

Expression of IL-6, IL-8, and RANTES on human bronchial epithelial cells, NCI-H292, induced by influenza virus A

Satoshi Matsukura, MD, Fumio Kokubu, MD, Hiromichi Noda, MD, Hisahiro Tokunaga, MD, and Mitsuru Adachi, MD Tokyo, Japan

Bronchial epithelial cells are primary sites of airway viral infection, and these cells may play an important role in the pathogenesis of respiratory diseases. It has recently been reported that bronchial epithelial cells express RANTES. RANTES attracts monocytes, T cells, eosinophils, and basophils; it can also activate eosinophils. To determine whether viral infection induces RANTES expression on bronchial epithelial cells, we infected a bronchial epithelial cell line, NCI-H292, with influenza virus A (H3N2). We then examined the concentration of RANTES in the culture medium of infected cells by ELISA and assessed expression of the gene for RANTES by the reverse-transcriptase polymerase chain reaction. We also investigated the concentrations of IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor in the medium of infected cells, because some virus infections have been reported to induce expression of these cytokines on bronchial epithelial cells, but there are few data concerning influenza virus infection. Small amounts of IL-6 and IL-8 were detected in the medium of uninfected cells. RANTES was not detected in the medium of uninfected cells. After influenza virus infection, significant amounts of IL-6, IL-8, and RANTES were released into the culture medium of infected cells, and RANTES messenger RNA was detected from infected cells. Granulocyte-macrophage colony-stimulating factor was not detected in the medium of uninfected and infected cells. These results suggest that influenza virus infection may stimulate production of IL-6, IL-8, and RANTES from human bronchial epithelial cells and that these cytokines may contribute to the pathogenesis of airway inflammatory diseases caused by influenza virus infection. (J Allergy Clin Immunol 1996;98:1080-7.)

Key words: IL-6, IL-8, RANTES, influenza virus, airway inflammation

Viral infection causes bronchial inflammation and exacerbates bronchial asthma because of the infiltration of inflammatory cells and increased hyperreactivity in the bronchial wall.¹ Infection with influenza virus type A (H3N2) is common and is a major precipitant of infection-induced asthma in adults.² The influenza virus primarily infects epithelial cells, from which various chemotactic factors may be released, followed by the development of bronchial inflammation and infiltration of inflammatory cells such as lymphocytes, histio-

Abbreviations used

FBS:	Fetal bovine serum
GM-CSF:	Granulocyte-macrophage colony-stimulating factor
MDCK:	Madin-Darby canine kidney
MEM:	Minimal essential media
PCR:	Polymerase chain reaction
PFU:	Plaque forming unit
RT-PCR:	Reverse-transcriptase polymerase chain reaction
TNF- α :	Tumor necrosis factor- α

From First Department of Internal Medicine, University of Showa School of Medicine, Tokyo.

Supported by the Pollution-Related Health Damage Compensation and Prevention Association.

Received for publication Oct. 17, 1995; revised May 10, 1996; accepted for publication May 17, 1996.

Reprint requests: Satoshi Matsukura, First Department of Internal Medicine, University of Showa School of Medicine, 1-5-8 Hatanodai Shinagawa-ku, Tokyo, 142, Japan.

Copyright © 1996 by Mosby-Year Book, Inc.

0091-6749/96 \$5.00 + 0 1/1/75056

cytes, and eosinophils to the bronchial wall. Therefore monitoring of epithelial cells may provide insight into the inflammation process.

It has recently been reported that bronchial epithelial cells express a chemokine, RANTES,^{3,4} as determined by in vitro and in vivo studies.^{5,6} RANTES is chemotactic for monocytes, T cells,

eosinophils, and basophils.⁷⁻¹⁰ RANTES may contribute to the accumulation of these inflammatory cells into the epithelium in viral infection, but there is no report concerning the expression of RANTES induced by viral infection. Therefore we investigated whether influenza virus infection induced RANTES expression on bronchial epithelial cell line. On the other hand, investigators have demonstrated that some viruses induce the production of cytokines from bronchial epithelial cells, such as IL-6, IL-8, and granulocyte-macrophage colony-stimulating factor (GM-CSF).¹¹⁻¹⁵ But there are few data concerning cytokine production from bronchial epithelial cells in influenza virus infection. Therefore we analyzed the production of IL-6, IL-8, and GM-CSF from bronchial epithelial cells that were infected with influenza virus.

METHODS

Cell cultures

The bronchial epithelial cell line, NCI-H292, is an established line of human lung mucoepidermoid carcinoma cells (American Type Culture Collection) and is not transformed with virus.¹⁶ This cell line can be used to cultivate paramyxoviruses and may be sensitive to the influenza virus.¹⁷ Therefore we selected NCI-H292 as a model for respiratory epithelium in this study. NCI-H292 cells were cultured in RPMI-1640 medium (Gibco, Grand Island, N.Y.) containing 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μ g/ml streptomycin. Cultures were incubated at 37°C in 5% CO₂, humidified air.

Madin-Darby canine kidney (MDCK) cells (American Type Culture Collection) were cultured for influenza virus propagation. MDCK cells were grown and maintained in Eagles Minimal Essential Media (MEM) (Gibco) containing 2% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. Stock cultures of NCI-H292 and MDCK cells were frozen at -80°C.

Virus stock

Influenza virus A/Sisen/2/92 (a kind gift from Dr. M. Toda of Showa University, Tokyo, Japan) was grown in MDCK cells in MEM containing 2% FBS at 33.5°C in 5% CO₂. The infected culture fluid was subjected to centrifugation to produce a clear liquid, which was stored in aliquots of 2 ml at -80°C.

Virus titration

MDCK cells were grown to confluence in 6-well culture plates (Falcon, Becton Dickinson and Company). Serial 10-fold dilutions of the virus stocks were prepared in MEM supplemented with 100 U/ml penicillin, 100 μ g/ml streptomycin, and 2 mg/ml trypsin. The supernatant of each culture plate was removed, and 100 μ l of each dilution was added to the plates. The plates

were rocked every 10 minutes for adsorption of virus to the cells. After 1 hour, the supernatant was removed and replaced with 2 ml of MEM containing 1% agarose, 1% diethylaminoethyl dextran, and 4 μ g/ml trypsin. Cultures were incubated for 3 to 4 days at 33.5°C in 5% CO₂. These cells were fixed with 10% formalin and stained with methylene blue. Plaques of each plate were then enumerated, and plaque forming units (PFU) were determined.

Infection of cell culture with influenza virus A

NCI-H292 cells were grown to confluence in sterile tissue culture plates (3.5 cm in diameter). One hundred microliters of 1×10^6 PFU/ml influenza virus A (H3N2) was added to the plates and allowed to adsorb to the cells for 1 hour with gentle rocking of the plates every 10 minutes. After 1 hour, the virus was removed and 2 ml of fresh RPMI-1640 media with 2% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin was added; and the infected cells were grown at 37°C in 5% CO₂ humidified air. (The multiplicity of infection was 0.05.) Culture medium was collected at 6, 24, 48, and 72 hours after infection for cytokine assay. For detection of messenger RNA, infected cells were harvested at 24 hours after virus inoculation.

Detection of RNA of influenza virus A

NCI-H292 cells were grown to confluence in culture plates with a diameter of 3.5 cm. One hundred microliters of viral stock (1×10^6 PFU/ml influenza virus A, H3N2) was added to the plates. At 10 minutes or at 1 hour after addition of viral stock to plates with rocking every 10 minutes, the supernatant of the plates was removed and 2 ml of fresh RPMI-1640 medium with 2% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin was added. At 10 minutes or 24 hours after viral inoculation, the cells were washed with PBS three times and were harvested with a scraper. Total RNA was extracted from samples of 2×10^6 cells by guanidium thiocyanate.¹⁸ First-strand complementary DNAs were synthesized by incubation of 2 μ g of RNA templates in the presence of Moloney-Murine Leukemia Virus reverse transcriptase (Pharmacia, Uppsala, Sweden), random primers, and reaction buffer at 37°C for 1 hour. After denaturation of cDNAs at 94°C for 5 minutes, the polymerase chain reaction (PCR) was performed by using 5 pmol 5'-primer (5'-TTGTTGAACGCAGCAAAGCT-3') and 5 pmol 3'-primer (5'-TCTAGTTTGTCTCTCTGGTA-3'), 5 μ l cDNA, 0.6 unit of Taq polymerase (Boehringer-Mannheim, Mannheim, Germany), 2.5 μ l PCR buffer (100 mmol/L Tris-HCl, 15 mmol/L MgCl₂, 500 mmol/L KCl [Boehringer-Mannheim]), and distilled water were added to the reaction volume of 25 μ l. PCR primers were synthesized according to the sequence of hemagglutinin gene type 3 (H3, position at 337-1063) of influenza virus A.¹⁹ The amplification reaction was performed for 30 cycles with dena-

turation at 94° C for 45 seconds, annealing at 61° C for 45 seconds, and extension at 72° C for 1 minute (Perkin-Elmer Cetus, Norwalk, Conn.).²⁰ After incubation at 72° C for 10 minutes, PCR-amplified products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining, followed by visualization with a UV transilluminator.

Stimulation of the epithelial cells by IL-1 β , tumor necrosis factor- α , IL-6, and IL-8

NCI-H292 cells were grown to confluence in 3.5 cm diameter culture plates and incubated in RPMI-1640 medium with 2% FBS, 100 U/ml penicillin, and 100 μ g/ml streptomycin. IL-1 β , tumor necrosis factor- α (TNF- α), IL-6, and IL-8 (R&D Systems, Minneapolis, Minn.) were added to the medium (10 ng/ml each). After 6, 24, 48, and 72 hours, the supernatants of the culture were collected for assay of cytokines. At 24 hours after stimulation, cultured cells were harvested with a scraper.

Assay of cytokines

Concentrations of IL-6, IL-8, and RANTES in the collected culture medium were determined with a commercially available system for ELISA (Amersham International Ltd., Little Chalfont, U.K.). First, the standards and the samples were added to the 96 wells of a microtiter plate coated with anti-cytokine antibody. After incubation at room temperature for 2 hours, each well was washed with wash buffer four times. Then, polyclonal antibody against cytokine, which was conjugated to horseradish peroxidase, was added to the well. After incubation at room temperature for 2 hours, each well was washed with wash buffer four times. Substrate solution (stabilized hydrogen peroxide and tetramethylbenzidine) was added to each well, and the plate was incubated at room temperature for 20 minutes. Then sulfuric acid was 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, and the concentration of each sample was calculated. The minimal detectable concentrations of each cytokine were as follows; 0.7 pg/ml for IL-6, 4.7 pg/ml for IL-8, 1.5 pg/ml for GM-CSF, and 2.5 pg/ml for RANTES. This concentration reflects the release of cytokine into 2 ml of culture medium over 6, 24, 48, and 72 hours after infection.

To confirm that cytokine production was indeed due to the effects of influenza virus infection and not to a contaminant present in the viral stock, we performed the following experiment. We incubated viral stock with influenza virus A (serotype H3N2) specific antiserum for 30 minutes and then added it to the plates in which NCI-H292 cells were cultured to confluence. After incubation for 10 minutes, the supernatant of the plates was removed, and fresh RPMI-1640 medium was added. Forty-eight hours later, the culture medium was col-

lected, and the concentration of each cytokine was determined by ELISA.

Expression of RANTES mRNA

RANTES gene was assessed by reverse-transcriptase (RT)-PCR. According to the RANTES cDNA library, 5'- and 3'-primers were synthesized.²¹ The sequence selected for amplification was the coding region of RANTES, which is located from position 27 to position 302 and encodes a protein with 92 amino acids. Control cells; cells stimulated with IL-1 β , TNF- α , or IL-6 plus IL-8 (10 ng/ml each); and cells infected with influenza virus A were harvested with a scraper at 24 hours after cytokine stimulation or virus infection. Total RNA was extracted from samples of 2×10^6 cells by guanidium thiocyanate.¹⁸ First-strand cDNAs were synthesized by incubation of 2 μ g of RNA templates in the presence of Moloney-Murine Leukemia Virus reverse transcriptase (Pharmacia), random primers, and reaction buffer at 37° C for 1 hour. After denaturation of cDNAs at 94° C for 5 minutes, the PCR was performed by using 5 pmol 5'-RANTES primer (5'-ATGAAGGTCTCCGCG-GCAGCCT-3'); and 5 pmol 3'-RANTES primer (5'-CTAGCTCATCTCCAAAGAGTTG-3'), 5 μ l cDNA, 0.6 unit of Taq polymerase, 2.5 μ l PCR buffer, and distilled water were added to the reaction volume of 25 μ l. The amplification reaction was performed for 30 cycles with denaturation at 94° C for 45 seconds, annealing at 61° C for 45 seconds, and extension at 72° C for 1 minute (Perkin-Elmer Cetus). The β -actin gene was amplified as mentioned previously by using 5'- β -actin primer (5'-GTGGGGCGCCCCAGGCACCA-3') and 3'- β -actin primer (5'-CTCCTTAATGTCACGCAC-GATTTC-3'). After incubation at 72° C for 10 minutes, PCR-amplified products were analyzed by 2% agarose gel electrophoresis and ethidium bromide staining, followed by visualization with a UV transilluminator.²⁰

Statistics

Analysis of variance was used to evaluate statistical significance of the data in the analysis of cytokine concentration in the culture medium. Data that appeared statistically significant were compared by using Student's *t* test for means of multiple groups and were considered significant if *p* values were less than 0.05 compared with control. Three or five separate experiments were performed.

RESULTS

Infection of epithelial cells by influenza virus A

At 24 hours after influenza virus inoculation, remarkable cytopathic change was not noted when compared with control. At 48 to 72 hours after virus inoculation, rounding of the cells, large nuclei, and prominent nucleoli was observed. Several cells revealed cellular fusion. At 72 to 120 hours

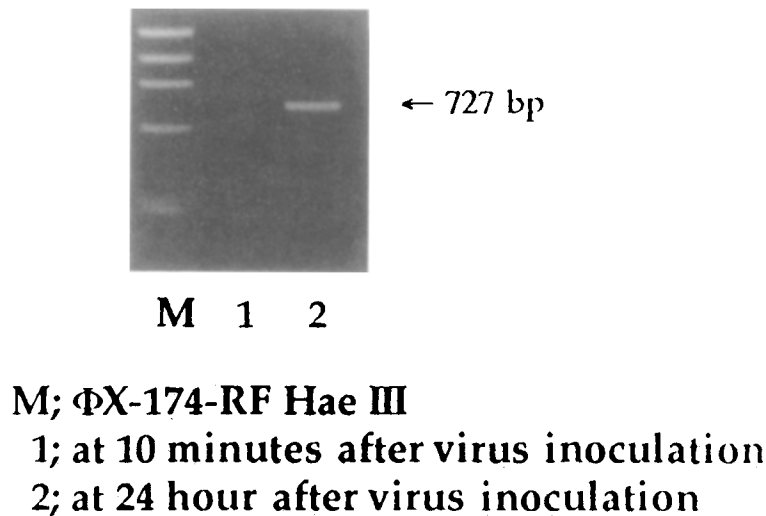


FIG. 1. Detection of viral RNA by RT-PCR. One hundred microliters of viral stock (1×10^8 PFU/ml influenza virus A, H3N2) was added to culture plates of NCI-H292 cells. At 10 minutes after addition of viral stock or at 1 hour after rocking of plates every 10 minutes, supernatant of the plates was removed and 2 ml of fresh RPMI-1640 medium was added. At 10 minutes or at 24 hours after viral inoculation, cells were harvested. Hemagglutinin 3 gene of influenza virus A was detected from collected cells by RT-PCR. M, Size marker; 1, cells collected at 10 minutes after virus inoculation; 2, cells collected at 24 hours after virus inoculation. Representative of three separate experiments.

after virus inoculation, cellular fusion and detachment were seen.

Detection of viral RNA by RT-PCR method

Influenza virus A H3 gene was not detected from cells immediately after 10 minutes of exposure to virus (Fig. 1, lane 1). On the other hand, the sample obtained from the cells that were collected at 24 hours after infection revealed a clear PCR product at 727 bp (Fig. 1, lane 2). These results imply that NCI-H292 cells were infected with influenza virus A (H3N2) and that the virus was replicating.

Production of IL-6, IL-8, and RANTES

IL-6 production was increased in the medium of the cells at 6 hours after stimulation with IL-1 β or TNF- α (408.3 ± 83.3 pg/ml or 441.7 ± 91.7 pg/ml, respectively, $p < 0.05$), compared with the production in the medium of control cells (30 ± 10.5 pg/ml). IL-6 production was increased in the culture medium of infected cells at 24 hours after infection (216.7 ± 125 pg/ml, $p < 0.05$), compared with the production in the medium of control cells (28.3 ± 19.5 pg/ml). The concentration of IL-6 in the culture medium of infected cells increased over time. At 72 hours after infection, the concentration of IL-6 in the medium of infected cells was 488.8 ± 41.7 pg/ml ($p < 0.05$). Five separate experiments

were performed in the assay of IL-6 concentration (Fig. 2, A). IL-6 concentration in the culture medium of the cells stimulated with IL-8 did not differ significantly from the concentration in the medium of control cells at any of four time points (data not shown).

IL-8 production was significantly increased in the medium of the cells at 6 hours after stimulation with IL-1 β or TNF- α (4.2 ± 0.4 ng/ml or 4.6 ± 0.7 ng/ml, respectively, $p < 0.05$), compared with the production in the medium of control cells (51 ± 22 pg/ml). IL-8 production was increased in the culture medium of infected cells at 24 hours after infection (2.1 ± 0.7 ng/ml, $p < 0.05$), compared with the production in the medium of control cells (85 ± 32 pg/ml). The concentration of IL-8 in the culture medium of the cells stimulated with IL-1 β or TNF- α or in the cells infected with influenza virus increased in a time-dependent manner. The concentration of IL-8 in the medium of the cells at 72 hours after stimulation with IL-1 β or TNF- α or in the cells at 72 hours after infection were 5.7 ± 0.5 ng/ml, 5.9 ± 0.5 ng/ml, and 4.5 ± 0.3 ng/ml, respectively. These IL-8 concentrations were higher ($p < 0.05$, respectively) than that of the culture medium of control cells at 72 hours (120 ± 46 pg/ml). Five separate experiments were performed in the assay of IL-8 concentration (Fig. 2, B). IL-8 concentration in the culture medium of

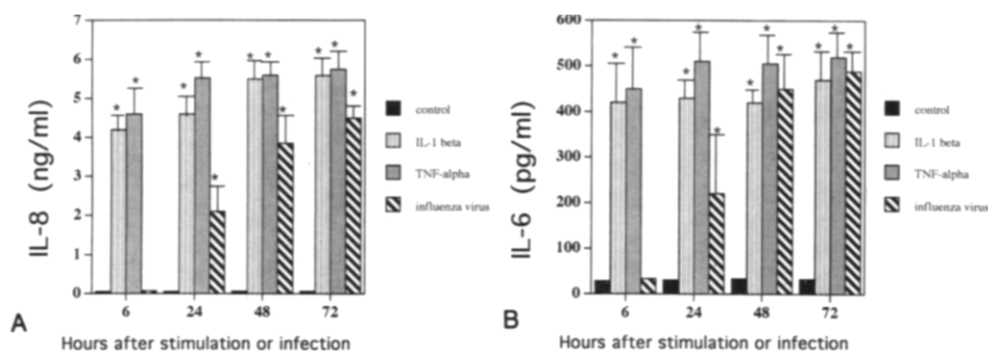


FIG. 2. Concentration of IL-6 and IL-8 in culture medium. Bronchial epithelial cells, NCI-H292, were incubated with 10 ng/ml IL-1 β or TNF- α . NCI-H292 cells were also infected with influenza virus A. Culture medium was collected at 6, 24, 48, and 72 hours after stimulation or infection. Concentrations of IL-6 and IL-8 in collected medium were determined by ELISA. **A**, IL-6 concentration. **B**, IL-8 concentration. * $p < 0.05$ compared with control. Five separate experiments were performed.

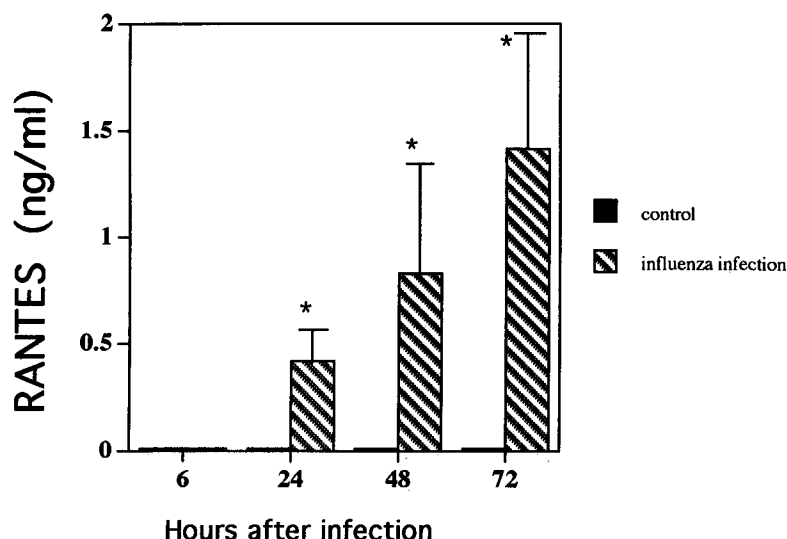


FIG. 3. Concentrations of RANTES in culture medium. Bronchial epithelial cells, NCI-H292, were infected with influenza virus A. Medium was collected 6, 24, 48, and 72 hours later. Concentrations of RANTES in medium of control cells and cells infected with influenza virus were determined by ELISA. * $p < 0.05$ compared with control. Five separate experiments were performed.

the cells stimulated with IL-6 did not differ significantly from the concentration in the medium of control cells at any of four time points (data not shown).

RANTES was not detected in the culture medium of control cells at any of four time points or in the medium of the cells at 6 hours after infection. At 24 hours after infection, RANTES was detected in the culture medium of infected cells (0.4 ± 0.1 ng/ml, $p < 0.05$). RANTES production in the culture medium of infected cells increased over time. The concentration of RANTES ob-

served at 72 hours after infection was 1.4 ± 0.6 ng/ml ($p < 0.05$). RANTES was not detected in the culture medium of cells stimulated with IL-1 β , TNF- α , or IL-6 plus IL-8 at any of four time points (data not shown). Five separate experiments were performed in the assay of RANTES concentration (Fig. 3).

GM-CSF was not detected in the culture medium of control cells; in the medium of cells stimulated with IL-1 β , TNF- α , IL-6, or IL-8; or in the medium of cells infected with influenza virus at any of the time points (data not shown).

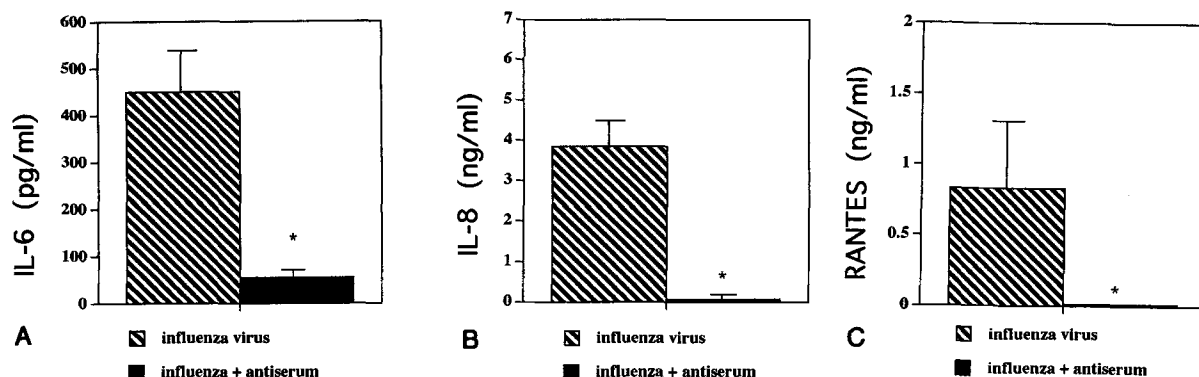


FIG. 4. Effects of antiserum on cytokine production. Viral stock was incubated with antiserum and then added to NCI-H292 cells. After incubation for 10 minutes, supernatant was removed and fresh RPMI-1640 medium was added. Forty-eight hours later, culture medium was collected, and concentration of each cytokine was determined by ELISA. **A**, Concentration of IL-6. **B**, Concentration of IL-8. **C**, Concentration of RANTES. Three separate experiments were performed.

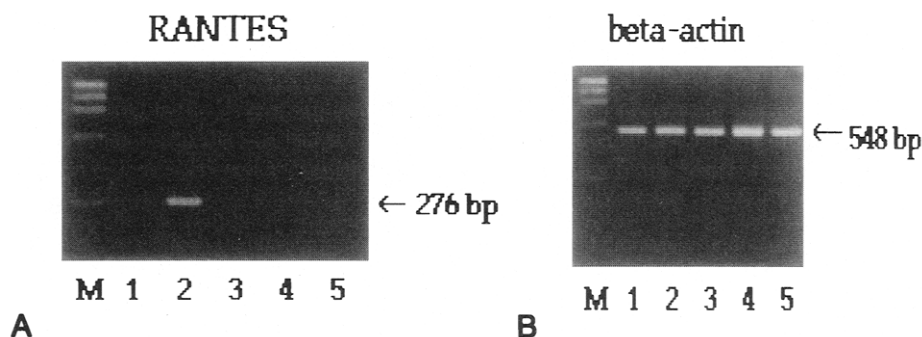


FIG. 5. RANTES mRNA detected by RT-PCR. NCI-H 292 cells were stimulated with IL-1 β , TNF- α , and IL-6 plus IL-8 (10 ng/ml each). NCI-H292 cells were also infected with influenza virus A. After 24 hours, epithelial cells were harvested and mRNA of RANTES (**A**) and β -actin (**B**) were assayed by RT-PCR. *M*, Size marker; 1, control cells; 2, cells infected by influenza virus A; 3, cells stimulated with IL-1 β ; 4, cells stimulated with TNF- α ; 5, cells stimulated with IL-6 plus IL-8. Representative of five separate experiments.

Infection was blocked in all three experiments by preincubation of the virus with antiserum to influenza virus A (H3N2). Cytopathic effect was not noted in the cells that were incubated with influenza virus A antiserum. The production of IL-6 and IL-8 in the medium of the cells was reduced to the same level as that of noninfected control cells by preincubation with antiserum (54 ± 14 pg/ml and 62 ± 21 pg/ml, respectively) (Fig. 4, *A* and *B*). RANTES was not detected in the medium of the cells treated with antiserum (Fig. 4, *C*).

Expression of RANTES mRNA

RANTES mRNA was detected by RT-PCR. The sample, obtained from epithelial cells that were harvested at 24 hours after influenza virus A

infection, revealed an amplified cDNA band compatible with the size of the PCR product (276 bp). No product was detected from control cells or from cells stimulated with IL-1 β , TNF- α , or IL-6 plus IL-8 (Fig. 5, *A*).

The β -actin PCR product (548 bp) was detected in samples from control cells; from cells stimulated with IL-1 β , TNF- α , and IL-6 plus IL-8; and from infected cells (Fig. 5, *B*).

DISCUSSION

Our studies have demonstrated that human bronchial epithelial cell line, NCI-H292, was infected with influenza virus A and that its infection induced the expression of RANTES on the NCI-H292 cells and increased the production of IL-6 and IL-8 from these cells. The production of these

cytokines was inhibited by antiserum against influenza virus A.

NCI-H292 is an established cell line of mucoepidermoid carcinoma cells derived from human lung tissue.¹⁶ This cell line expresses IL-6, IL-8, and intercellular adhesion molecule-1 constitutively; and the expression of these cytokines and adhesion molecules was increased by IL-1 β and TNF- α .²² These characteristics are similar to those of normal bronchial epithelial cells. Therefore we used this cell line as a model of airway inflammation in this study. However, we must acknowledge that NCI-H292 is a tumor cell line and may have some characteristics that are different from normal bronchial epithelium.

In this study we detected cytopathic effects such as rounding of the cells, large nuclei, prominent nucleoli, and cellular fusion in NCI-H292 cells after inoculation of influenza virus A. Influenza virus RNA was detected in the cells at 24 hours after virus inoculation, and it was not detected in the cells at 10 minutes after inoculation. These results indicate that NCI-H 292 cells were infected with influenza virus A and that influenza virus was replicating.^{11, 12, 23}

It has been reported that influenza virus infection increases the levels of cytokines such as IL-1 α , IL-1 β , IL-6, TNF- α , and GM-CSF in bronchoalveolar lavage from infected mice.²⁴ We thought that bronchial epithelial cells may be important in the inflammatory process, because they are the primary site of airway viral infection. Recently, it has been reported that cytokines such as IL-1 β , IL-6, and IL-8 were expressed in nasal epithelial cells obtained from patients during viral respiratory infection *in vivo*.²⁵ Investigators have reported that respiratory syncytial virus and human rhinovirus infection increased the production of IL-6, IL-8, and GM-CSF from bronchial epithelial cells in an *in vitro* study.¹¹⁻¹⁴ IL-8 expression in normal human bronchial epithelial cells by influenza virus infection has been reported.²⁶ Similarly, our study demonstrated enhanced production of IL-6 and IL-8 from a bronchial epithelial cell line, NCI-H292. IL-6 induces T-cell proliferation.²⁷ IL-8 is chemotactic for T cells and neutrophils.²⁸ These cytokines may contribute to the pathogenesis of airway inflammation in influenza virus infection.

RANTES is a member of the C-C chemokine family. It attracts monocytes, eosinophils, basophils, and T cells—in particular, CD4 T cells.⁷⁻⁹ RANTES can also stimulate the release of eosinophil cationic protein from eosinophils, and it can upregulate the expression of adhesion molecules

on eosinophils.¹⁰ To our knowledge, we demonstrated for the first time that viral infection induced the expression of RANTES. Influenza virus infection causes accumulation of inflammatory cells such as lymphocytes, histiocytes, and eosinophils in the airway²³; and influenza virus is a major pathogen of airway inflammatory disease. Our data suggest that RANTES may contribute to the accumulation of inflammatory cells to the bronchial wall in airway inflammatory diseases caused by influenza infection.

Recently, it was reported that bronchial epithelial cells in biopsy specimens obtained from patients with asthma express RANTES.^{5, 6} Examiners found that bronchial epithelial cell lines, such as A549 cells and BEAS-2B cells, expressed RANTES in response to IL-1, TNF, or interferon- γ .^{3, 4} In contrast, we found that neither IL-1 β nor TNF- α stimulated production of RANTES by NCI-H292 cells; only influenza virus infection stimulated these cells to produce RANTES. We examined expression of RANTES mRNA using RT-PCR and similarly found that only influenza virus infection induced RANTES mRNA. The mechanism of RANTES expression may differ between lines of bronchial epithelial cells, but we have recently demonstrated that normal bronchial epithelial cells express RANTES in response to influenza virus infection in the same manner as NCI-H292 cells (manuscript in preparation).

We detected high levels of IL-6 and IL-8 in the culture medium of the cells infected with influenza virus. To assure that these cytokines did not contribute to the expression of RANTES, we added purified cytokines of IL-6 and IL-8 in the culture medium of NCI-H292 cells and analyzed RANTES production and mRNA expression. However, IL-6 and IL-8 did not induce RANTES production or mRNA expression. The results of this study indicate that influenza virus infection may induce expression of RANTES on bronchial epithelial cells without intervention of these cytokines.

Phal and Baeuerle²⁹ have reported that influenza virus hemagglutinin activates transcription factor NF- κ B in HeLa cells. In our report we demonstrated that NCI-H292 cells were infected with influenza virus A and that its hemagglutinin gene was expressed in the infected cells. NF- κ B is a transcription factor of many cytokines including IL-6, IL-8, and RANTES.^{30, 31} Therefore it is possible that influenza virus A infection induced IL-6, IL-8, and RANTES expression in NCI-H292 cells by the intervention of NF- κ B, which was activated by the hemagglutinin gene.

The results of this study may contribute to the understanding of the mechanisms of airway inflammation. However, these results were obtained in vitro, and it is not known whether they precisely reflect in vivo airway inflammation. Further investigations are necessary to clarify the mechanism by which viral infection causes these epithelial cells to express cytokines.

We thank Drs. Bruce S. Bochner, David Proud, Hiroyuki Watanabe, Kunihiro Fukuchi, Kunio Gomi, Kazuo Kobayashi, Takeshi Kasama, Keita Kasahara, Masako Yasuda, Masashi Iwata, Hiroyuki Tsujiyama, and Masako Toda for helpful discussions and Hitomi Kubo, Michiko Okamoto, and Hiroko Takeuchi for skillful technical assistance.

REFERENCES

- Empey DW, Laitinen LA, Jacobs L, Gold WM, Nadel JA. Mechanisms of bronchial hyperreactivity in normal subjects following upper respiratory tract infection. *Am Rev Respir Dis* 1976;113:131-9.
- Hugel DW, Langston L Jr, Seliner JC, McIntosh K. Viral and bacterial infections in adults with chronic asthma. *Am Rev Respir Dis* 1979;120:393-7.
- Kwon OJ, Jose PJ, Robbins RA, Schall TJ, Williams TJ, Barnes PJ. Glucocorticoid inhibition of RANTES expression in human lung epithelial cells. *Am J Respir Cell Mol Biol* 1995;12:488-96.
- Stellato C, Beck LA, Gorgone GA, Proud D, Schall TJ, Ono SJ, et al. Expression of the chemokine RANTES by a human bronchial epithelial cell line. *J Immunol* 1995;155:410-8.
- Davies RJ, Wang JH, Trigg CJ, Devalia JL. Expression of granulocyte/macrophage-colony-stimulating factor, interleukin-8 and RANTES in the bronchial epithelium of mild asthmatics is down-regulated by inhaled beclomethasone dipropionate. *Int Allergy Immunol* 1995;107:428-9.
- Devalia JL, Wang JH, Sapsford RJ, Davies RJ. Expression of RANTES in human bronchial epithelial cells and the effect of beclomethasone dipropionate (BDP) [abstract]. *Eur Respir J* 1994;7(suppl. 18):98S.
- Kameyoshi Y, Dörschner A, Mallet AI, Christophers E, Schröder JM. Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J Exp Med* 1992;176:587-92.
- Schall TJ, Bacon K, Toy KJ, Goeddel DV. Selective attraction of monocytes of the memory phenotype by cytokine RANTES. *Nature* 1990;347:669-71.
- Schall TJ. Biology of the RANTES/SIS cytokine family. *Cytokine* 1992;3:165-83.
- Alam R, Stanford S, Forsythe P, Harrison R, Faubion D, Lett-Brown MA, et al. RANTES is a chemotactic and activating factor for human eosinophils. *J Immunol* 1993;150:3442-7.
- Subauste MC, Jacoby DB, Richards SM, Proud D. Infection of a human respiratory epithelial cell line with rhinovirus. *J Clin Invest* 1995;96:549-57.
- Noah TL, Becker S. Respiratory syncytial virus-induced cytokine production by a human bronchial epithelial cell line. *Am J Physiol* 1993;265:L472-478.
- Becker S, Koren HS, Henke DC. Interleukin-8 expression in nasal epithelium and its modulation by infection with respiratory syncytial virus and cytokines tumor necrosis factor, interleukin-1, and interleukin-6. *Am J Respir Cell Mol Biol* 1993;8:20-7.
- Arnold R, Humbert B, Werchau H, Gallati H, König W. Interleukin-8, interleukin-6, and soluble tumor necrosis factor receptor type I release from a human pulmonary epithelial cell line (A549) exposed to respiratory syncytial virus. *Immunology* 1994;82:126-33.
- Becker S, Soukup J, Yankaskas JR. Respiratory syncytial virus infection of human primary nasal and bronchial epithelial cell cultures and bronchoalveolar macrophages. *Am J Respir Cell Mol Biol* 1992;6:369-74.
- Carney DN, Gazdar AF, Bepler G, Guccion JG, Marangos PJ, Moody TW. Establishment and identification of small cell lung cancer cell lines having classic variant features. *Cancer Res* 1985;45:2913-23.
- Castells E, Velma GG, Hierholzer JC. NCI-H292 as an alternative cell line for the isolation and propagation of the human paramyxoviruses. *Arch Virol* 1990;115:277-88.
- Brenner CA, Tam AW, Nelson PA, et al. Message amplification phenotyping (MAPPING): a technique to simultaneously measure multiple mRNAs from small number of cells. *Biotechniques* 1989;7:1096-103.
- Verhoeven M, Fang R, Jou WM, et al. Antigenic drift between the haemagglutinin of the Hong Kong influenza strains A/Aichi/2/68 and A/Victoria/3/75. *Nature* 1980;286:771-6.
- Saiki RK, Gelfand DH, Stoffel S, et al. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988;239:487-91.
- Schall TJ, Jongstra J, Bradley BJ, et al. A human T cell-specific molecule is a member of a new gene family. *J Immunol* 1988;141:1018-25.
- Bloemen PGM, Tweel MC, Henricks PAJ, et al. Expression and modulation of adhesion molecules on human bronchial cells. *Am J Respir Cell Mol Biol* 1993;9:586-93.
- Walsh JJ, Dietlein LF, Low FN, Burch GE, Mogabgab WJ. Bronchotracheal response in human influenza. *Arch Intern Med* 1960;108:376-88.
- Hennet T, Ziltener HJ, Frei K, Peterhans E. A kinetic study of immune mediators in the lungs of mice infected with influenza A virus. *J Immunol* 1992;149:932-9.
- Noah TL, Henderson FW, Wortman IA, et al. Nasal cytokine production in viral acute upper respiratory infection of childhood. *J Infect Dis* 1995;171:584-92.
- Choi AMK, Jacoby DB. Influenza virus A infection induces interleukin-8 gene expression in human airway epithelial cells. *FEBS Lett* 1992;309:327-9.
- Helle M, Boeije L, Aarden LA. IL-6 is an intermediate in IL-1-induced thymocyte proliferation. *J Immunol* 1989;142:4335-8.
- Larsen CG, Anderson AO, Appella E, Oppenheim JJ, Matsushima K. The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. *Science* 1989;243:1464-6.
- Phal HL, Baeuerle PA. Expression of influenza virus hemagglutinin activates transcription factor NF-kappa B. *J Virol* 1995;69:1480-4.
- Baeuerle PA, Henkel T. Function and activation of NF-kB in the immune system. *Annu Rev Immunol* 1994;12:141-79.
- Nelson PJ, Kim HT, Manning WC, Goralski TJ, Krensky AM. Genomic organization and transcriptional regulation of RANTES chemokine gene. *J Immunol* 1993;151:2601-12.