal manner remains unclear. However, the present study showed that EM treatment reduced RV14-induced MUC5AC synthesis and secretion in RV14-infected cultured tracheal epithelial cells.

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SHORT COMMUNICATION

# Submucosal gland cells in human lower airways produce MUC5AC protein

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**Submucosal gland cells in human lower airways produce MUC5AC protein**
INOUE D, KUBO H, WATANABE M, SASAKI T, YASUDA H, NUMASAKI M, SASAKI H, YAMAYA M. *Respirology* 2008; **13**: 285–287
**Background and objective:** It is now considered that the major component of mucus, MUC5AC, is mainly produced by goblet cells but not submucosal glands, and the role of the submucosal glands in the production of MUC5AC is unclear. The aim of this study was to clarify whether human submucosal glands produce MUC5AC.
**Methods:** Immunohistochemical analysis with MUC5AC antibody of lower airways resected from six lung cancer patients and three patients with acute myocardial infarction was performed.
**Results:** The submucosal glands contained both MUC5AC-positive cells and non-positive cells.
**Conclusions:** These data suggest that MUC5AC protein is produced in both goblet cells and in airway submucosal glands.

**Key words:** airway mucus, MUC5AC protein, submucosal gland.

## INTRODUCTION

Mucus secretion is important because excess secretion leads to exacerbations of asthma and COPD.[1] In normal airways, secreted mucus is wafted towards the mouth by beating cilia, removing the particles trapped in the airways. This airway clearance is impaired when excessive secretion occurs, leading to airflow obstruction.[1,2]

The main component of mucus is mucin. MUC5AC and MUC5B are reported to constitute 95–98% of secreted mucin in the airways. Mucus with a high concentration of MUC5AC or MUC5B has a high viscosity and is likely to cause airway narrowing.[1]

In the airway, MUC5AC was thought to be produced by goblet cells rather than submucosal gland cells.[3–5] However, recent reports show that stimulated human nasal submucosal glands produce MUC5AC,[6] and that a low level of MUC5AC is also present in secretions from the human tracheal glandular ducts,[7] suggesting that submucosal gland cells are also the source of MUC5AC.

The aim of this study was to examine the sites of production of MUC5AC in the lower airways of Japanese patients.

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## METHODS

Human bronchi were obtained from patients with lung cancer who underwent lung surgery ($n = 6$, three male) and from patients who died with acute myocardial infarction ($n = 3$, two male). All of the lower airway samples from the lung cancer patients were obtained from the lobe in which the cancer was located. However, all of the cancer lesions were located peripherally and the large airway samples did not include any cancer lesions. The study was approved by the Institutional Review Board and informed consent was obtained as appropriate.

Immunohistochemical analysis of MUC5AC expression was performed as described previously.[8] Briefly, a lower airway sample from each patient was fixed in 4% paraformaldehyde at 4°C for 24 h and was embedded into a paraffin section. For MUC5AC staining, the sections (2 μm) were incubated with trypsin (0.25%) diluted with PBS, at 37°C for 45 min. After washing with PBS three times, the sections were incubated with 1% mouse-derived mAb to human

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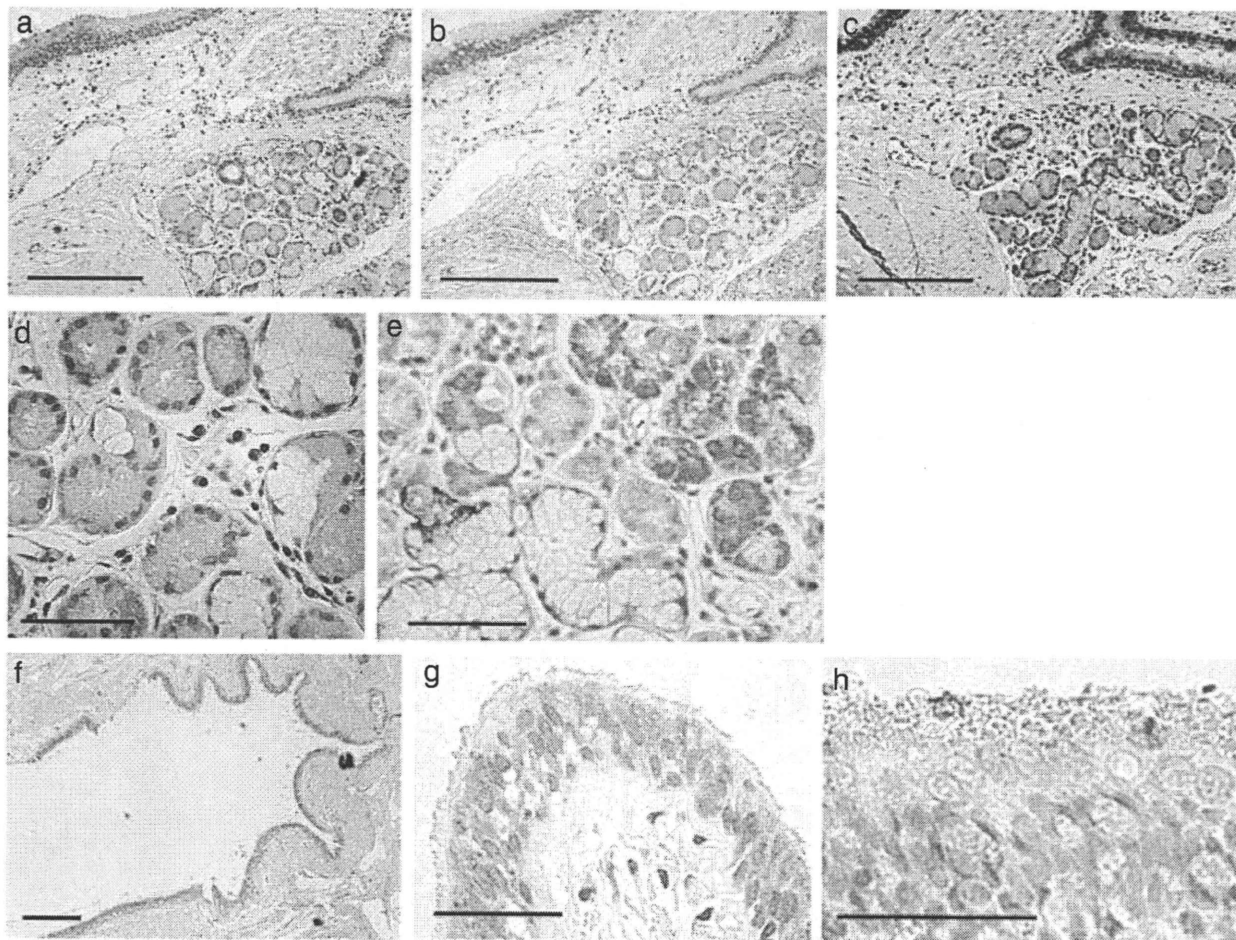


**Figure 1** MUC5AC was stained by 1-13M antibody. MUC5AC was seen in goblet cells (f–h) and submucosal glands (a, d) of the lower airway. Goblet cells also contained MUC5AC (g, h). Some submucosal glands contained MUC5AC. Most of the glands usually included both MUC5AC-positive cells and non-positive cells (a, d). Patients who died from acute myocardial infarction were similar (c). Staining with control mouse $IgG_1$ as well as antibody (45M1) verified specific staining (b). For panels a–c and f size bar = 200 μm; for panels d, e, g and h size bar = 50 μm.

MUC5AC (Clone 1-13M1, MS-551, Neomarkers, Fremont, CA, USA) overnight at 4°C. The sections were then incubated with 1% anti-mouse antibody labelled with histofine biotin (Nichirei, Tokyo, Japan) for 30 min at room temperature. After washing with PBS three times, sections were incubated with 3,3′-diaminobenzamide tetrahydrochloride (DAB; Dojin, Kumamoto, Japan) in Tris buffer for colour development. To confirm the reproducibility of the study with 1–13 antibodies, immunohistochemistry with another MUC5AC antibody (Clone 45M1, MS-553, Neomarkers) was performed using a different protocol as described previously.[9]

## RESULTS

### Immunohistochemical analysis with MUC5AC antibody in lower airways

To confirm the presence of MUC5AC in submucosal glands in the lower airways, immunohistochemical analysis was performed. Immunohistochemical analysis of submucosal glands revealed that there were two kinds of submucosal gland cells, those containing MUC5AC and those not containing MUC5AC (Fig. 1a,d). In some of the glands, all cells contained MUC5AC, while in others, none contained MUC5AC. There were characteristic 'mosaic like glands' that consisted of both MUC5AC-positive cells and non-positive cells. Both mucous gland cells and serous gland cells contained MUC5AC. These observations were the same in all the airway submucosal glands of the nine patients studied. The ratio of the number of MUC5AC-containing cells to non-containing cells was 62.6:37.4 on average as counted by microscopy. The average ratios in patients with lung cancer (64.7:35.3) and in patients with myocardial infarction (58.3:41.7) were not significantly different. MUC5AC was present in all goblet cells in the present study (Fig. 1f–h).

Immunohistochemical study using another MUC5AC antibody (45M1) was performed to confirm the specificity of the antibody and produced a similar result (Fig. 1c).

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## DISCUSSION

This study has reported that MUC5AC is present in submucosal gland cells, in both mucous and serous cells, from the lower airways in an Asian population. Until recently, it has been considered that MUC5AC is not produced by submucosal gland cells but by goblet cells in lower airways.[3–5] However, a recent report demonstrated that a small amount of MUC5AC was secreted from glandular ducts,[7] suggesting that an additional source of MUC5AC might be submucosal glands.

The reason for the discrepancy between the present findings and reports, which concluded that airway submucosal glands do not produce MUC5AC, might be racial differences.[3–5] A recent report from Korea, where the population is genetically similar to the Japanese, showed that nasal submucosal glands secreted MUC5AC.[6] Further studies on the impact of race on MUC5AC are needed to clarify the nature of the secretion from the airway submucosal glands.

The disease background, age, gender and smoking history had no impact on MUC5AC synthesis in submucosal glands. Because patients with myocardial infarction in this study had recently died, the bronchi obtained from these patients conceivably reflect a healthy population.

This study reports, for the first time, that submucosal glands in the human lower airways contain MUC5AC. Furthermore, there may be two different phenotypes; cells that secrete MUC5AC and those that do not produce the protein.

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Approval of the Tohoku University ethics committee was obtained for this study. We thank Mr Brandon Au (University of Hawaii, Manoa) for help with English.

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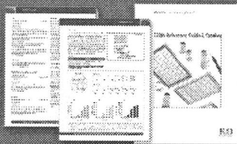
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# Identification of Pendrin as a Common Mediator for Mucus Production in Bronchial Asthma and Chronic Obstructive Pulmonary Disease

This information is current as of February 1, 2011

Isao Nakao, Sachiko Kanaji, Shoichiro Ohta, Hidetomo Matsushita, Kazuhiko Arima, Noriko Yuyama, Mutsuo Yamaya, Katsutoshi Nakayama, Hiroshi Kubo, Mika Watanabe, Hironori Sagara, Kumiya Sugiyama, Hiroyuki Tanaka, Shuji Toda, Hiroaki Hayashi, Hiromasa Inoue, Tomoaki Hoshino, Aya Shiraki, Makoto Inoue, Koichi Suzuki, Hisamichi Aizawa, Satoshi Okinami, Hiroichi Nagai, Mamoru Hasegawa, Takeshi Fukuda, Eric D. Green and Kenji Izuhara

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# Identification of Pendrin as a Common Mediator for Mucus Production in Bronchial Asthma and Chronic Obstructive Pulmonary Disease[1]

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**Excessive production of airway mucus is a cardinal feature of bronchial asthma and chronic obstructive pulmonary disease (COPD) and contributes to morbidity and mortality in these diseases. IL-13, a Th2-type cytokine, is a central mediator in the pathogenesis of bronchial asthma, including mucus overproduction. Using a genome-wide search for genes induced in airway epithelial cells in response to IL-13, we identified pendrin encoded by the *SLC26A4* (*PDS*) gene as a molecule responsible for airway mucus production. In both asthma and COPD mouse models, pendrin was up-regulated at the apical side of airway epithelial cells in association with mucus overproduction. Pendrin induced expression of MUC5AC, a major product of mucus in asthma and COPD, in airway epithelial cells. Finally, the enforced expression of pendrin in airway epithelial cells in vivo, using a Sendai virus vector, rapidly induced mucus overproduction in the lumens of the lungs together with neutrophilic infiltration in mice. These findings collectively suggest that pendrin can induce mucus production in airway epithelial cells and may be a therapeutic target candidate for bronchial asthma and COPD.** ***The Journal of Immunology,* 2008, 180: 6262–6269.**

Bronchial asthma and chronic obstructive pulmonary disease (COPD)[3] are chronic inflammatory airway diseases associated with infiltration of inflammatory cells and alterations of structural components (1). Mucus overproduction is often a common feature of bronchial asthma as well as COPD and is a major factor responsible for morbidity and mortality in these diseases (2–4). The precise mechanism of mucus overproduction in both diseases, however, is still poorly understood. Although a nonspecific antiinflammatory therapy such as inhalation of corticosteroids is effective for suppressing mucus overproduction and local inflammation in bronchial asthma (5), it is critical to find a molecular target to specifically control mucus overproduction in these disorders (6).

IL-13, a Th2-type cytokine, is a central mediator in the pathogenesis of bronchial asthma (7–9). Notably, IL-13 signals are capable of inducing mucus production in bronchial epithelial cells (10). However, the molecular mechanism of IL-13-induced mucus production is still poorly understood. Because chloride transport regulates mucus production (11), anion transporters are suggested to be involved in mucus hypersecretion in bronchial asthma. Consistent with this idea, niflumic acid, a broad inhibitor of anion transport, suppresses the development of asthma in mice (12). Previous reports showed that Th2-type cytokines including IL-13 induce expression of gob-5 (mCLCA3), which had been thought to be a chloride transporter, suggesting a critical role for gob-5 in mucus overproduction in bronchial asthma (13, 14). However, the role of gob-5 in asthma remains somewhat elusive at the moment, because this transporter is indeed a secretory, but not transmembrane, protein (15).

The *SLC26A4* (*PDS*) gene encoding pendrin was originally identified as the defective gene in Pendred syndrome, characterized by deafness and goiter (16). Pendrin is a multispanning transmembrane protein and acts as an anion transporter. It is expressed at the apical membrane of the follicular epithelium in thyroid, acting as a transporter of iodide (17). Additionally, pendrin is localized in cochlea; disruption of the *Slc26a4* (*Pds*) gene causes auditory dysfunction due to dysplasia of the cochlea (18, 19). To date, however, nothing is known about the role of pendrin in airway mucus production.

In this study, we identified the *SLC26A4* gene as an IL-13-inducible gene, using a microarray approach, and found that pendrin is highly expressed at the apical membrane of bronchial epithelial cells (BECs) in animal models of bronchial asthma and COPD.

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[3] Abbreviations used in this paper: COPD, chronic obstructive pulmonary disease; BEC, bronchial epithelial cell; BAL, bronchoalveolar lavage; EGFP, enhanced GFP; PAS, periodic acid-Schiff; Penh, enhanced pause.

Moreover, the enforced expression of pendrin in airway cells caused mucus overproduction in vitro and in vivo. These results suggest that pendrin is a critical mediator of mucus production in airway epithelial cells, and that it may serve as a potential therapeutic target candidate for bronchial asthma and COPD.

## Materials and Methods

### *Culture of freshly prepared human tracheal epithelial cells*

Trachea for cell culture were obtained 3–6 h after death from four patients (59–77 years of age, 3 men, 72 years of age, one woman) under a protocol passed by the Tohoku University Ethics Committee. None of the patients had a respiratory illness. Isolation and culture of human tracheal epithelial cells were performed as previously described (20). Briefly, the culture medium, composed of DMEM-Ham's F-12 medium (Invitrogen) containing 5% FCS, was added to both the mucosal surface side and the basolateral side (by immersion). This medium was replaced by DMEM-Ham's F-12 medium containing 2% ultoroser G (BioSepra) on the first day with an air-liquid interface fashion, and 10 ng/ml IL-13 was added in the medium of the basolateral side. Human tracheal epithelial cells were cultured for 3 or 7 days after IL-13 addition for mRNA extraction and for 3 wk for histochemical staining.

### *Probe preparation and microarray analysis*

The procedures of probe preparation and microarray analysis were performed as we previously reported (21), except that we performed the analysis with GeneChip Human Genome U95 Array (HG-U95, Affymetrix) representing ~10,000 full-length genes and expressed sequence tag clusters. The data were analyzed using GeneChip software (suite ver. 4.0, Affymetrix).

### *Culture of mouse trachea*

Culture of mouse trachea was performed based on the previous report with minor modifications (22). Briefly, tracheas were dissected from anesthetized mice and longitudinally cut on the ventral side. The dissected tracheas were placed with the lumen side up on stainless steel wire mesh screens suspended in a dish filled with the culture medium. Then the screens were placed in the culture wells such that the top of the trachea was bathed in the medium.

### *Cells*

TGMBE-02-3 cells and COS7 cells were cultured as previously described (23, 24). NCI-H292, a human lung carcinoma cell line (American Type Culture Collection, CRL-1848), was cultured with RPMI 1640 medium (Invitrogen) containing 10% FCS, 10 mM HEPES, 4.5 g/L glucose, 1.5 g/L $NaHCO_3$, 2 mM glutamine, 1.0 mM sodium pyruvate, 100 μg/ml streptomycin, and 100 U/ml penicillin G.

*SLC26A4* cDNA inserted in pEGFP-C1 (pEGFP-C1-PDS) was prepared as described before (25). The coding region of enhanced GFP (EGFP) and pendrin in this plasmid was inserted into pIRESneo2 (pIRESneo2-EGFP-PDS, Clontech). pEGFP-C1-PDS and pIRESneo2-EGFP-PDS were used for transient and stable transfection into the cells, respectively. Expression of pendrin into COS7 or NCI-H292 cells was performed by TransFast transfection reagent (Promega). NCI-H292 cells stably transfected with pendrin were selected and maintained with the culture medium containing 300 μg/ml G-418 sulfate (Invitrogen).

For stimulation, cells were cultured with 20 ng/ml human IL-4 or IL-13 (Peprotech) or 10 ng/ml mouse IL-4 or 50 ng/ml mouse IL-13 (R&D Systems) for the indicated period. In some experiments, cells were incubated with 250 μM of niflumic acid (Sigma-Aldrich) for 24 h. The indicated concentration of scramble or EGFP siRNA (iGENE Therapeutics) was transfected into NCI-292 cells with lipofectamine 2000 (Invitrogen). The culture medium was exchanged 6 h after the transfection, followed by incubation for 48 h.

### *Model mice*

Seven-week-old BALB/c mice (Japan SLC and Charles River Laboratories Japan) were used. Experiments were undertaken following the guidelines for the care and use of experimental animals of the Japanese Association for Laboratory Animal Science (1987).

Asthma model mice were generated as previously reported (26). Mice were actively sensitized by i.p. injections of 50 μg OVA (Seikagaku Kogyo) with 1 mg alum on days 0 and 12. Starting on day 22, they were exposed to 1% OVA for 30 min, three times every fourth day. Prednisolone (Shionogi) was administered orally once a day for 10 consecutive days from the day before the first Ag challenge. Histological examination was performed 24 h after the final Ag challenge.

Intratracheal administration of IL-13 into mice was performed as previously described (14).

For expression of Sendai virus vector-coding proteins, $5 \times 10^7$ cell infectious units (CIU) of the fusion (F) gene-deleted Sendai virus (SeV/ΔF) vectors carrying the *Slc26a4* gene between the hemagglutinin-neuraminidase (HN) and large protein (L) genes, that is, SeV(HNL)-mPend/ΔF and SeV(HNL)-mPend/ΔF-GFP, or the control vector SeV/ΔF ($SeV^{18+}$GFP/ΔF), in 50 μl 1% BSA/PBS was administered transnasally.

COPD model mice were generated as previously reported (27). Five units of porcine pancreas elastase (Elastin Products Company) in 50 μl PBS or 50 μl PBS alone was administered into mice via the trachea. Histological examination was performed after 1 or 2 wk.

### *RT-PCR*

Total RNA was extracted by either ISOGEN (Nippongene) or RNeasy Mini Kit (Qiagen). The RT reaction primed with random hexamer was performed using GeneAmp RNA PCR Kit (Applied Biosystems). Quantitative RT-PCR analysis was performed using the ABI PRISM 7700 sequence detection system (PerkinElmer), as previously described (21). Primer sequences and PCR conditions are available upon request.

### *In situ hybridization*

Paraffin-embedded mouse lung sections fixed with 4% paraformaldehyde (7 μm/section) were prepared for in situ hybridization. Hybridization was performed with 100 ng/ml digoxigenin-labeled anti-sense and sense probes corresponding to the 1605 to 2025 basic acids of the *Slc26a4* gene at 60°C for 16 h. The sections were washed with 5 × SSC at 60°C for 20 min, then with 2 × SSC in 50% formamide at 60°C for 20 min, followed by treatment of 50 μg/ml RNaseA for 30 min at 37°C. Then the sections were incubated with alkaline phosphatase-conjugated anti-digoxigenin Ab (Roche Diagnostics), and the signals were detected with nitro blue tetrazolium (NBT)/5-bromo-4-chloro-3-indoxyl phosphate (Roche Diagnostics) as substrates.

### *Immunohistochemical staining*

The synthetic peptide corresponding to aa 630–643 of human pendrin (PTKEIEIQVDWNSE) or 612–625 of rat pendrin (NNAFEPDEDVEEPE) was used to generate anti-pendrin serum. The peptides or recombinant GFP protein was injected together with complete Freund's adjuvant (Sigma-Aldrich) into New Zealand White rabbits.

Paraffin-embedded mouse lung tissues fixed with 3.7% formaldehyde were processed, and the Ag was detected using Histofine SAB-PO(R) Kit and anti-pendrin serum according to the manufacturer's procedure (Nichirei).

### *Recovery of Sendai virus vectors*

We constructed SeV(HNL)-mPend/ΔF and SeV(HNL)-mPend/ΔF-GFP according to the methods described previously (28–30), with minor modifications. As a control vector, the GFP gene carrying SeV/ΔF ($SeV^{18+}$GFP/ΔF, Ref. 30) was used. The virus vector titers were determined using infectivity and were expressed in CIU.

### *Western blotting*

Pendrin-expressing NCI-H292 cells were lysed by PBS containing 1% SDS, and the cell lysates were boiled with SDS sample buffer containing 7 M urea. The samples were applied to SDS-PAGE and then electrophoretically transferred to polyvinylidene difluoride membranes (Amersham Biosciences). The proteins were visualized by ECL (Amersham Biosciences).

### *Transport assay of iodide*

Iodide uptake and efflux were measured as previously described (31). COS7 cells ($5 \times 10^4$/well) transiently transfected with pEGFP-C1-PDS for 48 h or NCI-H292 cells ($5 \times 10^4$/well) incubated with $8 \times 10^5$ CIU/well of SeV(HNL)-mPend/ΔF or SeV/ΔF ($SeV^{18+}$GFP/ΔF) for 48 h were used. Chloride-free buffer consisting of 140 mM sodium gluconate, 5.4 mM potassium gluconate, 1.8 mM $CaOH_2$, 0.5% BSA, and 10 mM HEPES-NaOH (pH 7.4) was used for incubation and washing. The incubation buffer contained 1 mM $Na^{125}I$ (20 mCi/mmol).

### *ELISA*

As for ELISA for Muc5ac/MUC5AC, we coated 96-well plates with 0.2 μg of anti-MUC5AC Ab (45M1, Lab Vision/Neomarkers) overnight at 4°C. After blocking with buffer (PBS containing 2% BSA and 0.05% Tween 20) for 1 h at room temperature, the indicated dilutions of bronchoalveolar lavage (BAL) fluid or supernatants were incubated for 1 h at room temperature. After extensive washing, 5 μg/ml peroxidase-conjugated soybean agglutinin (Sigma-Aldrich) was incubated for 1 h at room temperature, and then the signals were developed by the ABTS Peroxidase Substrate System

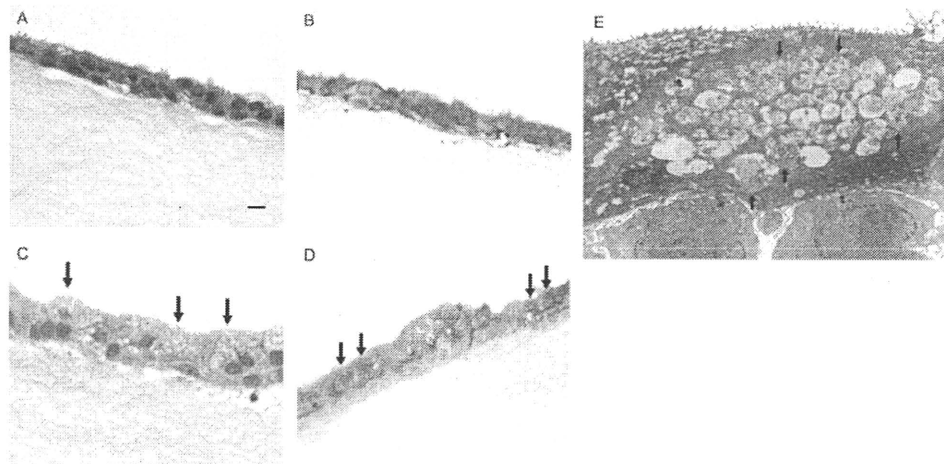


**FIGURE 1.** Freshly prepared human primary tracheal epithelial cells were differentiated into mucus-producing cells by IL-13. Freshly prepared human primary tracheal epithelial cells cultured in the absence (*A* and *B*) or presence (*C* and *D*) of 10 ng/ml IL-13 for 3 wk by air interface were stained with H&E (*A* and *C*) or PAS/Alcian blue (*B* and *D*) or subjected to the electron microscopic analysis (*E*). The arrows indicate the mucus produced in the cells (*C* and *D*) or the mucus granules produced in the cells (*E*); the bar in *A* indicates 10 $\mu$m.

(KPL). Cxcl1 and Cxcl2 were quantitated using Quantikine Kits (R&D Systems), according to the manufacturer's instructions.

### *Measurement of airway reactivity*

We measured airway reactivity in response to methacholine inhalation in mice by whole-body plethysmography using Buxco Electronics hardware and software 48 h after SeV(HNL)-mPend/ΔF or SeV/ΔF ($SeV^{18+}$GFP/ΔF) was exposed to mice. Mice were placed in chambers and exposed to aerosols of PBS or increasing concentrations of methacholine (0.25, 0.5, and 1 mg/ml) for 8 min. The average values of the enhanced pauses (Penh) between 3 and 6 min for each concentration were calculated.

### *Statistical analysis*

Expression of pendrin, gob-5, MUC5AC, Cxcl1 and Cxcl2, the iodide transport, and Penh were compared by the two-sided, unpaired Student *t* test or the two-sided Welch test. A *p* value of $<0.05$ was considered to be statistically significant.

## Results

### *Identification of pendrin as an IL-13-inducible molecule in human airway epithelial cells*

To identify molecules involved in IL-13-induced mucus production, we used freshly prepared human primary tracheal epithelial cells (20), culturing them with IL-13 for 3 wk in an air-liquid interface fashion. As shown in Fig. 1, treatment of epithelial cells with IL-13 resulted in mucus production that was brightly colored by H&E staining and positive by PAS/Alcian blue staining. Additionally, electron microscopic analysis verified the existence of mucus granules at the apical side of the cytoplasm (Fig. 1*E*). These results suggest that IL-13 stimulation alone causes differentiation of airway epithelial cells into mucus-producing cells, as previously reported (32, 33). We then cultured freshly prepared primary tracheal epithelial cells derived from two different donors with IL-13 for 3 or 7 days and subjected them to microarray analysis. Among a number of IL-13-inducible genes, we were particularly intrigued with the *SLC26A4* gene encoding pendrin (expression compared with control: 18.8- and 118.3-fold at day 3 and 20.1- and 58.2-fold at day 7 for each donor). The *SLC26A4* gene was the most significantly induced gene encoding the ion channel family in our microarray analysis. We thus evaluated the potential role of pendrin in mucus overproduction in a bronchial asthma setting.

### *Expression of pendrin in the lungs of allergic airway disease*

We first tested whether IL-4 or IL-13 can induce expression of pendrin in various airway epithelial cells. As shown in Fig. 2*A–C*, both IL-4 and IL-13 were capable of induction of pendrin in freshly prepared human primary tracheal epithelial cells as well as in mouse cultured trachea, NCI-H292 cells (human lung carcinoma cell line), and TGMBE-02-3 cells (mouse tracheal epithelial cell line), in parallel with gob-5, a well-known IL-13-inducible product (14). Intratracheal administration of IL-13 into BALB/c mice up-regulated expression of pendrin, and the induction was abolished in $STAT6^{-/-}$ mice, supporting the dependency of induction of pendrin on the IL-13/STAT6 pathway (Fig. 2*D*). To test whether expression of pendrin is induced in vivo in the pathophysiological setting of asthma, we used the OVA inhalation model (26). Mice inhaling OVA presented symptoms consistent with allergic airway disease, including mucus hyperproduction, infiltration of inflammatory cells including eosinophils into the bronchial epithelium (Fig. 3*A*), and enhanced airway hyperreactivity evaluated by acethylcholine-induced bronchial construction (26). Treatment of such mice with prednisolone abrogated these symptoms (Fig. 3*A*). The epithelium collected from these OVA-induced asthmatic lungs produced a high amount of pendrin, whereas its level decreased in the presence of prednisolone as well as gob-5 (Fig. 3*B*). To perform immunohistochemical analysis, we generated anti-pendrin Ab, confirming the specificity of this Ab using pendrin-expressing NCI-H292 cells (Fig. 3*C*). Immunohistochemical staining and also in situ hybridization showed that the expression of pendrin was up-regulated, particularly at the apical side of BECs (Fig. 3*D* and data not shown). Immunohistochemical analyses using a limited number of human samples also showed that pendrin was expressed in BECs of asthma patients (data not shown). These results suggest a possible link between the induction of pendrin by IL-4/IL-13 in BECs and the overproduction of mucus in bronchial asthma.

### *Expression of pendrin in the lungs of elastase-induced lung injury*

Mucus hypersecretion is also a feature of COPD, a disease that can be the result of excess elastase activity. We thus tested the expression of pendrin in an experimental model (27) of mucus overproduction, inflammation, and alveolar wall destruction induced by administration of elastase. As shown in Fig. 4, intratracheal administration of pancreatic elastase induced inflammation, mucus overproduction, and increased expression of pendrin. Expression of the *Slc26a4* gene was detectable after a week and increased thereafter (Fig. 4*B*). Immunohistochemical analysis showed that pendrin was highly induced at the apical side of BECs (Fig. 4*C*). These results indicate an association between increased pendrin expression and mucus induction in the lungs by elastase, an inflammatory stimulus other than IL-13.

### *Pendrin induces mucus production in airway epithelial cells*

To directly examine the function of pendrin in airway epithelial cells, we transfected NCI-H292 cells with a pendrin-expressing

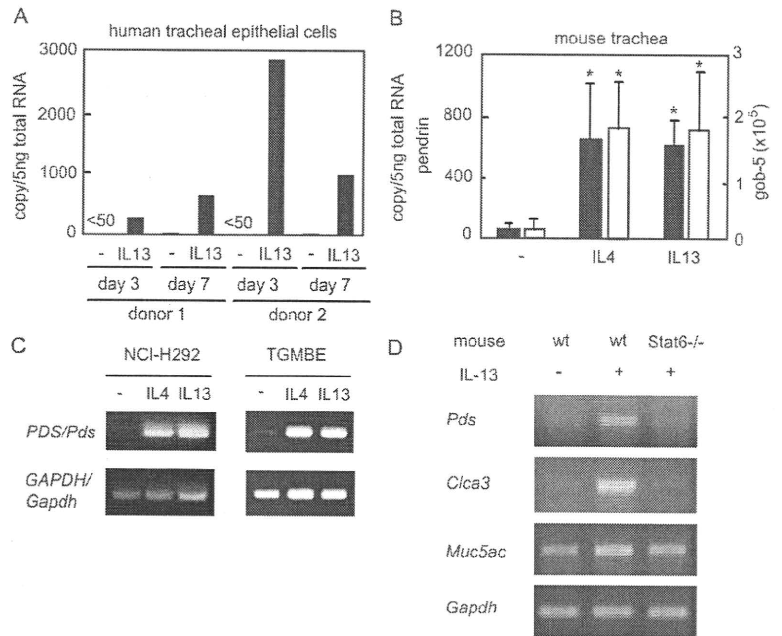


**FIGURE 2.** Pendrin was induced in airway epithelial cells by IL-4 or IL-13. mRNA was extracted from freshly prepared human primary tracheal epithelial cells (*A*), mouse cultured trachea (*B*), or NCI-H292 cells or TGMBE-02-3 cells (*C*) cultured with IL-4 or IL-13 for 3 or 7 days (human tracheal epithelial cells), 24 h (mouse trachea), 48 h (NCI-H292 and TGMBE-02-3 cells), or lung tissues of intratracheally IL-13-administered to wild-type or Stat6$^{-/-}$ mice (*D*) ($n = 4$). The quantitative RT-PCR analysis (*A* and *B*) and RT-PCR (*C* and *D*) for *SLC26A4/Slc26a4* (*PDS/Pds*), *Clca3*, *Muc5ac*, and *Gapdh* genes are depicted. In *A* and *B*, filled and open boxes represent *PDS/Pds* and *Clca3* genes, respectively. *, $p < 0.001$ (vs no stimulant).

vector. Pendrin can transport iodide, bicarbonate, and chloride (17, 34, 35). We first confirmed that pendrin expressed on plasma membranes acted as an anion transporter, using COS-7 cells and NCI-H292 cells (data not shown). Both cells transiently expressing pendrin showed enhanced uptake and efflux activities for iodide. We then generated the NCI-H292 cells stably expressing EGFP-pendrin. Two clones of pendrin-expressing transfectants (no. 1 and no. 2–3) were generated; higher expression of pendrin (no. 2–3)

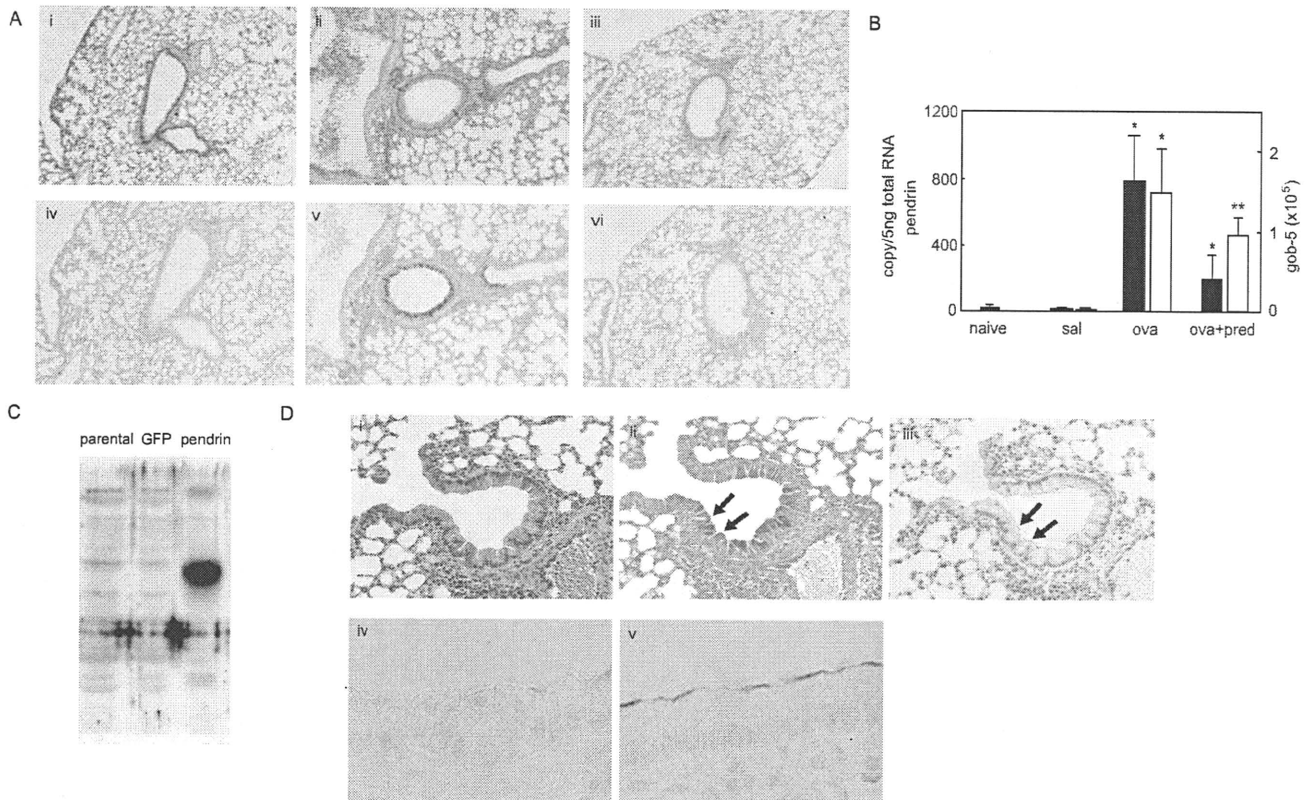


**FIGURE 3.** Pendrin was induced in asthmatic lung. *A*, The lung tissues of saline-inhaled mice (*i* and *iv*) or OVA-inhaled mice without (*ii* and *v*) or with treatment of prednisolone (*iii* and *vi*) were stained with H&E (*i–iii*) or PAS (*iv–vi*). *B*, mRNA was extracted from the lungs of naive or saline- or OVA-inhaled mice without or with treatment of prednisolone. Quantitative RT-PCR analysis for *Slc26a4* (■) or *Clca3* (□) gene is depicted. *, $p < 0.001$; **, $p < 0.05$ (vs saline- or OVA-inhaled mice). *C*, Western blotting using anti-pendrin Ab in parental NCI-H292 cells or NCI-H292 cells incubated with SeV/ΔF (SeV$^{18+}$GFP/ΔF) (GFP) or SeV(HNL)-mPend/ΔF (pendrin) is depicted. *D*, The lung tissues of OVA-inhaled (*i–iii* and *v*) or saline-inhaled (*iv*) mice stained with H&E (*i*) or PAS (*ii*) or immunostaining with anti-pendrin serum (*iii–v*) are depicted. The arrows indicate PAS-positive or pendrin-expressing cells; *iv* and *v* show high-magnitude views.

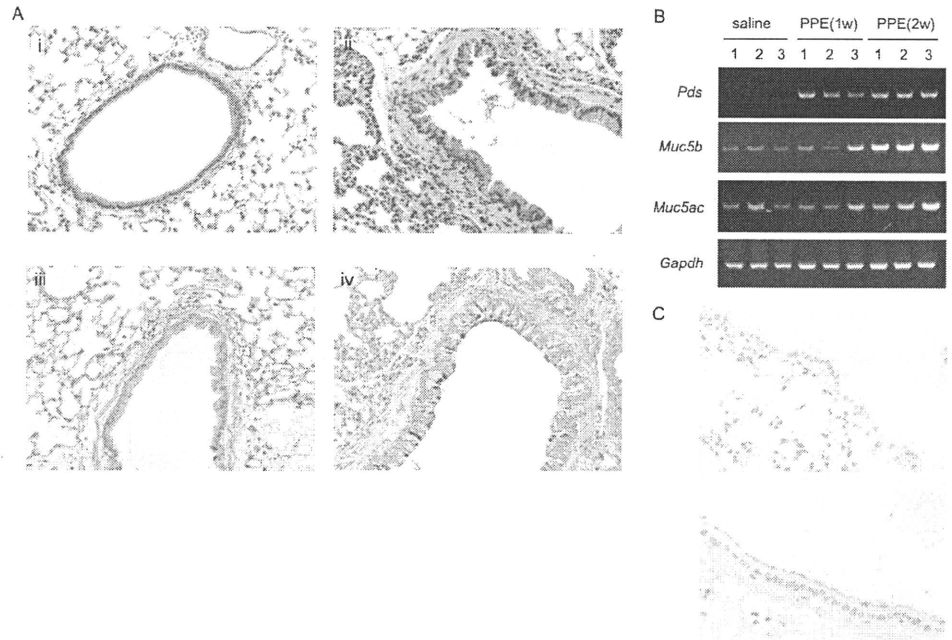


**FIGURE 4.** Pendrin was induced in the lungs of elastase-inhaled mice. *A*, Lung tissues of saline-inhaled mice (*i* and *iii*) or porcine pancreas elastase (PPE)-inhaled mice for 1 wk (*ii* and *iv*) were stained with H&E (*i* and *ii*) or PAS (*iii* and *iv*). *B*, mRNA was extracted from the lungs of saline- or PPE-inhaled mice (1 or 2 wk). RT-PCR for *Slc26a4* (*Pds*), *Muc5b*, *Muc5ac*, and *Gapdh* is depicted. *C*, Immunohistochemical staining of the lungs of saline- (*top*) or PPE-inhaled (*bottom*) mice for 1 wk by anti-pendrin serum is depicted.

correlated with more pronounced iodide uptake (Figs. 5*A*–*C*). These transfectants up-regulated the expression of MUC5AC, a major mucus protein generated in asthma and COPD patients (36), at both the mRNA and protein levels, in parallel with the expression level of pendrin (Fig. 5*D*). Because pendrin expressed in these transfectants was a fusion protein with EGFP, transfection with

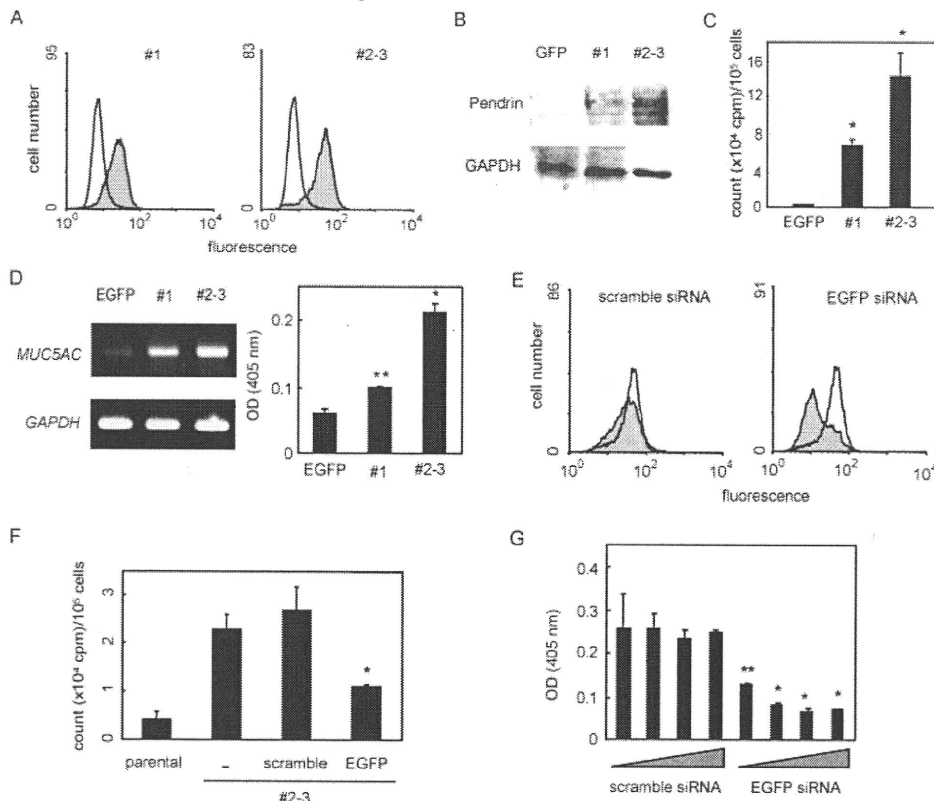


**FIGURE 5.** Mucus production was induced in pendrin-expressing transfectants of NCI-H292 cells. *A*, Expression of EGFP by flow cytometric analysis was shown in two clones (no. 1 and no. 2–3) of NCI-H292 cells stably expressing EGFP-PDS. Gray and white areas represent expression of the transfectants and parental cells, respectively. *B–D*, Western blotting of pendrin and GAPDH (*B*) or iodide uptake at 1 min (*C*) or RT-PCR for *MUC5AC* and *GAPDH* (*D*, *left*) or the amounts of MUC5AC in the cell lysates (*D*, *right*) in NCI-H292 cells stably expressing EGFP alone and EGFP-PDS (no. 1 and no. 2–3) are depicted. *E* and *F*, Expression of EGFP by flow cytometric analysis (*E*) or iodide uptake at 1 min (*F*) in NCI-H292 cells stably expressing EGFP-PDS (no. 2–3) transfected with either scramble or EGFP siRNA (10 pmol) is depicted. Gray and white areas represent expression of the transfectants with or without siRNA, respectively. *G*, The relative amounts of MUC5AC in the cell lysates of NCI-H292 cells stably expressing EGFP-PDS (no. 2–3) transfected with either scramble or EGFP siRNA (1–50 pmol) are depicted. *, $p < 0.01$; **, $p < 0.05$ (vs EGFP or scramble).

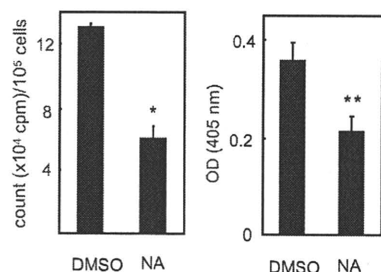


**FIGURE 6.** Niflumic acid inhibited mucus production by pendrin. Iodide uptake at 1 min (*left*) and the amounts of MUC5AC (*right*) in the cell lysates of NCI-H292 cells stably expressing EGFP-PDS (no. 2–3) incubated with either DMSO alone or niflumic acid (NA, 250 $\mu$M) are depicted. *, $p < 0.01$; **, $p < 0.05$ (vs DMSO).

EGFP siRNA significantly knocked down expression of pendrin on the plasma membrane, followed by down-regulation of its anion transport activity (Fig. 5, *E* and *F*). Down-regulation of pendrin expression attenuated MUC5AC production in a dose-dependent manner (Fig. 5*G*). Thus, expression of pendrin directly induced enhanced mucus production in airway epithelial cells.

### *Effects of niflumic acid on mucus production by pendrin*

Niflumic acid, a well-known blocker of anion transporters, has recently been shown to act as an inhibitor of pendrin (37). We thus investigated whether niflumic acid inhibits anion transport by pendrin, followed by attenuated mucus production (Fig. 6). Addition of niflumic acid partially inhibited iodide transport at ~250 $\mu$M; a higher concentration of niflumic acid showed cytotoxicity (data not shown). Treatment of pendrin-expressing transfectants with niflumic acid suppressed MUC5AC production by almost 50%, in parallel with the levels of anion transport. These results suggest that mucus production by anion transporters including pendrin is dependent on its anion transport activity and that niflumic acid acts as a weak inhibitor against such anion transporters. Alternatively, this result may suggest that an anion transporter insensitive to niflumic acid is involved in mucus production.

### *Induction of mucus production by pendrin in mouse lungs*

To examine the pathological role of pendrin in airway epithelial cells in vivo, we directly transfected the target gene specifically into the bronchial epithelial cells. To do so, we established the *Slc26a4* gene-expressing Sendai virus vector, because this vector possesses highly efficient gene transfer capability into airway epithelial cells in vivo (38). Inhalation of the vector induced expression of the coding product, specifically in lung epithelial cells (data not shown). Mice inhaling the pendrin vector developed periodic acid-Schiff (PAS)-positive mucus exudates containing cell components in the lumens of the lungs, whereas mice transfected with the control GFP vector did not display such abnormalities (Fig. 7*A*). Additionally, the expression of pendrin also caused

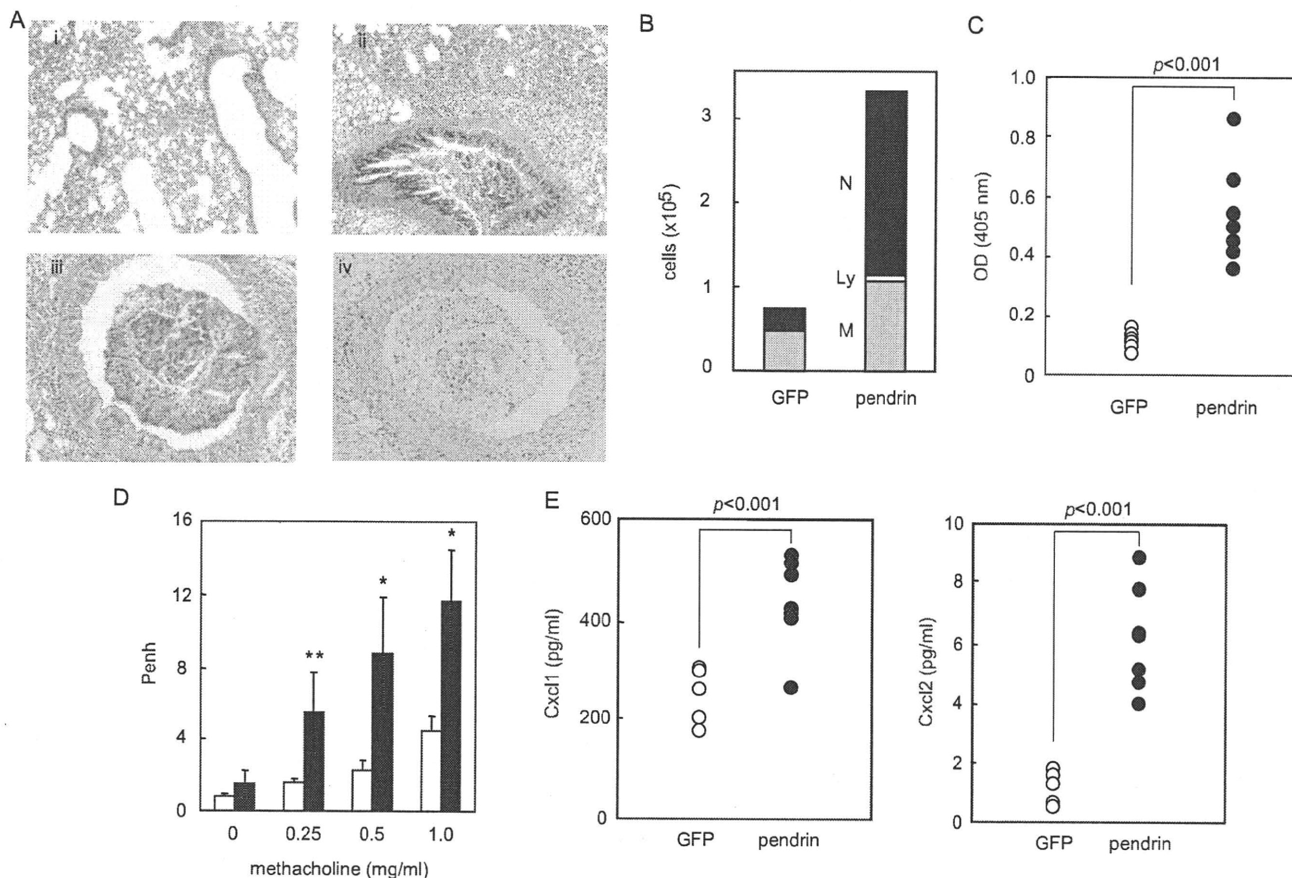


**FIGURE 7.** Enforced expression of pendrin caused mucus production in mouse lungs. Forty-eight hours after $5 \times 10^7$ CIU of SeV/ΔF (SeV$^{18+}$GFP/ΔF) or SeV(HNL)-mPend/ΔF was administered into mouse lungs, histochemical analysis (*A*), cell counts (*B*), ELISA for Muc5ac in the BAL fluid (*C*), analysis of airway reactivity (*D*), and ELISA for Cxcl1 and Cxcl2 in the BAL fluid (*E*) were performed. H&E staining (*i* and *ii*) or PAS staining (*iii*) or immunostaining with anti-pendrin serum (*iv*) of GFP-expressing (*i*) or pendrin-expressing (*ii–iv*) mouse lungs are depicted in *A*. N, Ly, and M represent neutrophil, lymphocyte, and macrophage, respectively, in *B*. EGFP-expressing mice ($n$ = 5 or 6) and pendrin-expressing mice (filled boxes, $n$ = 6 or 7) were used. Four identical experiments were performed, and the representative data are shown. *, $p < 0.01$; **, $p < 0.05$ (vs GFP-expressing mice).