Expression of RANTES by Normal Airway Epithelial Cells after Influenza Virus A Infection

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The chemokine regulated on activation, normal T cells expressed and secreted (RANTES), is a C-C chemokine and a potent chemoattractant for monocytes, T lymphocytes, basophils, and eosinophils. Its expression by human airway epithelium has been demonstrated both *in vitro* and *in vivo*. We investigated whether RANTES is expressed by normal human airway epithelial cells after influenza viral infection and examined its bioactivity. Epithelial cells were obtained from bronchial tissue or nasal polyps of patients who had undergone lobectomy for lung cancer or polypectomy for nasal polyps. These cells were cultured by the outgrowth method. Cultured cells were infected with influenza virus A (subtype H3N2) after which the supernatants and the cells were collected 8 to 72 h after infection. RANTES mRNA (messenger RNA) was analyzed by the reverse transcriptase-polymerase chain reaction and Southern blot analysis of its product. Concentrations of RANTES in the supernatants were analyzed by enzyme-linked immunosorbent assay. RANTES protein and mRNA were not detected in the media of uninfected cells. PCR products for RANTES were clearly detected in nasal and bronchial epithelial cells 24 h after infection. Southern blot analysis confirmed that the PCR products were indeed specific for RANTES mRNA. Twenty-four to 72 h after infection, significant levels of RANTES protein were detected in culture media. We also investigated the chemotactic activity of the supernatant of cultured cells. The supernatant of the cells 48 h after infection had potent chemotactic activity for eosinophils, which was attenuated by the addition of anti-RANTES antibodies. These findings suggest that influenza virus infection may induce expression of bioactive RANTES by normal human bronchial and nasal epithelial cells. **Matsukura, S., F. Kokubu, H. Kubo, T. Tomita, H. Tokunaga, M. Kadokura, T. Yamamoto, Y. Kuroiwa, T. Ohno, H. Suzaki, and M. Adachi. 1998. Expression of RANTES by normal airway epithelial cells after influenza virus A infection. Am. J. Respir. Cell Mol. Biol. 18:255–264.**

The chemokine regulated on activation, normal T cells expressed and secreted (RANTES), is a member of the C-C chemokine family whose first two cysteine residues are adjacent (1). RANTES is a chemoattractant for monocytes, T lymphocytes, basophils, and eosinophils. It is expressed by T lymphocytes, endothelial cells, fibroblasts, platelets, mesangial cells, renal tubular epithelial cells, and eosinophils (2–6). Immunohistochemical studies have demonstrated that nasal polyp epithelium and bronchial tissue from patients with asthma express RANTES (7–9). It has been reported that RANTES might be expressed by bronchial epithelial cell lines and normal human bronchial epithelial cells *in vitro* (10–12).

Viral infection induces inflammatory cells to infiltrate the nasal and bronchial epithelium and causes various respiratory diseases. Viral infection also induces airway epithelial cells to express cytokines, including interleukin (IL)-1, IL-6, IL-8, granulocyte-macrophage colony-stimulating factor (GM-CSF), and tumor necrosis factor α (TNF-α). These cytokines may influence airway inflammation caused by viral infection (13–19). However, little is known about RANTES expression in airway viral infection. We previously observed

(*Received in original form November 4, 1996 and in revised form July 7, 1997*)

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Abbreviations: enzyme-linked immunosorbent assay, ELISA; granulocyte-macrophage colony-stimulating factor, GM-CSF; interferon γ, IFN-γ; interleukin, IL; platelet-activating factor, PAF; regulated on activation, normal T cells expressed and secreted, RANTES; reverse transcriptase-polymerase chain reaction, RT-PCR; tumor necrosis factor α, TNF-α.

that influenza virus can induce RANTES expression in the NCI-H292 lung cancer cell line (20). Influenza virus is an important cause of airway viral infection and airway inflammation (21, 22). In response to influenza viral infection, normal airway epithelial cells might express RANTES. In this study, we cultured human bronchial and nasal epithelial cells obtained from surgical specimens and investigated their ability to express RANTES mRNA (messenger RNA) and to release immunoreactive RANTES. We also analyzed the eosinophilic chemotactic activity of the supernatant of cultured cells and investigated the bioactivity of RANTES in culture media.

Materials and Methods

Culture of Nasal Epithelial Cells and Normal Human Bronchial Epithelial Cells

Normal airway epithelial cells were cultured by a modification of the explant method of Lechner and coworkers (23). Nasal tissue was obtained from 10 nonallergic patients who had undergone surgery to relieve nasal obstruction. Bronchial tissue was obtained from 10 patients who had undergone lobectomy for lung cancer. Tissues were cut into 0.5 × 0.5 cm pieces and explanted to six-well culture plates. Two milliliters of LHC-1 medium (MCDB 151 with the Ca^{2+} concentration increased to 0.1 mM and supplemented with 5 μg/ml insulin, 5 ng/ml epidermal growth factor, 10 μg/ml transferrin, 5×10^{-4} mM each of phosphoethanolamine and ethanolamine, 5×10^{-4} mM hydrocortisone, and 50 μg/ml each of gentamicin and kanamicin) (Sigma Chemical Co., St. Louis, MO) with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY) was then added to the plates, which were then incubated in a humidified 5% CO_2 incubator at 36.5°C.

After 7 to 11 d, outgrowths of epithelial cells were cultured in serum-free LHC-1 medium. After 7 d, cells were dissociated with trypsin (Böehringer-Mannheim, Mannheim, Germany) and inoculated into six-well culture plates coated with collagen (Böttger GmbH, Berlin, Germany). The identity of cultured cells was confirmed on the basis of morphologic characteristics and results of immunocytochemical staining with antibodies against cytokeratin, fibroblasts, and macrophages (Dako Japan, Kyoto, Japan) (24). The morphologic characteristics and positive staining for cytokeratin indicated that more than 96% of cells were epithelial cells. However, less than 3% of cells reacted with antibodies against fibroblasts and macrophages.

Virus Stock

Influenza virus A/Sisen/2/92 (a kind gift from Dr. M. Toda, Showa University, Tokyo, Japan) was grown in Madin–Darby canine kidney (MDCK) cells (American Type Culture Collection, Rockville, MD) in minimal essential medium containing 2% FBS at 33.5°C under 5% CO_2. The infected culture fluid was subjected to centrifugation to produce a clear fluid, which was stored in aliquots at −80°C.

Infection of Cell Culture with Influenza Virus A

Epithelial cells were cultured to near confluence in six-well tissue culture plates. Influenza virus A (H3N2) was added to the plates at a multiplicity of infection of 0.2 (this viral titer was selected because severe cell damage was not observed and epithelial cells could produce cytokines with this viral titer) and allowed to be adsorbed by the cells for 1 h with gentle rocking of the plates every 10 min. After 1 h, virus was removed and 2 ml of serum-free LHC-1 medium was added. Infected cells were incubated at 36.5°C under 5% CO_2 in humidified air. For analysis of viral RNA, infected cells were harvested 1, 8, 24, 48, and 72 h after infection. For analysis of RANTES mRNA, nasal and bronchial epithelial cells, which were either infected or uninfected, were collected 24 h after infection or when media were exchanged. Culture media were collected 8, 24, 48, and 72 h after infection for enzyme-linked immunosorbent assay (ELISA).

Stimulation of Cell Culture with Cytokines

Nasal and bronchial epithelial cells were cultured to near confluence in 24-well tissue culture plates. To analyze whether inflammatory cytokines could stimulate RANTES production, cytokines were added to the plates and the culture media were collected 24 h after stimulation to assay RANTES concentration. Added cytokines were as follows: 10 ng/ml, IL-1β; 100 U/ml, TNF-α; 100 U/ml, interferon γ (IFN-γ); and cytokine mix (a mixture of these three cytokines).

Analysis of Viral RNA by Reverse Transcription-Polymerase Chain Reaction

Total RNA was extracted with guanidium thiocyanate from samples of about 5×10^5 cells (25). First-strand complementary DNAs (cDNAs) were synthesized by means of incubation of 1-μg RNA templates in the presence of Moloney murine leukemia virus reverse transcriptase (Pharmacia, Tokyo, Japan), random primers, and reaction buffer at 37°C for 1 h. After cDNAs were denatured at 94°C for 5 min, the polymerase chain reaction (PCR) was performed with 5 pmol each of the 5′ and 3′ primers, 5 μl of cDNA, 0.6 U of *Taq* polymerase (Böehringer-Mannheim), and 2.5 μl of PCR reaction buffer (100 mmol/liter, Tris-HCl; 15 mmol/liter, $MgCl_2$; 500 mmol/liter, KCl; Böehringer-Mannheim), distilled water was added until the reaction volume was 25 μl. The PCR primers were synthesized according to the sequence of hemagglutinin gene type 3 (H3, positions 337–1063) of influenza virus A (26). The sequences of the primers were 5′-TTGTTGAACGCAGCAAAGCT-3′ and 5′-TCTAGTTTGTTTCTCTGGTA-3′. The amplification reaction was performed for 25 cycles with denaturation at 94°C for 45 s, annealing at 61°C for 45 s, and extension at 72°C for 1 min (Perkin-Elmer Cetus, Norwalk, CT) (27). After incubation at 72°C for 10 min, PCR-amplified products were analyzed with 1.8% agarose gel electrophoresis and ethidium bromide staining followed by visualization with an ultraviolet transilluminator.

Analysis of mRNA for RANTES by RT-PCR

Total RNA was extracted from 5×10^5 airway epithelial cells. The RT-PCR was performed using the method described previously. The PCR primers were as follows (1):

RANTES: 5′-ATGAAGGTCTCCGCGGCACGCCT-3′
5′-CTAGCTCATCTCCAAAGAGTTG-3′

β-actin: 5′-GTGGGGCGCCCCAGGCACCA-3′
5′-CTCCTTAATGTCACGCACGATTTC-3′

The amplification reaction was performed for 30 cycles with denaturation at 94°C for 45 s, annealing at 61°C for 45 s, and extension at 72°C for 1 min.

Southern Blot Hybridization of PCR Products

After incubation at 72°C for 10 min, PCR-amplified products were analyzed by 1.8% agarose gel electrophoresis. The gel was soaked in a denaturing solution containing 1.5 M sodium chloride and 0.5 M sodium hydroxide for 45 min with gentle shaking, then soaked in a neutralizing solution containing 1.5 M sodium chloride and 1 M Tris-EDTA, pH 7.4, for 45 min. The PCR products were transferred onto a nitrocellulose filter in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 10 h with capillary action. The filter was dried at 80°C for 1 h. Prehybridization was carried out at 65°C for 2 h in a solution containing 6× SSC, 0.5% sodium dodecyl sulfate (SDS), 5× Denhardt's solution, and 100 μg/ml salmon sperm DNA. Hybridization was carried out at 65°C for 10 h in a solution containing 5× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 0.2% SDS, 5× Denhardt's solution, 100 μg/ml salmon sperm DNA, and the probes for RANTES and β-actin, which encoded the internal region of the PCR products (R&D Systems, Minneapolis, MN) and were end labeled with ^{32}P by means of polynucleotide kinase. The filter was washed twice with 2× SSC and 0.1% SDS at room temperature for 30 min and then washed at 65°C for 2 h with gentle shaking in 0.1× SSC and 0.1% SDS. The filter was dried and used to expose autoradiography film at −80°C (28).

Assay of RANTES Released into Culture Medium

Concentrations of RANTES in the collected culture medium were determined with a commercially available system for ELISA (Amersham Life Science, Amersham, UK). The standards and samples were added to the 96 wells of a microtiter plate coated with anti-RANTES antibody. After incubation at room temperature for 2 h, each well was washed with wash buffer four times. Then, polyclonal antibody against RANTES, which was conjugated to horseradish peroxidase, was added to the well. After incubation at room temperature for 2 h, each well was washed with wash buffer four times. Substrate solution (stabilized hydrogen peroxide and tetramethylbenzidine) was added to each well, after which the plate was incubated at room temperature for 20 min. Sulfuric acid was then added to each well, and the absorbance was measured at 450 nm.

A standard curve was generated by plotting the logarithm of the optical density against the logarithm of the concentration of the standard; the concentration of each sample was then calculated. The minimal detectable concentration was 2.5 pg/ml.

Assay of IL-1β, TNF-α, and IFN-γ in the Medium

To determine whether inflammatory cytokines mediated the RANTES production, concentrations of IL-1β, TNF-α, and IFN-γ in the culture medium collected 24 h after infection were also analyzed using commercially available ELISA kits (IL-1β and TNF-α [R&D Systems]; IFN-γ [Genzyme, Cambridge, MA]) with the same method as described previously. The minimal detectable concentration was as follows: IL-1β, 1 pg/ml; TNF-α, 4.4 pg/ml; and IFN-γ, 3 pg/ml.

Blocking Assay to IL-1β and TNF-α

We also examined a blocking assay to analyze the intervention of IL-1β and TNF-α in RANTES production induced by influenza virus. Anti-IL-1β antibodies (40 μg/ml; R&D Systems) and anti-TNF-α antibodies (40 μg/ml; R&D Systems) were added at the time of inoculation of influenza virus. Twenty-four hours after, the medium of the cells was collected and the concentrations of RANTES in the medium were analyzed by ELISA.

Eosinophil Chemotaxis

The peripheral blood of healthy volunteers was heparinized and overlaid onto Mono-Poly solution (Dai Nippon Pharmaceutical Co., Ltd., Osaka, Japan) (29) and then centrifuged at 400 × *g* at room temperature for 20 min. The polymorphonuclear leukocyte-rich fraction was aspirated and washed twice with phosphate-buffered saline (centrifugation at 400 × *g* for 10 min). Eosinophils were purified by negative selection with immunomagnetic microbeads coated with antibody against CD16 (30). Fifty microliters of MACS CD16 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) per 5×10^7 cells was added to the cell pellets, after which the cells were incubated for 30 min at 6°C. The cells were suspended in 1 ml of phosphate-buffered saline, and the cell suspension was applied to the column in a MACS separator. The CD16-negative cells were collected and the eosinophil purity was determined by microscopic examination of Giemsa-stained cytospin preparations.

Eosinophil chemotaxis was assayed by means of a modification of Junger's method (31) using a 96-well chamber (Neuro Probe, Inc., MD). Eosinophils were suspended at 2.5×10^5 cells/ml in the LHC medium. Each supernatant of cultured epithelial cells (uninfected cells and cells infected with influenza virus A) with or without anti-RANTES neutralizing antibodies (50 μg/ml, R&D Systems), a volume of 410 μl, was added to each well of a flat-bottom 96-well microtiter plate (Neuro Probe, Inc.). The microtiter plate was placed into the bottom tray of the chamber and covered with a polycarbonate filter (5-μm pore size, Neuro Probe, Inc.). Eosinophil suspension (200 μl) was then added to the upper wells after the chamber was tightened and allowed to migrate for 60 min at 37°C in 5% CO_2 atmosphere. The upper wells were then emptied by suction, and the microtiter plate with the attached filter was removed. The upper surface of the filter was wiped with a tissue to remove residual cell suspension, and the cells in the microtiter plate were sedimented by centrifugation at 250 × *g* for 15 min. The filter was detached and sedimented cells were counted by phase-contrast microscopy. The numbers of cells were counted in five random fields (one field = 1 mm^2) of each duplicate well. LHC medium

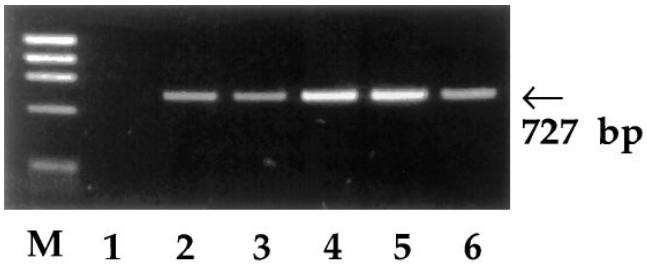


Figure 1. Example of RT-PCR amplification of hemagglutinin gene in airway epithelial cells. Normal human bronchial epithelial cells were infected with influenza virus A (H3N2) and collected 1, 8, 24, 48, and 72 h after infection. Hemagglutinin gene in the cells was amplified with RT-PCR, and the PCR products were analyzed by 1.8% agarose gel electrophoresis and ethidium bromide staining followed by visualization with an ultraviolet transilluminator. *Lane M*, size marker; *lane 1*, product from uninfected cells (negative control); *lanes 2–6*, products from cells collected 1, 8, 24, 48, and 72 h after infection.

only was used as a negative control, and platelet-activating factor (PAF) (10^{-7} M; Sigma) or recombinant RANTES (1, 10, and 100 ng/ml; R&D Systems) was used as a positive control for eosinophil chemotaxis.

Data Analysis

Analysis of variance was used to evaluate the statistical significance of the data in the analysis of cytokines concentration in the culture medium and eosinophil chemotaxis. Differences with P values less than 0.05 were considered significant.

Results

Expression of Hemagglutinin Gene in Infected Cells

The hemagglutinin (H3) gene of influenza virus A was detected by RT-PCR in bronchial and nasal epithelial cells after virus inoculation. The PCR product was not detected in the samples obtained from uninfected cells (negative control, Figure 1, *lane 1*). Samples obtained from cells collected 1 h after infection yielded a thin PCR product at 727 bp (Figure 1, *lane 2*). Samples from cells collected 24 to 48 h after infection yielded a clear PCR product (Figure 1, *lanes 4* and *5*).

Expression of mRNA for RANTES in Infected Cells

RANTES mRNA was detected by RT-PCR. Samples obtained from nasal epithelial cells (Figure 2a, *lane 2*) and bronchial epithelial cells (Figure 2a, *lane 4*) harvested 24 h after influenza virus A infection yielded amplified cDNA bands whose size was compatible with that of the PCR product (276 bp). No product was detected from uninfected nasal and bronchial epithelial cells (Figure 2a, *lanes 1* and *3*). The PCR product for β-actin (548 bp) was detected in each sample (Figure 2b, *lanes 1–4*). Southern blot analysis confirmed that the PCR products were indeed specific for RANTES mRNA and β-actin (Figure 3).

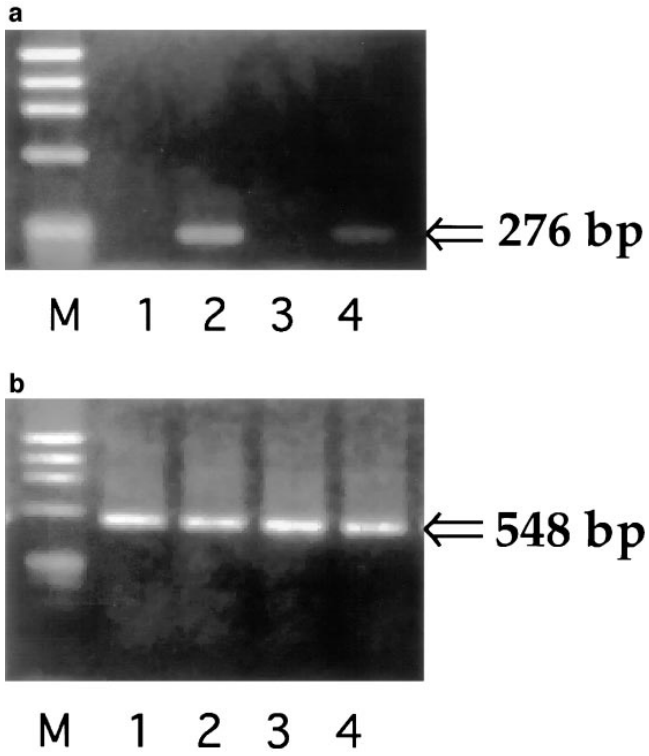


Figure 2. Detection of RANTES mRNA by RT-PCR. Normal human bronchial and nasal epithelial cells were infected with influenza virus A (H3N2). The infected cells were collected 24 h after infection. Uninfected cells were also collected 24 h after the exchange of medium. RANTES mRNA and β-actin in these cells were analyzed by RT-PCR. The PCR products were analyzed by 1.8% agarose gel electrophoresis and ethidium bromide staining followed by visualization with an ultraviolet transilluminator. (*a*) Products of RANTES mRNA. (*b*) Products of β-actin. *Lane M*, size marker; *lane 1*, product of sample from uninfected nasal epithelial cells; *lane 2*, nasal epithelial cells infected with influenza virus; *lane 3*, uninfected bronchial epithelial cells; *lane 4*, bronchial epithelial cells infected with influenza virus. (Examples of five separate experiments.)

Presence of RANTES Protein in the Medium of Infected Nasal Epithelial Cells

RANTES was not detected in the culture medium of uninfected nasal epithelial cells at any of the four times examined. However, RANTES was detected in the culture medium of infected cells 24 h after infection (298 ± 173 pg/ml, mean ± standard deviation, $*P < 0.05$) and increased in concentration until 48 h (806 ± 158 pg/ml; Figure 4a).

Production of RANTES Protein in the Medium of Infected Bronchial Epithelial Cells

As was the case with nasal epithelial cells, immunoreactive RANTES was not detected in the medium of normal bronchial epithelial cells at any of the times examined. However, RANTES was detected at low concentrations (24.5 ± 0.7 pg/ml) in the medium of bronchial epithelial cells 8 h after influenza viral infection. The concentration

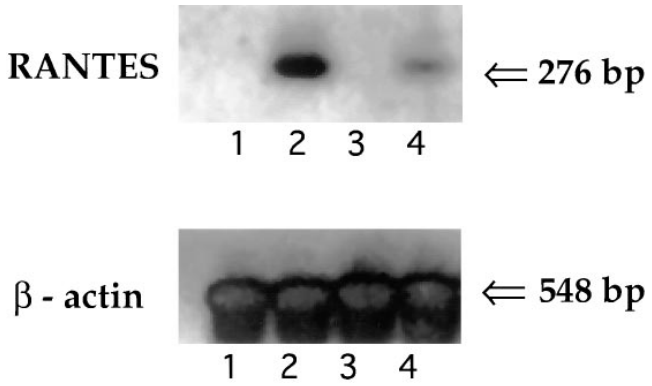


Figure 3. Southern blot analysis of PCR products. The PCR products (Figure 2) were blotted and hybridized with probes for RANTES and β-actin. *Lane 1,* Southern blot analysis of product from uninfected nasal epithelial cells; *lane 2,* nasal epithelial cells infected with influenza virus; *lane 3,* uninfected bronchial epithelial cells; *lane 4,* bronchial epithelial cells infected with influenza virus. (Examples of five separate experiments.)

of RANTES increased with time and peaked 72 h after infection (506.8 ± 134.2 pg/ml; Figure 4b).

Production of RANTES Protein in the Medium of Epithelial Cells Stimulated by Cytokines

Small amounts of RANTES were detected in the medium of nasal epithelial cells (Figure 5, *open bars*) 24 h after stimulation by each cytokine (9.7 ± 7.2 pg/ml by IL-1β, 4.3 ± 4.0 pg/ml by TNF-α, 5.5 ± 3.7 pg/ml by IFN-γ). Its production significantly increased 24 h after stimulation with cytokine mix (133.2 ± 68.1 pg/ml).

As was the case with nasal epithelial cells, small amounts of RANTES were detected in the medium of bronchial epithelial cells (Figure 5, *closed bars*) 24 h after stimulation by each cytokine (14.7 ± 9.6 pg/ml by IL-1β, 12.7 ± 8.6 pg/ml by TNF-α, 23.1 ± 21.3 pg/ml by IFN-γ). However, its production significantly increased 24 h after stimulation with cytokine mix (119.7 ± 19.4 pg/ml; Figure 5).

Concentration of IL-1β in the Medium of Epithelial Cells

Small amounts of IL-1β were detected in the medium of uninfected nasal epithelial cells (4.7 ± 1.9 pg/ml). Its concentration increased 24 h after infection with influenza virus (67.3 ± 45.7 pg/ml). As was the case for nasal epithelial cells, uninfected bronchial epithelial cells produced small amounts of IL-1β (2.0 ± 1.7 pg/ml). Its production increased 24 h after infection (34.7 ± 25.7 pg/ml; Figure 6a).

Concentration of TNF-α in the Medium of Epithelial Cells

TNF-α was not detected in the media of uninfected nasal and bronchial epithelial cells. However, it was detected in the medium of nasal epithelial cells 24 h after infection with influenza virus (38.7 ± 21.1 pg/ml). It was also detected in the medium of bronchial epithelial cells 24 h after infection (9.8 ± 3.0 pg/ml; Figure 6b).

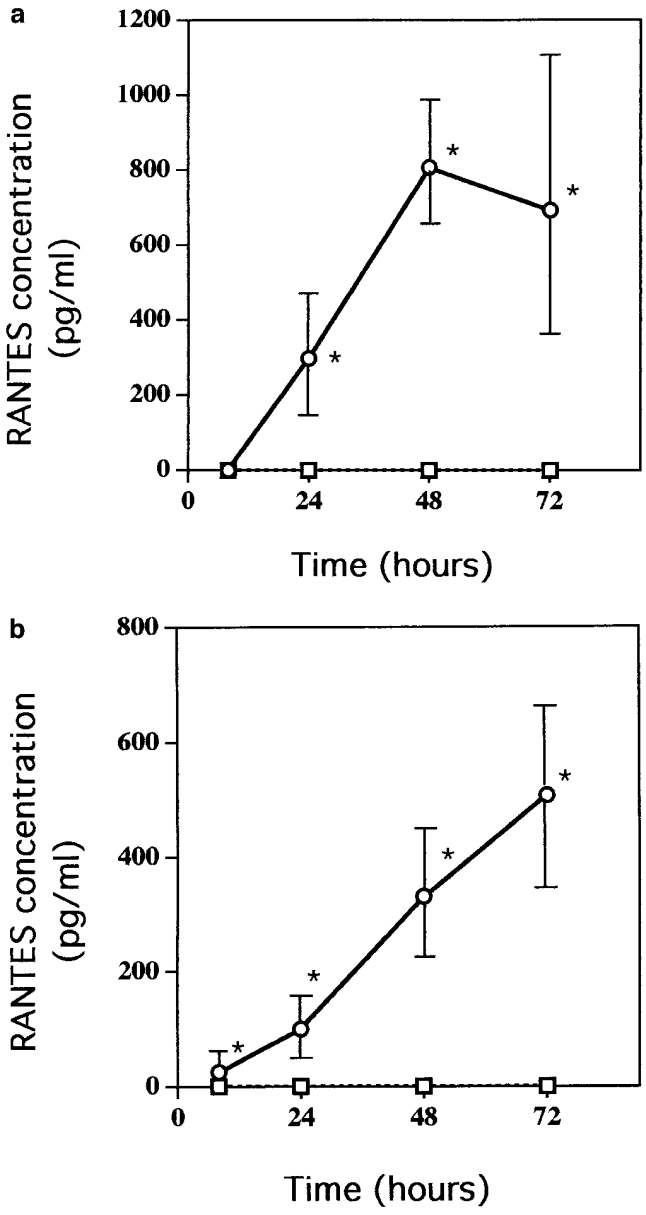


Figure 4. Assay of RANTES in media of cultured airway epithelial cells, which were infected with influenza virus. Media of uninfected cells and cells infected with influenza virus were collected, and RANTES was detected by ELISA. (*a*) Time course of RANTES concentration in the medium of nasal epithelial cells. (*b*) Time course of RANTES concentration in the medium of normal bronchial epithelial cells. *Circles* represent data of infected cells. *Squares* represent data of uninfected cells. $*P < 0.05$ compared with uninfected cells. Five separate experiments were performed.

Concentration of IFN-γ in the Medium of Epithelial Cells

IFN-γ was not detected in the media of all experiments (data not shown).

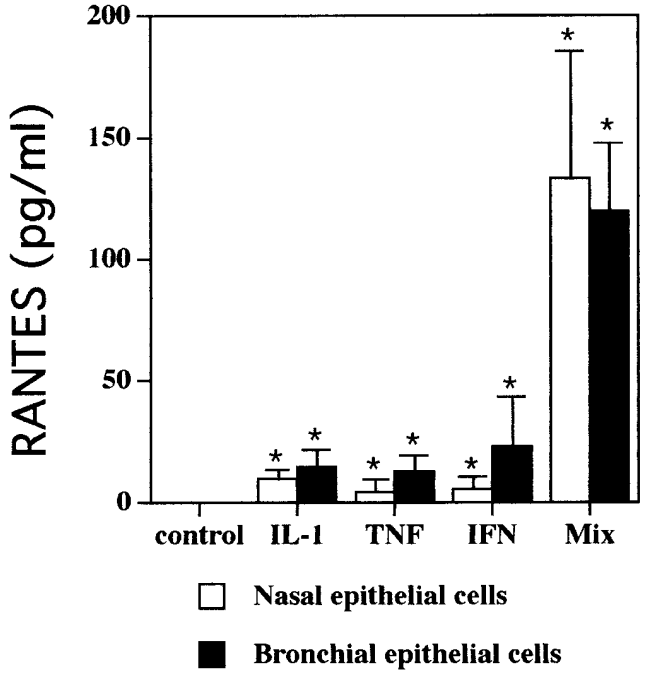


Figure 5. Assay of RANTES in medium of epithelial cells, which were stimulated by cytokines. Media of the cells stimulated with 10 ng/ml IL-1β, 100 U/ml TNF-α, 100 U/ml IFN-γ, and a mixture of these cytokines were collected 24 h after stimulation, and RANTES was detected by ELISA. *Open bars* represent data on nasal epithelial cells. *Closed bars* represent data on bronchial epithelial cells. $*P < 0.05$ compared with unstimulated cells (control). Four separate experiments were performed.

Effect of Anti-IL-1β and Anti-TNF-α Antibodies

Anti-IL-1β and TNF-α antibodies did not suppress the production of RANTES in nasal and bronchial epithelial cells (Figure 7).

Eosinophil Chemotaxis

As positive controls, both PAF (0.1 μM) and recombinant RANTES (10 and 100 ng/ml) had significant chemotactic activity for eosinophils (PAF: 0.1 μM, 6.5 ± 0.7 cells/field; RANTES: 10 ng/ml, 4.5 ± 0.7 cells/field; 100 ng/ml, 7 ± 1.4 cells/field). However, as negative controls, the chemotactic activity of culture medium alone (2 ± 0.4 cells/field) and recombinant RANTES (1 ng/ml, 3.0 cells/field) did not differ significantly (Figure 8a).

The medium of uninfected nasal epithelial cells (6 ± 2.2 cells/field) had significant chemotactic activity for eosinophils; however, it was not suppressed by anti-RANTES antibodies. There was no significance between the activity in the medium of uninfected cells and that in the medium of the cells 24 h after infection (6.2 ± 2.1 cells/field). The activity of the medium in the cells 24 h after infection was not suppressed by anti-RANTES antibodies (Figure 8b). However, the medium of nasal epithelial cells 48 h after infection had potent chemotactic activity for eosinophils (12.5 ± 0.7 cells/field). This activity was suppressed by

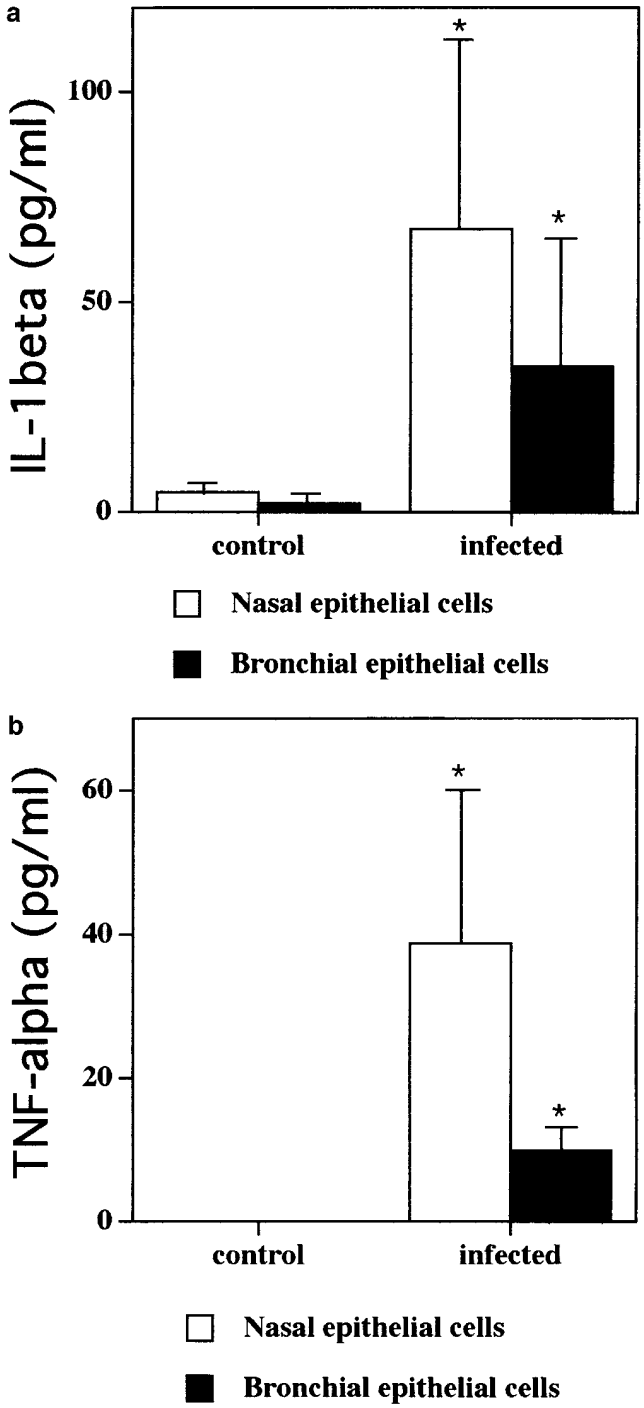


Figure 6. Assay of IL-1β and TNF-α in medium of cultured airway epithelial cells, which were infected with influenza virus. Media of uninfected cells (control) and cells infected with influenza virus were collected 24 h after infection, and concentrations of IL-1β (*a*) and TNF-α (*b*) were determined by ELISA. *Open bars* represent data on nasal epithelial cells. *Closed bars* represent data on bronchial epithelial cells. $*P < 0.05$ compared with uninfected cells (control). Four separate experiments were performed.

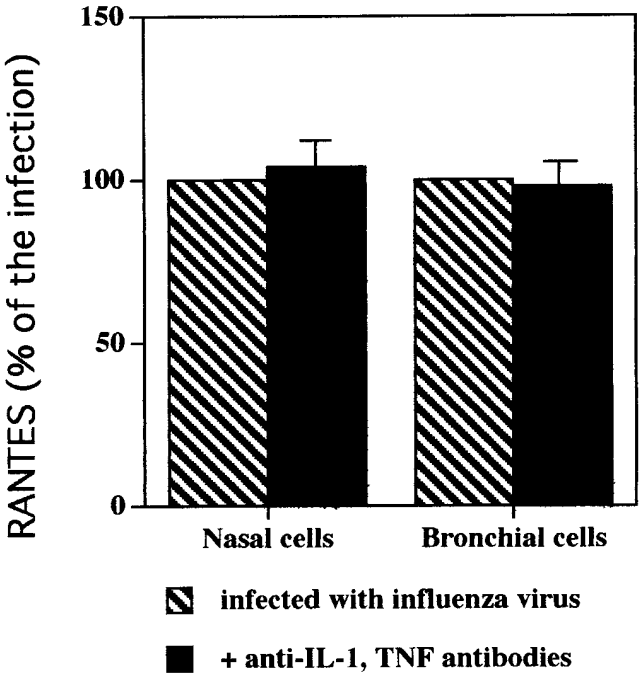


Figure 7. The effect of anti-IL-1β and anti-TNF-α antibodies on the production of RANTES in the media of infected cells. Media of infected cells with or without anti-IL-1β and anti-TNF-α antibodies were collected 24 h after infection and RANTES was detected by ELISA. *Hatched bars* represent RANTES concentration in the media without antibodies. *Closed bars* represent RANTES in the media with antibodies. Data shown as a percentage of RANTES concentration in the media without antibodies. Four separate experiments were performed.

anti-RANTES antibodies to the level of uninfected cells (5.5 ± 0.7 cells/field) (Figure 8c).

As was the case with nasal epithelial cells, the medium of uninfected normal bronchial epithelial cells was chemotactic for eosinophils (6.0 ± 1.0 cells/field); however, it was not suppressed by anti-RANTES antibodies. There was no statistical significance between the activity in the medium of uninfected cells and that in the medium of the cells 24 h after infection (6.8 ± 1.3 cells/field). The activity of the medium in the cells 24 h after infection was not suppressed by anti-RANTES antibodies (Figure 8d). However, the chemotactic activity of the medium of bronchial epithelial cells 48 h after infection was approximately three times greater than that of the medium of uninfected cells (17.3 ± 2.1 cells/field) and was significantly suppressed when anti-RANTES antibodies were added (7.0 ± 2.0 cells/field) (Figure 8e).

Discussion

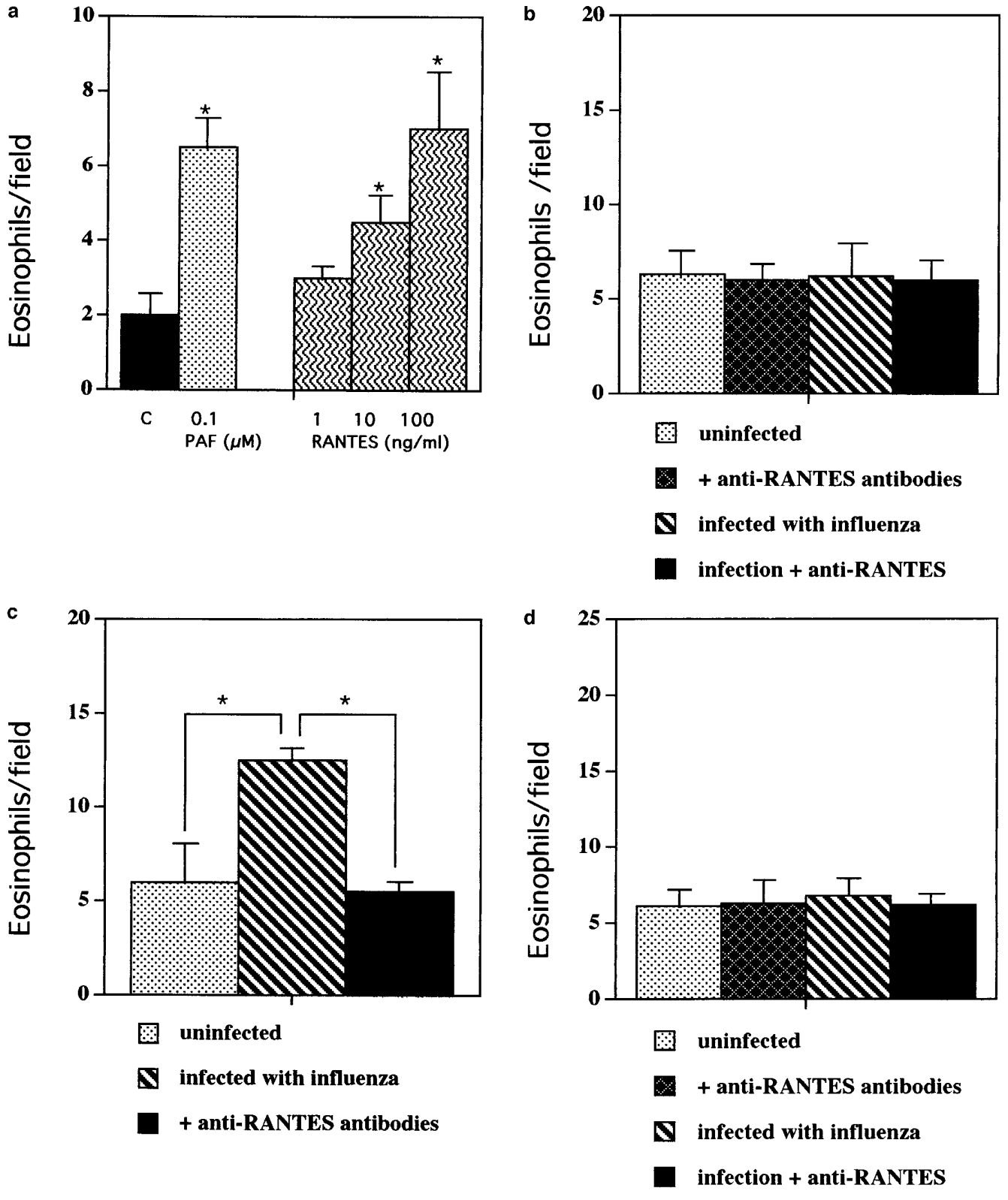
This study demonstrates that cultured normal airway epithelial cells may be infected with influenza virus A, that the viral RNA replicates in the cells, and that influenza viral infection induces these cells to express RANTES mRNA and produce RANTES. This study also demonstrates that the supernatant of cells infected with influenza virus has strong chemotactic activity for eosinophils that is suppressed to the level of uninfected cells by anti-RANTES antibodies.

RANTES is chemotactic for T lymphocytes and eosinophils and also activates eosinophils. It has been reported that RANTES is expressed in the bronchial mucosa of patients with asthma. Therefore, RANTES may play an important role in the pathogenesis of airway hypersensitivity that is caused by the accumulation of inflammatory cells, such as eosinophils and lymphocytes, in the bronchial mucosa. In this study, we demonstrated that RANTES is produced by normal airway epithelial cells after influenza virus infection and that it has strong chemotactic activity for eosinophils. Respiratory viral infection causes airway inflammation and persistent airway hypersensitivity, such as in asthma. Influenza viral infection may induce RANTES production by airway epithelium, continuously attracts eosinophils into the bronchial mucosa, and causes airway hypersensitivity. Some investigators have reported that viral infection induces the infiltration of eosinophils into the airway (32, 33). Our findings may support these reports.

Although we found that recombinant RANTES (10 and 100 ng/ml) caused chemotaxis of eosinophils, the chemotactic activities of medium only and of the medium of 1 ng/ml recombinant RANTES did not differ significantly. However, RANTES produced by epithelial cells that had been infected with influenza virus 48 h previously had strong chemotactic activity for eosinophils at concentrations less than 1 ng/ml. This activity may be due to the synergistic effects of other chemokines for eosinophils, which are produced by airway epithelial cells and may be upregulated by viral infection (20, 34). In addition, RANTES derived from airway epithelial cells might have greater chemotactic activity than recombinant RANTES, even if there were no effects of other cytokines in the media.

Although nasal epithelial cells, 48 h after infection, produced the higher concentration of RANTES compared with bronchial epithelial cells, the chemotactic activity for eosinophils in the medium of nasal epithelial cells was less than that in the medium of bronchial epithelial cells. The chemotactic activity in the medium of infected cells may be dependent on the level of RANTES concentration, because the medium of epithelial cells 24 h after infection (containing a low level of RANTES) did not have significant activity. Therefore, there is a possibility that bronchial epithelial cells may produce more bioactive RANTES compared with nasal epithelial cells.

Although some investigators have demonstrated that unstimulated cultured normal human bronchial epithelial cells produce RANTES (12, 34, 35), in this study we did not detect RANTES mRNA or RANTES protein produced by uninfected epithelial cells. Considering the effect of hydrocortisone, we cultured these airway epithelial cells in medium without hydrocortisone but could not detect RANTES production or mRNA expression (data not shown). Because the amount of RANTES produced by unstimulated normal airway epithelial cells may be extremely small, this discrepancy may be due to differences in the sensitivities of protein assay systems. In addition, the differences in culture conditions and samples (tissue of patients) may influence the results of experiments.



The mechanism by which RANTES expression is induced by influenza virus infection has not been clarified. As in the report of Berkman and coworkers (35), we demonstrated that IL-1β, TNF-α, and IFN-γ each induced production of small amounts of RANTES protein by nasal and bronchial epithelial cells and that a combination of these three cytokines induced production of greater amounts of RANTES. We hypothesized that these cytokines may con-

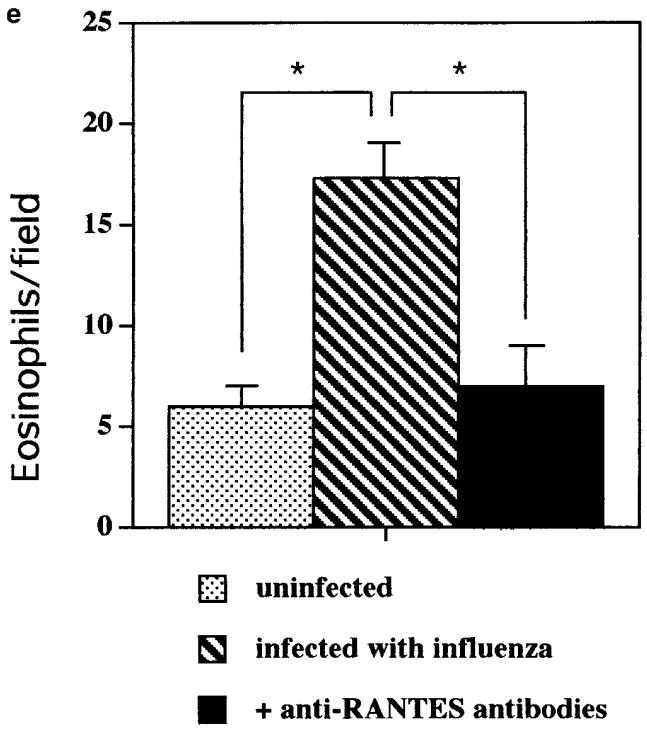


Figure 8. Assay of eosinophil chemotaxis. (*a*) The effect of PAF and recombinant RANTES on eosinophil chemotaxis. C, LHC medium only was used as negative control. (*b*) The effect of the supernatant of cultured nasal epithelial cells, which were collected 24 h after infection or medium exchange, on eosinophil chemotaxis. (*c*) The effect of the supernatant of nasal epithelial cells, collected 48 h after infection or medium exchange, on eosinophil chemotaxis. (*d*) The effect of the supernatant of cultured bronchial epithelial cells, which were collected 24 h after infection or medium exchange, on eosinophil chemotaxis. (*e*) The effect of the supernatant of bronchial epithelial cells, collected 48 h after infection or medium exchange, on eosinophil chemotaxis. $*P < 0.05$. Four separate experiments were performed.

tribute to RANTES expression in viral infection. However, we could not detect IFN-γ in the culture media and the detected concentrations of IL-1β and TNF-α in the medium of infected cells were under 100 pg/ml. These low levels of IL-1β and TNF-α could not induce RANTES in the epithelial cells (data not shown). Also, anti-IL-1β and anti-TNF-α antibodies did not suppress RANTES production after influenza infection. Therefore, we thought that a mechanism other than the intervention of cytokines might play an important role in RANTES expression by influenza virus.

Some transcription factors may contribute to the RANTES expression induced by viral infection. Phal and coworkers report that hemagglutinin of influenza virus activates transcription factor NF-κB in HeLa cells (36). Similarly, hemagglutinin may activate NF-κB in airway epithelial cells. NF-κB is one of the transcription factors that upregulate RANTES (37). Because, as shown in the present study, the hemagglutinin gene is expressed in airway epithelial cells infected with influenza virus, influenza virus might regulate RANTES expression in these cells through the activation of NF-κB.

The present study demonstrates that cultured nasal and normal bronchial epithelial cells can express and secrete bioactive RANTES. These findings may contribute to the understanding of the mechanisms of airway inflammation. However, because these findings were obtained *in vitro*, whether they precisely reflect *in vivo* airway inflammation is not known. Further investigations are necessary to clarify the mechanism by which viral infection causes airway inflammatory disease.

Acknowledgments: The authors thank Drs. Bruce S. Bochner, David Proud, Robert P. Schleimer, Hiroyuki Watanabe, Kunihiko Fukuchi, Kunio Gomi, Kazuo Kobayashi, Takeshi Kasama, Keita Kasahara, Toshimichi Imai, Masako Yasuda, Masashi Iwata, Hiroyuki Tsujiyama, and Masako Toda for their helpful comments and Midori Hayashida, Kaoru Saijyou, Michiko Okamoto, and Hiroko Takeuchi for their skillful technical assistance. This study was supported by a grant from the Pollution-Related Health Damage Compensation and Prevention Association.

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