

Influenza virus A stimulates expression of eotaxin by nasal epithelial cells

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Summary

Background Respiratory virus is one of the most common causes of airway inflammation, but its pathogenic mechanisms are not well understood. Eotaxin is a potent eosinophil chemoattractant and is a selective agonist for C-C chemokine receptor 3 (CCR3). Although it has recently been demonstrated that epithelial cells express eotaxin, both *in vivo* and *in vitro*, there are few data concerning the expression in viral infection.

Objects We hypothesized that eotaxin may play an important role in attracting inflammatory cells into the airway after viral infection and analysed whether viral infection induces eotaxin in nasal epithelial cells *in vitro*.

Methods Nasal epithelial cells obtained from polypectomy for nasal polyp were infected with influenza virus A (subtype H3N2). The cells and supernatants were collected 8, 24 and 48 h after infection. Eotaxin mRNA was analysed by RT-PCR. Eotaxin concentration in the supernatants was analysed by enzyme-linked immunosorbent assay. We also examined a blocking assay to analyse the intervention of pro-inflammatory cytokines, TNF- α and IL-1 β in eotaxin production induced by influenza virus.

Results The results showed that eotaxin was expressed constitutively in uninfected cells, but was up-regulated for both mRNA and protein levels in infected cells. Blocking experiments using anti-TNF- α and anti-IL-1 β antibodies showed no effects of these agents on the level of eotaxin. In addition, UV-inactivated virus did not enhance the expression of eotaxin.

Conclusions These results suggest that influenza virus A infection in nasal epithelial cells stimulates the expression of eotaxin, and may play an important role in the pathogenesis of airway inflammation by inducing eotaxin.

Keywords: airway inflammation, eotaxin, influenza virus, nasal epithelial cells

Clinical and Experimental Allergy, Vol. 31, pp. 873–880. Submitted 28 February 2000; revised 12 July 2000; accepted 15 November 2000.

Introduction

Persistent infiltration of the nasal epithelium by inflammatory cells such as eosinophils, lymphocytes and neutrophils, is thought to contribute to the underlying pathophysiology of allergic disease, such as allergic rhinitis [1–5]. Activated

eosinophils and the cytokines produced by them induced epithelium desquamation, subepithelial fibrosis and hyper-responsiveness characterizing allergic inflammation [6–8]. Although the local production of several cytokines may regulate allergic mucosal inflammation, little is known about the mechanism of infiltration of eosinophils in nasal epithelium.

Eotaxin has selective chemotactic activity for eosinophils. Indeed, eotaxin increases the chemotaxis of

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eosinophils, but not of neutrophils and monocytes, *in vitro* [9]. Airway epithelial cells is a major cell source of eotaxin [10–12]. These cells produce several classes of pro-inflammatory molecules when exposed to various stimuli, such as viral infection.

Airway epithelial cells are the primary sites of airway viral infection. Respiratory viral infection causes airway inflammation and production of many cytokines. Viral infection induces airway epithelial cells to express various cytokines, chemokines and adhesion molecules, including interleukin (IL)-1, IL-6, IL-8, IL-11, granulocyte/macrophage colony-stimulating factor (GM-CSF), regulated on activation, normal T cells expressed and secreted (RANTES), and intercellular adhesion molecule-1 (ICAM-1) [13–16].

Influenza virus A (H3N2) infection is frequently associated with allergic diseases [17,18]. The influenza virus infects airway epithelial cells, from which various chemotactic factors may be released, which is followed by airway inflammation and infiltration of inflammatory cells into the airway. Therefore, characterization of virus-infected airway epithelial cells may provide insight into the mechanisms of allergic inflammation by viral infection.

In this study we investigated whether influenza viral infection induces eotaxin expression in human nasal epithelial cells.

Materials and methods

Culture of nasal epithelial cells

Nasal epithelial cells were cultured using the outgrowth method [19]. They were obtained from 22 non-allergic patients who had undergone nasal polypectomy. These tissues were cut into 0.5 × 0.5 cm pieces and explanted on to six-well culture plates. Then, 2 mL of LHC-1 medium (MCDB 151) with the Ca²⁺ concentration increased to 0.1 mM and supplemented with 5 µg/mL insulin, 5 ng/mL epidermal growth factor, 10 µg/mL transferrin, 5 × 10⁴ mM each of phosphoethanolamine and ethanolamine, 5 × 10⁴ mM hydrocortisone, and 50 µg/mL each of gentamicin and kanamycin (Sigma Chemical Co., St Louis, MO, USA) with 10% fetal bovine serum (FBS) (GIBCO, Grand Island, NY, USA), were added to the plates, followed by incubation of the cells in a humidified 5% CO₂ incubator at 36.5 °C.

After 7–10 days, epithelial cells were cultured in serum-free LHC-1 medium. Cells were dissociated with trypsin (Boehringer-Mannheim, Mannheim, Germany), and inoculated into six-well culture plates coated with collagen (Bottger GmbH, Berlin, Germany). The identity of cultured cells was confirmed on the basis of morphological characteristics and the results of immunocytochemical

staining with antibodies against cytokeratin, fibroblasts and macrophages (Dako Japan, Kyoto, Japan). The morphological 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 fibroblast and macrophage-associated markers.

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, USA) in minimal essential medium (MEM) containing 2% FBS at 33.5 °C in 5% CO₂. The infected culture fluid was centrifuged at 300 g to remove cellular debris and stored in aliquots at – 80 °C.

Virus titration

MDCK cells were grown to confluence in six-well culture plates. A serial 10-fold dilution of the virus stocks was prepared in MEM supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin and 2 mg/mL trypsin. The supernatant was removed and 100 µL of each diluted virus stock was added to the wells. The plates were rocked every 10 min to facilitate absorption of the virus by the cells. After 1 h, the supernatant was removed and replaced with 2 mL of MEM containing 1% agarose, 1% diethylaminoethyl dextran and 4 µg/mL of trypsin. Cultures were incubated for 3–4 days at 33.5 °C in 5% CO₂. The cells were fixed with 10% formalin and stained with methylene blue. Plaques of each plate were then enumerated, and plaque-forming units (PFU) were measured.

Infection of cell culture with influenza virus A

Nasal epithelial cells (1 × 10⁶ cells/well) in six-well tissue culture plates were infected with influenza virus A (100 µL of 1 × 10⁷ PFU/mL viral stock) at a multiplicity of infection of 1.0, and the virus was absorbed by the cells for 1 h with gentle agitation of the plates every 10 min. After 1 h, virus was removed and 1 mL of serum-free LHC-1 medium was added.

Infected cells were incubated at 36.5 °C under 5% CO₂ in humidified air. For analysis of viral RNA, infected cells were harvested 1, 8, 24 and 48 h after infection. For analysis of eotaxin mRNA, nasal cells, either infected or uninfected, were collected 24 h after infection or exchange of media. Supernatants were collected 8, 24 and 48 h after infection and subjected to enzyme-linked immunosorbent assay (ELISA) for measuring eotaxin level.

Analysis of viral RNA with RT-PCR

Total RNA was extracted with guanidium thiocyanate from samples of about 1×10^6 cells. First-strand complementary DNAs (cDNAs) were synthesized with incubation of 1 µg of RNA templates in the presence of Moloney-Murine leukaemia virus reverse transcriptase (Pharmacia, Tokyo, Japan), random primer and reaction buffer at 37 °C for 1 h. After cDNAs had been denatured at 94 °C for 5 min, an aliquot (5 µL) of the resultant cDNA was subjected to PCR with 5 pmol each of 5' and 3' primers, 0.6 U Taq polymerase (Boehringer-Mannheim), 2.5 µL PCR reaction buffer (Tris-HCL, 100 mmol/L; MgCl₂, 15 mmol/L; KCL, 500 mmol/L; Boehringer-Mannheim). Distilled water was added until the reaction volume was 25 µL. The PCR primers were synthesized according to the sequence of haemagglutinin gene type 3 (H3, position at 337–1063) of influenza virus A [20]. The sequence of primers was 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, USA). After incubation at 72 °C for 1 min, PCR-amplified products were analysed with 2.0% agarose gel electrophoresis and ethidium bromide staining, followed by visualization with an ultraviolet transilluminator.

Analysis of eotaxin mRNA with RT-PCR

Total RNA was extracted from 1×10^6 cells. RT-PCR was performed with the method described above. The PCR primers were as follows:

eotaxin:

5'-CCAACCACCTGCTGCTTTAACCTG-3'

5'-GGCTTTGGAGTTGGAGATTTTGG-3'

β-actin:

5'-GTGGGGCGCCCCAGGCACCA-3'

5'-CTCCTTAATGTCACGCACGATTTC-3'

The amplification reaction was performed for 35 cycles with denaturation at 94 °C for 1 min, annealing at 57 °C for 1 min and extension at 72 °C for 45 s. PCR products were detected by ethidium bromide staining and quantified by video densitometry using a gel documentation system that was configured by UVP (San Gabriel) with a computer containing Image 1.61 software (NIH Public Software, National Institutes of Health, Bethesda, MD, USA). The level of eotaxin gene expression was quantified by calculating the ratio of densitometric readings of the band intensity for eotaxin and β-actin from the same cDNA sample.

Eotaxin concentration into culture supernatants

Eotaxin protein levels in the collected supernatants were

determined with a commercially available ELISA kit (R & D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, 50 µL of the standard and samples were added to the 96 wells of a microtitre plate coated with an anti-eotaxin antibody. After incubation at 25 °C for 2 h, each well was washed with wash buffer three times. Then, a polyclonal antibody against eotaxin, which was conjugated to horseradish peroxidase, was added to each well. After incubation at 25 °C for 1 h, each well was washed with wash buffer three times. Substrate solution (stabilized hydrogen peroxide and tetramethylbenzidine) was added to each well, after which the plate was incubated at 25 °C for 1 h. Then, sulphuric 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 concentration of the standard, and then the concentration of each sample was then calculated. The minimal detectable concentration was 5 pg/mL.

Stimulation of cells with cytokines

When nasal epithelial cells were cultured to near confluence in six-well culture plates, cytokines were added to the plates to determine whether inflammatory cytokines can stimulate production of eotaxin. The cells and culture media were collected 24 h after stimulation. The cytokines 100 U/mL TNF-α, 10 ng/mL IL-1β and 100 U/mL interferon γ (IFN-γ) were added alone or in a combination of all three cytokines. Eotaxin protein levels were measured with ELISA as described above.

Blocking assay to TNF-α and IL-1β

We performed a blocking assay to analyse whether endogenous TNF-α and IL-1β play a role in eotaxin production induced by influenza virus. Saturating amounts of anti-TNF-α antibodies (40 µg/mL; R & D Systems) and anti-IL-1β antibodies (40 µg/mL; R & D Systems) were added at the time of influenza viral infection. The medium was collected 24 h after stimulation and eotaxin protein levels in the medium were analysed by ELISA.

Effect of ultraviolet (UV) radiation

UV-inactivated virus was prepared by exposing of UV light (60 W, 312 nm, 2 cm distance) for 1 h, and the inactivated virus titre was less than 10^2 PFU/mL by the titration as described above. This virus was absorbed by nasal epithelial cells for 1 h and these cells were collected 24 h after inoculation. Eotaxin and haemagglutinin mRNA were

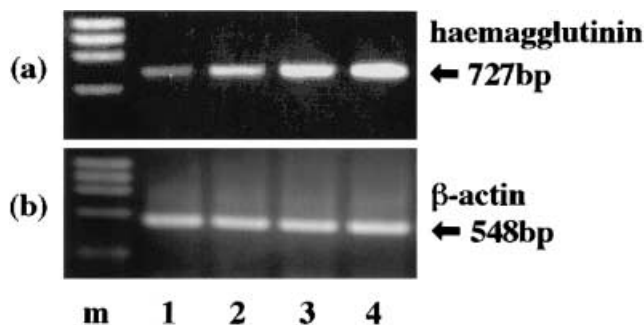


Fig. 1. Detection of haemagglutinin gene expression by RT-PCR. Nasal epithelial cells were infected with influenza virus A and collected 1, 8, 24 and 48 h after infection. Panel (a) shows the products of haemagglutinin mRNA (772 bp). Panel (b) shows the products of β -actin mRNA (548 bp). M, DNA size marker; 1–4, products from cells collected 1, 8, 24 and 48 h after infection.

analysed by RT-PCR and eotaxin concentrations were measured with ELISA.

Data analysis

Analysis of variance (ANOVA) was used to evaluate eotaxin concentrations in the supernatants from multiple groups. In addition, the Mann–Whitney test was performed to evaluate statistical significance between two groups and the Kruskal–Wallis test was performed to evaluate statistical significance between multiple groups. Any difference with *P*-values less than 0.05 was considered significant. Results were expressed as mean $\pm$ SEM.

Results

Expression of the haemagglutinin gene in infected cells

The haemagglutinin (H3) gene of influenza virus A was detected with RT-PCR in nasal epithelial cells after virus inoculation. Samples obtained from cells collected 1 h after infection yielded a RT-PCR product at 727 bp (Fig. 1, lane 1). Samples from cells collected 8–48 h after infection showed enhancement of H3 gene expression (Fig. 1, lanes 2–4).

Expression of eotaxin mRNA in infected cells

Eotaxin mRNA was weakly expressed in uninfected cells (Fig. 2a, lane 1). Moreover, the intensity of PCR product was significantly increased in nasal epithelial cells collected 24 h after influenza virus A infection ($n = 6$) (Fig. 2b). Similar intensity of the PCR amplification products for β -actin were detected in both samples (Fig. 2a, lanes 3 and 4).

Eotaxin concentration in the culture supernatants after infection

Eotaxin was not detected in the supernatants of nasal epithelial cells 8 h after infection or exchange of media, and small amounts of eotaxin protein were induced in uninfected nasal epithelial cells after 24 and 48 h (9.1 ± 1.4 pg/mL, 9.4 ± 2.1 pg/mL, $n = 6$, respectively). In contrast, eotaxin concentration at 24 h (30.1 ± 9.7 pg/mL, $n = 6$) and 48 h (48.9 ± 12.2 pg/mL, $n = 6$) time-points after viral infection were significantly higher than those in uninfected cells (Fig. 3b).

Cytokine-induced eotaxin concentration in the culture supernatants

Eotaxin concentration in the supernatants was not enhanced 24 h after stimulation with IFN- γ (9.7 ± 1.2 pg/mL, $n = 4$, Fig. 4), but was significantly increased 24 h after stimulation with either TNF- α or IL-1 β (21.2 ± 7.4 pg/mL, 16.6 ± 4.9 pg/mL, $n = 4$, respectively) compared with those in unstimulated cells, and, moreover, the combination of all three cytokines (34.9 ± 2.9 pg/mL, $n = 4$) was significantly increased compared with those in the cells stimulated with either TNF- α or IL-1 β .

Effect of anti-TNF- α and anti-IL-1 β antibodies

Anti-TNF- α and anti-IL-1 β antibodies did not affect the eotaxin concentration in virus-infected nasal epithelial cells (data not shown).

Effect of UV radiation

Haemagglutinin mRNA was not detected in nasal cells infected with UV-inactivated virus and eotaxin mRNA was weakly expressed in them (Fig. 5a). UV-inactivated virus did not enhance the eotaxin concentration (9.2 ± 3.7 pg/mL, $n = 6$). There was no significant difference in its concentration between UV-inactivated viral infection and non-infection (8.7 ± 3.1 pg/mL, $n = 6$) (Fig. 5b).

Discussion

Eotaxin is a potent eosinophil chemoattractant protein that plays a major role in eosinophil recruitment in allergic airway inflammation. Our studies showed that while eotaxin is constitutively expressed *in vitro* in primary nasal epithelial cells, it is kinetically increased in the level of eotaxin mRNA expression and secreted proteins from the cells following live influenza A virus infection, concomitant with an increase in viral haemagglutinin gene expression.

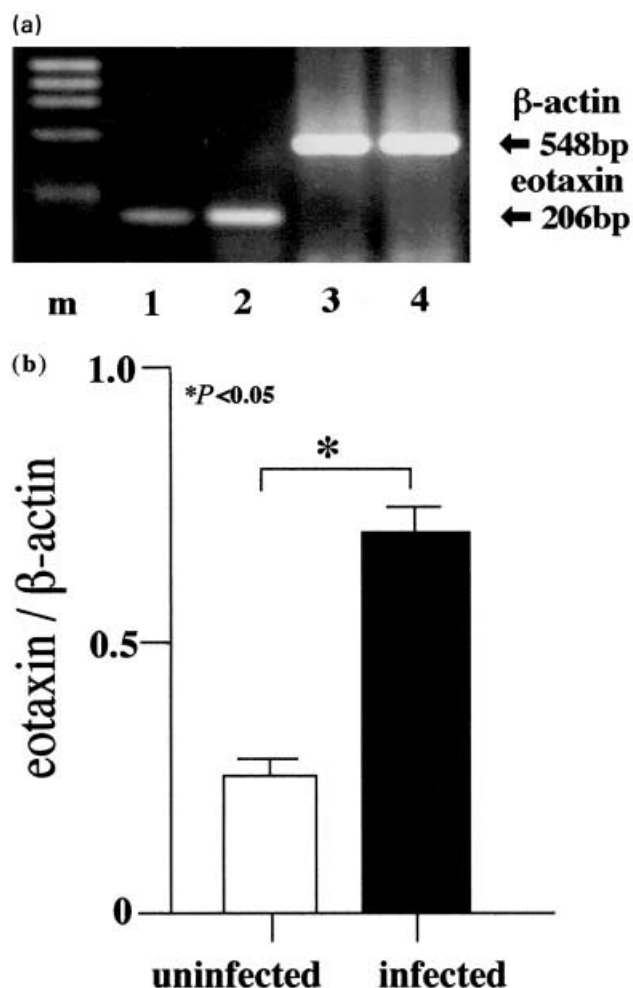


Fig. 2. Detection of eotaxin gene expression by RT-PCR. Nasal epithelial cells were infected with influenza virus A. The infected cells were collected 24 h after infection. Uninfected cells were also collected 24 h after exchange of medium. M, DNA size marker; 1, eotaxin mRNA (206 bp) in uninfected cells; 2, eotaxin mRNA in infected cells; 3, β-actin mRNA in uninfected cells; 4, β-actin mRNA in infected cells (a). The eotaxin/β-actin ratio was determined by densitometric analysis from the same cDNA. $*P < 0.05$ was considered significant by the Mann–Whitney test ($n = 6$, Fig. 2b).

In airway epithelial cells, the pro-inflammatory cytokines, IL-1β and TNF-α, induced eotaxin mRNA expression and protein production [21], and IFN-γ was synergistic with TNF-α and IL-1β on the regulation of eotaxin gene expression [9]. In the present study, we also reported that production of eotaxin protein by nasal epithelial cells was induced by either TNF-α or IL-1β, but not by IFN-γ alone, and secretion of eotaxin protein was further increased in the presence of all three cytokines. It has previously been shown that virus-infected cells secrete less than 100 pg/mL

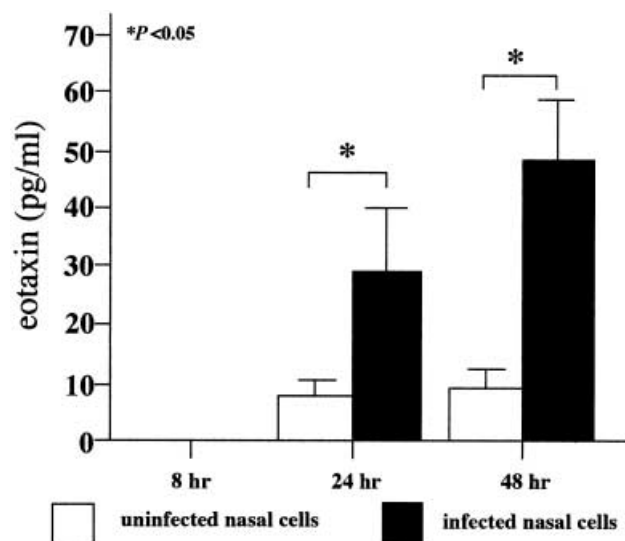


Fig. 3. Eotaxin concentration in the supernatants of the nasal epithelial cells infected with influenza virus A. The supernatants were collected 8, 24 and 48 h after infection, and those of uninfected cells were also collected at the same time-points after exchange of medium. Eotaxin concentration in the supernatants was analysed by ELISA. $*P < 0.05$ was considered significant by the Kruskal–Wallis test ($n = 6$).

of TNF-α and IL-1β [22]. We found that such low levels of cytokines did not induce eotaxin production (data not shown). Moreover, treatment of virus-infected cells with neutralizing anti-TNF-α and anti-IL-1β mAbs did not inhibit eotaxin production, suggesting that the induction of eotaxin expression and secretion from infected cells is independent of endogenously produced cytokines.

Furthermore, an increase in viral haemagglutinin gene expression was seen in the cells after 24 and 48 h viral infection. In contrast, the cells infected with UV-inactivated virus did not show a detectable level of haemagglutinin gene expression, and there was no increase in the level of eotaxin 24 h after infection of the cells with UV-inactivated virus. Therefore, regulation of eotaxin expression probably requires replication of influenza viral genes or protein in nasal epithelial cells.

At present, it is not known which transcription factors contribute to eotaxin expression induced by viral infection. However, various stimuli, including cytokines, can activate nuclear factor-kappa B (NF-κB) [23,24]. In addition, several viruses, such as adenovirus, rhinovirus, respiratory syncytial virus and influenza virus, can up-regulate NF-κB activation [25–30]. On the other hand, expression of eotaxin was regulated by NF-κB in airway epithelial cells [31]. Therefore it is tempting to speculate that activation of NF-κB by viral haemagglutinin is an important factor in influenza virus-induced transcriptional regulation of eotaxin, as suggested by a recent study of virus-infected

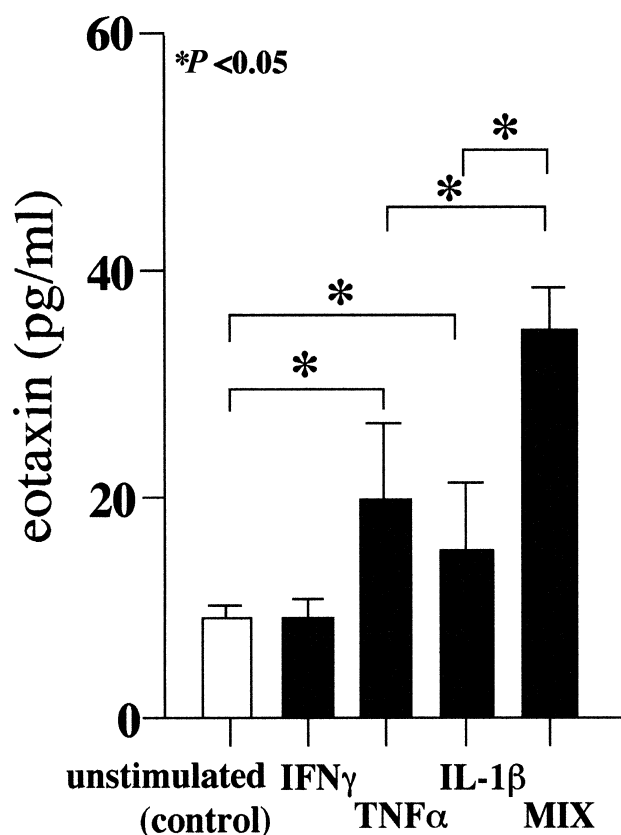


Fig. 4. Assay of eotaxin production stimulated by cytokines. Panel shows production of eotaxin concentration 24 h after stimulation with 100 U/mL IFN- γ , 10 ng/mL IL-1 β , 100 U/mL TNF- α and a combination of all three cytokines. $*P < 0.05$ was considered significant by the Kruskal–Wallis test ($n = 4$).

Hela cells [30] and by our findings that haemagglutinin gene expression is enhanced in nasal epithelial cells infected with influenza virus. Work is in progress to determine the mechanisms of virus-induced regulation of eotaxin gene expression.

Eotaxin plays a key role in the pathogenesis of airway inflammation caused by accumulation of eosinophils, basophils and T helper 2 cells, which express CCR3, a high-affinity receptor for eotaxin [32–34]. Eotaxin is frequently associated with several allergic diseases, such as bronchial asthma and allergic rhinitis. It was expressed in the bronchial mucosa of patients with bronchial asthma [10,35] and the number of cells expressing eotaxin mRNA correlated with symptom scores, with the number of eosinophils in the bronchial tissue, and with the level of airway hyper-responsiveness as measured by the histamine provocative concentration causing a 20% fall in FEV₁ [11]. In addition, eotaxin was expressed in nasal mucosal cells of patients with allergic rhinitis [12,36], and its expression was locally up-regulated with allergen challenge in the

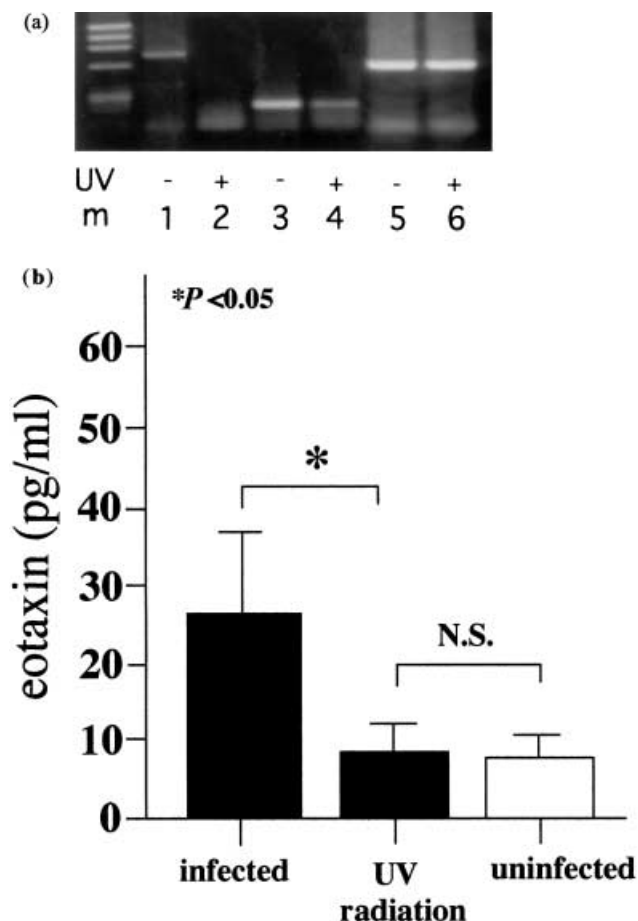


Fig. 5. Effect of UV exposure to influenza virus on eotaxin expression and production. Panel (a) shows expression of haemagglutinin and eotaxin mRNA in nasal epithelial cells 24 h after viral infection with or without UV-inactivation. M, DNA size marker; 1–2, the products of haemagglutinin mRNA; 3–4, the products of eotaxin mRNA; 5–6, the products of β -actin mRNA. Panel (b) shows production of eotaxin protein 24 h after exchange of medium (uninfected) and viral infection with or without UV-inactivation. $*P < 0.05$ was considered significant by the Kruskal–Wallis test ($n = 6$).

nasal mucosa of patients with atopy. [12]. In an *in vivo* study, experimental nasal inoculation of human rhinovirus induced mucosal output of eotaxin in patients with seasonal allergic rhinitis, which was associated with cold symptoms [37].

In our previous study [22], influenza virus A also stimulated RANTES production. A recent study demonstrated that RANTES did not correlate with numbers of eosinophils and markers of asthma severity [35]. On the other hand, eotaxin expression correlated with clinical parameters of asthma severity [10,11] and chemotactic activity of eotaxin was much stronger than that of RANTES

[32]. These findings suggest that eotaxin is involved in the pathogenesis of airway inflammation. At this point, eotaxin induced by influenza virus may be valuable to consider the mechanisms of airway inflammation by viral infection.

However, because these findings were obtained *in vitro* and the amount of eotaxin protein was much smaller than that of the CC chemokine RANTES [22], whether they precisely reflect airway inflammation, such as rhinitis, *in vivo* is not known.

The present study shows that cultured nasal epithelial cells can express and secrete eotaxin following influenza virus infection. Although these findings may contribute to the understanding of the mechanisms of eosinophilic airway inflammation with viral infection, our findings were obtained from nasal polyps instead of nasal epithelium. Therefore, further investigations are necessary to clarify the mechanism by which viral infection causes nasal inflammatory disease.

Acknowledgements

We thank Drs Takeshi Kasama, Keita Kasahara, Toshimichi Imai and Masako Toda for their helpful comments and Hiroko Takeuchi for skilful technical assistance.

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