

Human airway submucosal glands augment eosinophil chemotaxis during rhinovirus infection

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Summary

Background Asthma exacerbations are frequently associated with rhinovirus (RV) infections. However, the contribution of airway submucosal gland (SMG) to exacerbations of asthma in RV respiratory infection has not been studied.

Objective This study was undertaken to examine whether RV-infected human respiratory SMG cells produce pro-inflammatory cytokines and chemokines for eosinophils, and augment eosinophil transmigration across human airway epithelium.

Methods We infected cultured human tracheal SMG cells with RV14, collected culture media at 1, 3, and 5 days after infection, and measured the chemotactic activity for eosinophils in the culture supernatant using a 48-well microchemotaxis chamber and a ^{51}Cr -labelled eosinophil transmigration assay.

Results Exposing a confluent human tracheal SMG cell monolayer to RV14 consistently led to infection. Human SMG cells with RV infection secreted soluble factors activating human eosinophil chemotaxis into the culture supernatant in a time-dependent manner, and the culture supernatant significantly augmented the transmigration of ^{51}Cr -labelled eosinophils through human airway epithelial cell layers from the basal to mucosal side. These effects were completely abolished by a mixture of a monoclonal antibody regulated on activation, normal T cells expressed and secreted (RANTES) and an antibody to granulocyte macrophage-colony stimulating factor (GM-CSF).

Conclusion These results suggest that human respiratory SMG cells may augment eosinophil transmigration across the airway epithelium through the secretion of RANTES and GM-CSF after RV infection, and may contribute to exacerbations of asthma.

Keywords airway lining layer, airway submucosal gland, common cold, eosinophil transmigration, epithelial damage, exacerbations of asthma, GM-CSF, RANTES, rhinovirus infection

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Introduction

Asthma is a chronic inflammatory airway disease, characterized by episodic exacerbations of symptoms and accompanied by transient airway obstruction and an increase in airway hyper-responsiveness to specific or non-specific stimuli [1]. Although airway inflammation in asthma involves multiple cell types and mediators, the eosinophil may have particularly significant roles [2]. Pulmonary infiltration with eosinophils is a common characteristic of asthma, and eosinophils have a potent respiratory burst activity and generate high levels of superoxide anions, which may contribute to airway epithelial damage in asthma [2].

Rhinoviruses (RVs) are the major cause of the common cold, and the most common acute infectious illness in humans

[3]. RVs are the pathogen most frequently associated with exacerbations of asthma in susceptible children and adults [4–6]. The reports suggest RV infection in the lower respiratory tract as well as upper respiratory tract in humans [6–9]. On the other hand, it remains to be established whether RV infections cause wheezing by direct effects on the lower airway or whether there are alternate mechanisms through which upper airway viral infections can cause changes in lower airway function, leading to bronchoconstriction and airway obstruction [6]. The experimental RV infections induce an increase in airway inflammation, characterized by cellular infiltration of the airway wall, particularly with eosinophils and lymphocytes [6, 8]. However, the mechanistic basis of an accumulation of these inflammatory cells into the airway after RVs infection is not fully understood.

Human respiratory submucosal glands (SMGs) distribute extensively along the bronchial trees and secrete mucous and serous fluid to the airway lumen, which is a main constituent of the airway lining layer and is widespread on the airway

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surface. The mucous layer covered on the airway epithelium provides a protective barrier against pathogenic and noxious agents, and participates in the mucosal response to inflammation and infection [10]. Eosinophils are recruited to mucosa and submucosa of the lower airway in patients with atopic asthma after inoculation of RV16 [8], suggesting the production and secretion of eosinophil-activating factors from epithelial cells, mesenchymal cells, and possibly SMG cells. Although the respiratory SMGs produce airway secretions 40 times more than goblet cells [11], little is known about the contribution of SMG cells to both eosinophil recruitment and activation in the RV respiratory infection and in the subsequent exacerbations of asthma. In the present study, we hypothesized that respiratory SMG cells infected with RV may secrete pro-inflammatory cytokines and chemokines that activate eosinophils, and may contribute to exacerbations of asthma.

To test this hypothesis, we inoculated human RV14 to the cultured human tracheal SMG cells, collected cultured supernatant at differing time points, and evaluated the chemotactic activity for eosinophils in the supernatant. Furthermore, we examined whether the supernatant actually augments eosinophil transmigration across the airway epithelium.

Materials and methods

Media components

Reagents for cell culture media were obtained as follows: Eagle's minimum essential medium (MEM), Dulbecco's modified Eagle's medium (DMEM), Ham's F-12 medium, fetal calf serum (FCS), and ultra low gamma globulin calf serum (ultra low GGCS) were from GIBCO-BRL Life Technologies (Palo Alto, CA, USA); hydrocortisone-water soluble, trypsin, EDTA, dithiothreitol, Sigma type XIV protease, human placental collagen, penicillin, streptomycin, gentamicin, and amphotericin B were from Sigma Chemical (St Louis, MO, USA); Ultrosor G serum substitute (USG) was from BioSeptra (Marlborough, MA, USA).

Culture of human SMG cells

Tracheas were obtained from 34 patients (mean age 56 years; range, 39–76 years) without overt pulmonary disease and allergy at autopsy between 2 and 9 h after death under a protocol permitted by the Tohoku University Ethics Committee. Culture methods have been previously described in detail [12]. In brief, after stripping the surface epithelium off the trachea, gland-rich submucosal tissue was dissected from the cartilage and the adventitia, immersed in fresh phosphate-buffered saline (PBS), and minced with scissors. Tissue fragments were recovered by centrifugation (200 g for 10 min) and placed in Hanks' buffered salt solution containing 20 mM HEPES buffer (pH 7.4), crude collagenase type IV (500 U/mL), pancreatic porcine elastase (6 U/mL), hyaluronidase (200 U/mL), and deoxyribonuclease (10 U/mL). After 12–16 h of disaggregation in a trypsinizing flask on an orbital shaker [240 round per minute (r.p.m.)] at room temperature, disaggregated tissue was decanted, recovered by centrifugation (200 g for 10 min), washed twice, and suspended in a

mixture of 40% Ham's F-12 medium, 40% DMEM, and 20% FCS (F-12–DMEM–FCS), plated into two T₂₅ tissue culture flasks (Corning, NY, USA), and incubated at 37 °C in 5% CO₂–95% air. The fragments of submucosal tissue remaining in the trypsinizing flask were again exposed to enzymatic digestion as above, and the second collection of dispersed gland acini was combined with the suspension of unattached acini from the two T₂₅ flasks. The combined acini from these two sources were spun down, suspended in fresh F-12–DMEM–FCS, and replated in the two T₂₅ flasks containing the attached acini from the first plating. The following morning, F-12–DMEM–FCS was replaced with F-12–DMEM with 0.1% USG and the following growth factors: 10 µg/mL of insulin, 5 µg/mL of transferrin, 20 ng/mL of triiodothyronine, 0.36 µg/mL of hydrocortisone, 7.5 µg/mL of endothelial cell growth supplement, 25 ng/mL of epidermal growth factor, 0.1 µM retinoic acid, and 20 ng/mL of cholera toxin. It took 14–21 days to achieve confluency, at which time the cells were collected by trypsinization (0.05% trypsin and 0.02% EDTA). The cells were pelleted (200 g for 10 min) and suspended in an F-12–DMEM–FCS medium. Cell counts were made with a haemocytometer, and estimates of viability were carried out with trypan blue and by measuring the amount of lactate dehydrogenase in the medium as previously reported [12]. The cells were then plated at 5×10^5 viable cells/mL in glass tubes. This medium was replaced with F-12–DMEM supplemented with 0.1% USG and growth factors on the first day after plating. The glass tubes were sealed with rubber plugs and cultured at 37 °C. The cell culture medium was supplemented with 10⁵ U/L of penicillin, 100 mg/L of streptomycin, 50 mg/L of gentamicin, and 2.5 mg/L of amphotericin B. All culture vessels were coated with human placental collagen (20 µg/cm²). The absence of fibroblasts in the glass tubes was confirmed with an inverted microscope (MIT-2, Olympus, Tokyo, Japan). Under an inverted microscope, the cultured human tracheal SMG cells had a cuboidal or round-shaped appearance of confluent sheets. In contrast, the fibroblasts had a spindle-shaped appearance. Furthermore, to determine whether cultured cells can form tight junctions, we performed parallel cultures of human tracheal SMG cells on Millicell CM inserts (0.45 µm pore size and 0.6 cm² area; Millipore Products Division, Bedford, MA, USA) to measure electrical resistance and short-circuit current with Ussing chamber methods. When cells cultured under these conditions form tight junctions without contamination by fibroblasts, they have values of $>40 \Omega \text{ cm}^2$ for resistance and $>2 \mu\text{A/cm}^2$ for short-circuit current [12]. Therefore, cultured human SMG cells able to form tight junctions without contamination by fibroblasts were used for the following experiments when the cultured cells in the tubes had a cuboidal or round-shaped appearance and the cells on the Millicell CM inserts had resistance higher than $40 \Omega \text{ cm}^2$ and short-circuit current higher than $2 \mu\text{A/cm}^2$. To determine whether cultured cells possess immunologic characteristics of SMG cells, the expression of serous and mucous gland cell secretory antigen was examined by using immunocytochemical methods as previously described [12]. The cells cultured on glass coverslips were fixed with 4% paraformaldehyde and 0.5% Triton X-100 in 0.1 M phosphate buffer for 15 min at room temperature and were then washed with PBS. Immunocytochemistry with monoclonal antibodies directed

against mucous (A1F8) and serous (B1D8) gland cells of the original tissues and antibodies against lysosome and lactoferrin, both markers for serous cells, was performed with a modification of a biotin-avidin procedure to further confirm that there is no contamination of both fibroblasts and surface epithelial cells in the cultured SMG cells.

Viral infection of airway SMG cells

RV14 was prepared in our laboratory from patients with a common cold. Stocks of RV14 were generated by infecting human embryonic fibroblast cells cultured in glass tubes in 1 mL of MEM supplemented with 2% ultra low GGCS, 50 U/mL of penicillin, and 50 µg/mL of streptomycin at 33 °C [12].

The medium was removed from the confluent monolayers of human tracheal SMG cells and replaced with 1 mL of F-12-DMEM supplemented with 0.1% USG and growth factors. RV was added at a concentration of 10^5 50% tissue culture infectious dose (TCID₅₀) U/mL as previously described [12]. After 1 h incubation at 33 °C, the viral solution was removed, and the cells were rinsed one time with 1 mL of PBS. The cells were then fed with F-12-DMEM containing 0.1% USG and growth factors supplemented with 10^5 U/L of penicillin, 100 mg/L of streptomycin, 50 mg/L of gentamicin, and 2.5 mg/L of amphotericin B and cultured at 33 °C with rolling in an incubator (HDR-6-T, Hirasawa, Tokyo, Japan) [12]. The supernatants were removed immediately before RV infection (cultured for 1 day before infection) and at day 1 (cultured for 1 day after infection), day 3 (cultured from days 1 to 3 after infection), and day 5 (cultured from days 3 to 5 after infection) after RV infection and were stored at -80 °C for all the following experiments. As a control, viral stock solution without RV was exposed to human SMG cells and the supernatants were collected at the same interval (sham infection).

Cytokine assays

In the preliminary study, we found that the mRNAs of IL-1 α , IL-1 β , IL-6, IL-8, TNF- α , regulated on activation, normal T cells expressed and secreted (RANTES) and granulocyte macrophage-colony stimulating factor (GM-CSF), but neither IL-3, IL-5 nor eotaxin were expressed in the cultured SMG cells before and after RV14 infection [12]. To determine the effects of RV14 infection on the production of eosinophil-activating cytokines, we therefore measured the amount of IL-8, GM-CSF, and RANTES in the cultured supernatant of SMG cells collected for 1 day before RV infection and for day 1, days 1–3, and days 3–5 after RV infection as described above. The proteins of IL-8, GM-CSF, and RANTES were measured by specific ELISAs. Sensitivities of the assay were 10 pg/mL for the IL-8 ELISA kit (Toray Co., Tokyo, Japan), 2 pg/mL for the GM-CSF ELISA kit (Genzyme, Cambridge, MA, USA), and 5.6 pg/mL for the RANTES ELISA kit (Ohtsuka, Tokushima, Japan). We used an average value of replicate cultures from the same trachea ($n = 3$ donors) for the analysis of cytokine production.

Effects of antibodies to intercellular adhesion molecule-1 (ICAM-1) on cytokine production

To determine whether cytokine production was associated with RV infection, pre-treatment with an antibody to ICAM-1, a major receptor for RV, was performed before RV infection. Thereafter, RV14 infection was performed and the cultured supernatants of SMG cells were collected for 1 day before infection and for day 1, days 1–3, and days 3–5 after infection for cytokine measurement as described above. Confluent human SMG cells were incubated for 30 min with medium alone, with a medium containing a mouse monoclonal anti-human antibody to ICAM-1 [84H10 (100 µg/mL; Immunotech, Marseilles, France)], or a medium containing an isotype-matched mouse IgG1 control monoclonal antibody (100 µg/mL; Chemicon International, Temecula, CA, USA) at 37 °C before RV14 exposure.

Preparation of eosinophils

Blood was drawn into a heparinized syringe from 16 mild-to-moderate asthma patients [age, 37 (mean) $\pm$ 6 (SE) years: five females and 11 male] with an eosinophilia and separated by sedimentation with Dextran for 45 min at room temperature as described previously [13, 14]. The leucocyte-rich plasma was layered on a cushion of Ficoll-Hypaque (Histopaque-1077, Sigma) and centrifuged at 300 *g* for 20 min. Erythrocytes in the granulocyte-rich pellet were lysed by suspending the cells in a solution consisting of 0.1% KHCO₃ and 0.83% NH₄Cl, and the suspension was then centrifuged at 400 *g* for 10 min [13]. Eosinophils were purified by negative selection with immunomagnetic microbeads coated with antibody against CD16 as described previously [14]. Briefly, 50 µL of MACS CD16 microbeads (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany) per 5×10^7 cells were 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 PBS, 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 Diff-Quik (American Scientific Products, McGraw Park, IL, USA)-stained cytocentrifugation preparations. The cells were suspended in PBS containing 2% bovine serum albumin (BSA) (Sigma) at pH 7.2 to give a final concentration of 3.0×10^6 cells/mL for eosinophil chemotaxis.

Measurement of eosinophil chemotaxis

The chemotaxis assay was performed using a 48-well microchemotaxis chamber (Neuroprobe, Cabin John, MD, USA) as previously described [13]. The bottom wells of the chamber were filled with 25 µL of tested solution with or without chemotactic stimulus in triplicate. A polycarbonate filter with a pore size of 5 µm (Nucleopore Corp., Pleasanton, CA, USA) was placed over the bottom wells. The silicon gasket and upper piece of the chamber were attached and the entire assembly was pre-incubated at 37 °C in humidified air for 15 min before filling the upper wells with 50 µL of cell suspension. Finally, the chamber was incubated in humidified air in 5% CO₂ at 37 °C for 90 min. After incubation, the chamber was disassembled and the filter was removed. The filter was then fixed, stained with Diff-Quik, and mounted on

a glass slide. Eosinophils that completely migrated through the filter were counted in 10 random high-power fields (HPF) ($\times 1000$) per well. Chemotactic activity was expressed as the mean number of migrated cells per HPF from triplicate wells. When data were obtained from cultures from different autopsies, the means of the chemotaxis experiments were used as single data points. Culture medium alone was used as a negative control, and platelet-activating factor (PAF) (10^{-7} M; Sigma) was used as a positive control for eosinophil chemotaxis. The supernatants collected for 1 day before infection and for day 1, days 1–3, and days 3–5 after infection, the corresponding time point to the cytokine assay experiments, were used for this experiment. Human tracheal SMG culture supernatant with sham infection was also used to determine background eosinophil migration (control).

Culture of human airway epithelium

Culture methods have been described elsewhere in detail [15, 16]. In brief, the surface epithelium of the trachea was scored into longitudinal strips and pulled off the submucosa. The tissue strips were rinsed five times in PBS containing 5 mM dithiothreitol and PBS alone, and then incubated at 4 °C overnight in PBS-containing protease (Sigma type XIV, 0.4 mg/mL). The following day, the enzyme was competitively inhibited by the addition of FCS to a final concentration of 2.5%, and small sheets of cells were dislodged from the epithelial strips by vigorous agitation. The denuded strips were removed, and the sheets of cells remaining were dispersed by repeated aspiration in a 10-mL pipette. Cells were pelleted (200 g for 10 min) and suspended in a 1:1 mixture of DMEM and Ham's F12 medium containing 5% FCS. Cell counts and estimates of viability were then made using trypan blue and a haemocytometer, and cells were seeded at 10^6 viable cells/cm² onto human placental collagen (Sigma type IV)-coated polyester membrane inserts (Transwell[®], 3 µm pore size, Ø 6.5 mm; Corning Inc., Corning, NY, USA) placed in 12-well plates and cultured with 2% USG supplemented with penicillin (10^5 U/L), streptomycin (100 mg/L), gentamicin (50 mg/L), and amphotericin B (2.5 mg/L) by an air-liquid interface method until they showed confluence [15, 16]. The inserts with cultured epithelial cells were placed in the wells of 12-well tissue culture plates to separate the upper chamber from the lower chamber by the cultured epithelial cell layer. Prior to experiments, cultured epithelial cells were confirmed to retain high transepithelial resistance ($150 \Omega \text{ cm}^2$ or greater) [16].

Eosinophil transmigration across airway epithelial layer

Isolated eosinophils were labelled with ⁵¹Cr by using a method described previously [16]. In brief, 75 or 150 µCi of ⁵¹Cr (1 mCi/mL) were added as sodium chromate (specific activity, 250–500 mCi/mg Cr; Amersham Japan, Tokyo, Japan) to the suspended eosinophils in 150 and 300 µL of PBS. The cell suspension was then incubated at 37 °C in 5% CO₂–95% air in PBS for 1 h. The cells were washed four times and resuspended in PBS at 5×10^5 /mL. Two hundred microlitres of ⁵¹Cr-labelled eosinophils (10^5 cells/well) were put in the lower chamber of the well, and tested solutions containing 10^{-5} M phorbol myristate acetate (PMA) were

added to the upper chamber as previously described [16]. Medium alone or 10^{-7} M of PAF was used as a negative and a positive control, respectively. After incubation for 4 h at 37 °C in 5% CO₂–95% air, the medium of the upper chamber was collected and the radioactivity (c.p.m.) of the collected medium was then measured using an auto-gamma counter (ARC-500; Aloka, Tokyo, Japan). The eosinophil penetration rate (%) was calculated as follows: (count of ⁵¹Cr-labelled eosinophils removed from the upper chamber/count of ⁵¹Cr-labelled eosinophils originally added to the lower chamber) $\times 100$ [16].

Effect of neutralizing antibodies to RANTES and GM-CSF on eosinophil chemotactic activity and eosinophil transmigration across airway epithelium

To determine the effects of antibodies to RANTES, GM-CSF, and IL-8 on the eosinophil chemotactic activity in the cultured supernatant after RV14 infection, mouse monoclonal anti-RANTES, anti-GM-CSF, and anti-IL-8 antibodies (100 µg/mL, respectively PharMingen International, San Diego, CA, USA) were added to the tested supernatants in both chemotaxis experiments in microchemotaxis chamber and transmigration experiments across cultured human tracheal epithelial cell layers. An isotype-matched mouse monoclonal antibody to human leucocyte antigen (HLA) class I (100 µg/mL; Immunotech) was used as a control antibody.

Statistical analysis

The results are expressed as means $\pm$ SE; *n* is the number of donors from which cultured SMG cells were used. Statistical analysis was performed with a two-way repeated measures ANOVA. Bonferroni's test was used to estimate the level of significance of differences between means. For all analyses, values of *P* < 0.05 were assumed to be significant.

Results

Characteristics of the cultured human tracheal SMG cells

Immunocytochemical analysis revealed that more than 96% of the cells of the confluent monolayers of SMG cells reacted with the monoclonal antibody A1F8 directed against mucous cells and with the monoclonal antibody B1D8 directed against serous cells, although there was cell-to-cell variability in the staining as described previously [12].

RV infection of human tracheal SMG cells

Exposing confluent human tracheal SMG cell monolayers to RV14 (10^5 TCID₅₀/mL) consistently led to infection. Collection of culture medium at differing times after viral exposure revealed no detectable virus 1 h after infection. RV14 was detected in culture medium 8 h after infection, and the viral content progressively increased between 8 and 24 h after infection, as shown in Fig. 1. Evidence of continuous viral production was obtained by demonstrating that the viral titres in supernatants collected for 1–3 and 3–5 days after infection each contained significant levels of RV14 (Fig. 1).

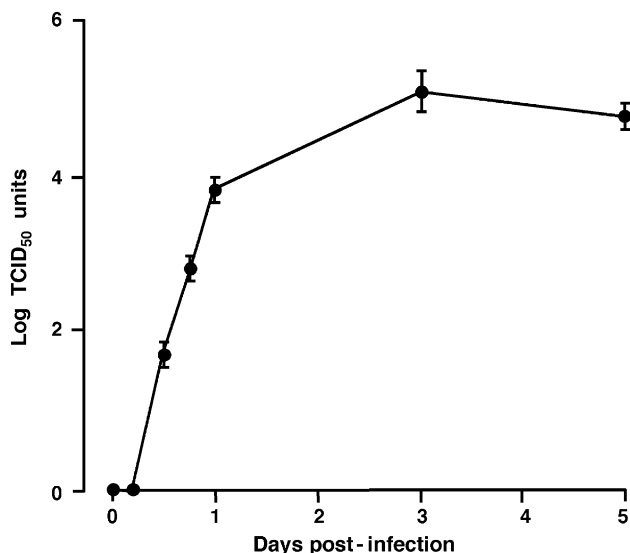


Fig. 1. Viral titres in supernatants of human tracheal submucosal gland cells were obtained at sequential times during the first 24 h, for 1–3 and 3–5 days after exposure to 10^5 50% tissue culture infectious dose (TCID₅₀) U/mL of RV14 (closed circle). Five separate experiments were performed.

Analysis of the levels of cell-associated virus (the virus detectable in sonicates of the human tracheal SMG cells) followed a time course similar to that observed in the supernatant as previously described (data not shown) [12].

Production of cytokines in cultured supernatants from RV-infected SMG cells

Figure 2 shows the time course of RANTES (Fig. 2a), GM-CSF (Fig. 2b), and IL-8 (Fig. 2c) production in the

supernatants of human tracheal SMG cells after RV14 infection. Because viral infection did not alter cell numbers [12], all cytokine values are reported in pg/mL of supernatant. Although basal secretion of IL-8 was extremely high, IL-8 production was significantly increased quite early in response to RV infection. By contrast, basal secretion was quite low with RANTES and GM-CSF, whereas secretion of RANTES and GM-CSF increased time dependently in response to RV infection (Fig. 2). An antibody to ICAM-1 inhibited RV infection of SMG cells and limited the increased production of IL-8, GM-CSF, and RANTES in the culture supernatant collected for days 3–5 after infection to 4%, 6%, and 2% of those without antibody pre-treatment, respectively. However, pre-treatment with an isotype-matched control monoclonal antibody was without effect on the production of these cytokines (data not shown).

Release of eosinophil chemotactic factors from RV-infected SMG cells

The chemotactic activity for eosinophils in response to positive control was 28.3 ± 5.3 cells/HPF for PAF. In contrast, the chemotactic activity in the culture medium alone was minimal (1.2 ± 0.2 cells/HPF). Figure 3 shows the time course of chemotactic activity for eosinophils in the supernatants of cultured human SMG cells after RV14 infection. Although uninfected SMG cells release eosinophil chemotactic activity in the baseline culture condition (maximum 4.4 ± 0.5 cells/HPF for 3–5 days), RV infection caused a significant increase in the chemotactic activity in a time-dependent manner (maximum 18.4 ± 1.6 cells/HPF for 3–5 days after infection, $P = 0.0012$ compared with baseline values). The chemotactic activity in the supernatants collected

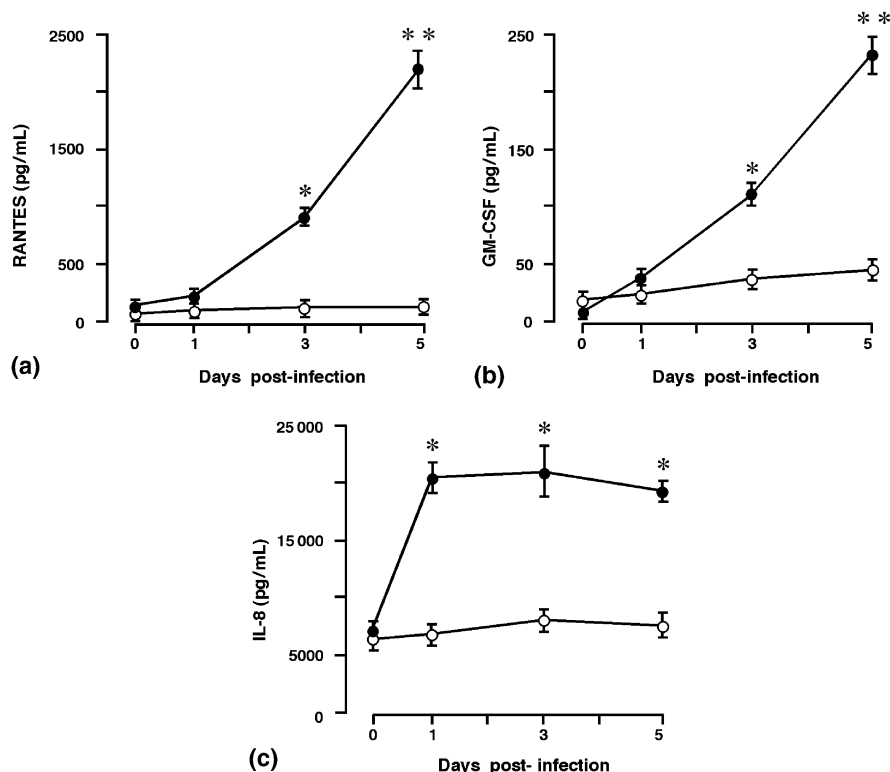


Fig. 2. Time course of release of cytokines into supernatants of human tracheal submucosal gland cells after rhinovirus (RV)14 infection (closed circle). Data from the culture supernatants collected for 1 day before RV infection and for day 1, days 1–3, and days 3–5 after RV infection were plotted onto days 0, 1, 3, and 5 of Fig. 2, respectively. Open circles represent sham infection (control). GM-CSF (b), granulocyte-macrophage colony-stimulating factor; RANTES (a), regulated on activation, normal T cells expressed and secreted. Results are means $\pm$ SE from three separate experiments. Significant difference from corresponding control value: * $P < 0.05$, ** $P < 0.01$ compared with the supernatants with sham infection.

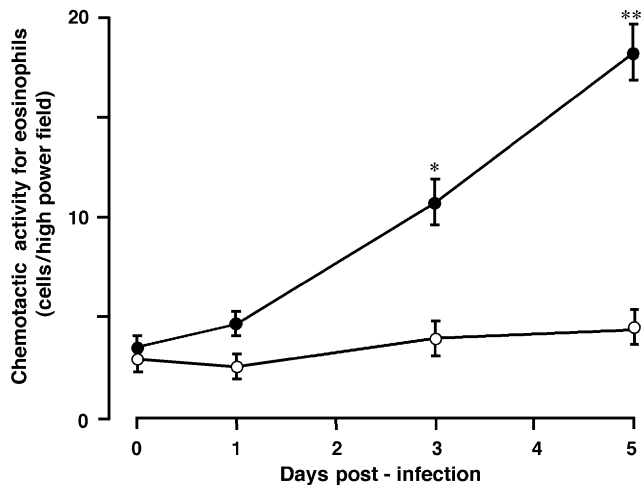


Fig. 3. Time course of release of eosinophil chemotactic activity into supernatants of human tracheal submucosal gland cells after rhinovirus (RV)14 infection (closed circle). The eosinophil chemotactic activity increased in a time-dependent manner after RV14 infection. Data from the culture supernatants collected for 1 day before RV infection and for day 1, days 1–3, and days 3–5 after RV infection were plotted onto days 0, 1, 3, and 5 of Fig. 2, respectively. Open circles represent sham infection (control). Results are means $\pm$ SE from eight separate experiments. * $P < 0.05$, ** $P < 0.01$ compared with sham infection.

for 3–5 days after RV infection was significantly inhibited either by an anti-RANTES antibody or by an anti-GM-CSF antibody (Fig. 4). Furthermore, the combination of anti-RANTES antibody and anti-GM-CSF antibody inhibited the chemotactic activity induced by RV14 to the level of uninfected cells (Fig. 4). In contrast, an anti-IL-8 antibody or a mouse IgG1 control monoclonal antibody did not alter the chemotactic activity in the supernatants collected for days 3–5 after RV infection (Fig. 4).

Effect of supernatants from RV-infected SMG cells on eosinophil transmigration across airway epithelial cell layers

The supernatants collected for days 3–5 after sham or RV infection were used for this experiment, because they showed

maximum eosinophil chemotactic activity as shown in Fig. 3. The control (sham infected) radioactive counts in the upper and lower chambers were 1080 ± 50 c.p.m. (range, 800–1400) and $61\,200 \pm 7400$ c.p.m. (range, 50 000–80 000 c.p.m.), respectively. Addition of the culture supernatant of human SMG cells collected for days 3–5 after RV infection into the upper chamber significantly increased the eosinophil penetration across epithelial layers from basal to mucosal direction compared with controls ($7.9 \pm 0.7\%$ vs. $1.7 \pm 0.3\%$, $P = 0.008$) (Fig. 5). Addition of either anti-RANTES antibody or anti-GM-CSF antibody to the tested supernatants significantly inhibited the eosinophil penetration ($4.1 \pm 0.5\%$ and $4.3 \pm 0.3\%$, $P < 0.05$, respectively) induced by RV14 infection, as shown in Fig. 5. Furthermore, the combination of an anti-RANTES antibody and an anti-GM-CSF antibody inhibited the supernatant-induced increase in eosinophil penetration across the epithelial cell layers to the control level (from $7.9 \pm 0.7\%$ to $2.1 \pm 0.2\%$, $P = 0.012$). However, adding an isotype-matched mouse IgG1 monoclonal antibody or an anti-IL-8 antibody did not alter the supernatant-induced increase in the eosinophil penetration ($8.3 \pm 0.6\%$ and $8.1 \pm 0.4\%$, respectively).

Discussion

In the present study, we showed that RV-infected human airway SMG cells secrete chemotactic factors for eosinophils into the culture supernatant, and these effects on eosinophil chemotaxis were completely abolished by an addition of anti-RANTES and anti-GM-CSF antibodies. Moreover, we detected a significant amount of RANTES and GM-CSF in the culture supernatant of RV-infected human tracheal SMG cells. We also showed that an antibody to ICAM-1 completely inhibited virally induced cytokine production, suggesting that cytokine production requires active RV infection. These findings suggest that RV infection may activate eosinophils to accumulate into airway mucosa and submucosa [17] through the production of RANTES and

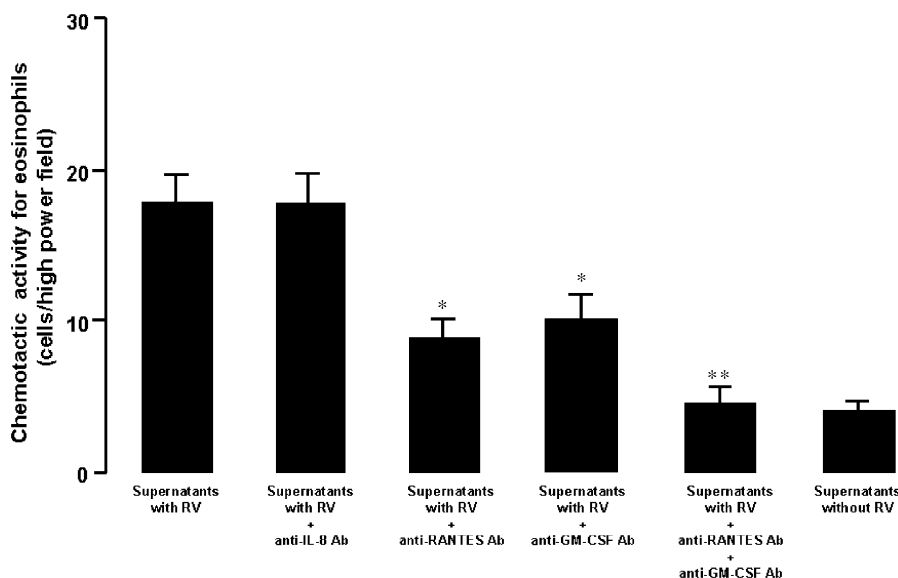


Fig. 4. Effect of neutralizing antibodies to regulated on activation, normal T cells expressed and secreted (RANTES), granulocyte-macrophage colony-stimulating factor (GM-CSF), and IL-8 on eosinophil chemotactic activities in the supernatant of the human tracheal submucosal gland cells during 3–5 days after rhinovirus (RV)14 infection. Results are means $\pm$ SE from five separate experiments. * $P < 0.05$ and ** $P < 0.01$ compared with the supernatants with RV infection.

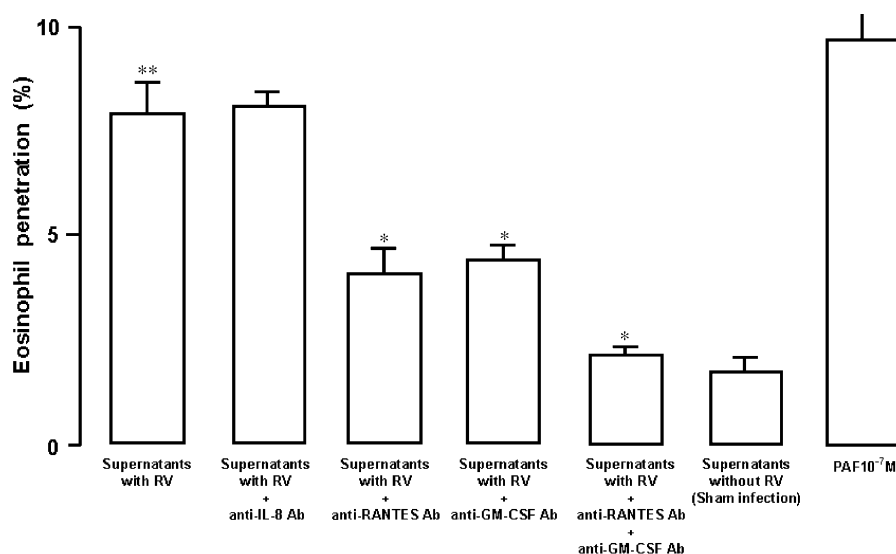


Fig. 5. Eosinophil transmigration activity across airway epithelium in culture supernatants of human tracheal submucosal gland cells after rhinovirus (RV)14 or sham infection in the presence or absence of a monoclonal antibody to either IL-8, granulocyte-macrophage colony-stimulating factor (RANTES) or granulocyte-macrophage colony-stimulating factor (GM-CSF), or the combination of antibodies to RANTES and GM-CSF. The supernatant obtained from the human tracheal submucosal gland (SMG) cells during 3–5 days after RV14 infection induced a significant increase in the penetration, which was blocked by adding an anti-RANTES antibody or by an anti-GM-CSF antibody. PAF was used as a positive control. Results are means $\pm$ SE from four separate experiments. * $P < 0.05$ compared with the supernatant of RV-infected SMG cells without any blocking antibodies. ** $P < 0.01$ compared with sham infection (control).

GM-CSF by airway SMG cells. RANTES is a chemoattractant for eosinophils and memory T cells [18]. The reason why GM-CSF in the cultured supernatant from RV-infected SMG cells enhanced eosinophil chemotaxis is uncertain. However, it is reported that GM-CSF promotes eosinophil chemotaxis in the presence of other cytokines [19], which might partly explain the eosinophil chemotactic activity of GM-CSF in this study. Furthermore, GM-CSF improves eosinophil survival and increases adhesion molecule expression of the cells, and is a cofactor for eosinophil superoxide production and degranulation [20]. The production and secretion of these cytokines by SMG cells may therefore contribute to exacerbations of asthma. By contrast, IL-8 is a potent chemoattractant for and activator of neutrophils, and also has chemotactic activity for lymphocytes, the two predominant cell types in the nasal mucosa during the early stage of RV infection [21]. According to our results, human SMG cells secrete IL-8 immediately after RV infection, which might contribute to the early-stage neutrophilic inflammation of the airway.

Many eosinophils are observed in the sputum and bronchoalveolar lavage fluid from patients with asthma, and eosinophils are considered to have an important role in the pathophysiology of asthma [17]. Calhoun et al. [22] reported that experimental inoculation of RV augments antigen-induced eosinophil recruitment to the lower airway. Furthermore, both eosinophils and T cells are recruited to mucosa and submucosa of the lower airway in normal subjects and patients with atopic asthma after inoculation of RV16 [8], suggesting the presence of factors released from activated epithelial cells, mesenchymal cells, and possibly SMG cells, which might be associated with the activation of these inflammatory cells. Our results might provide a possible explanation for an increased recruitment of eosinophils and T cells to the lower airway during RV infection.

Although several investigators have reported airway bronchial and nasal epithelial cells infected with respiratory viruses, including RV secrete eosinophil activating chemokines such as GM-CSF, IL-8, and RANTES [23–28], the direct action of these cytokines upon eosinophil activation

and transmigration across airway epithelium has not been demonstrated. In the present study, we showed, probably for the first time, that RANTES and GM-CSF released from RV14-infected SMG cells actually augmented eosinophil transmigration across airway epithelium treated with PMA, a protein kinase C activator. We have previously reported an increase in the transepithelial migration of eosinophils after treatment of epithelial cells with PMA [16]. Although the precise reason why an increased eosinophil penetration was not observed across the airway epithelial cell layers untreated with PMA is still uncertain, eosinophils might penetrate through the opened tight junctions via the activation of protein kinase C.

Airway epithelial injuries and/or damages that are characteristic pathologic features of asthma may require a contact between eosinophils and epithelial cells [16]. Use of our simple *in vitro* models allows for a detailed examination of the interactions among RV-infected SMG cells, eosinophils and airway epithelial cells. We have previously shown that the barrier function of the airway epithelium infected with RV is altered and the infected epithelium is more susceptible to chemical oxidant, which is released from inflammatory cells, such as eosinophils and neutrophils [29]. Furthermore, we have shown that RV14 infection induces the expression of ICAM-1 in the cultured human tracheal epithelial cells, a ligand for the adhesion molecule, CD18/CD11, on eosinophil surface membranes [15, 29]. Thus, an increase in adhesion molecule expression, together with RV-induced chemokine release from SMG cells, vigorously recruits eosinophils to the airway. These chemokines could increase binding of eosinophils to epithelial cells and further augment eosinophil transmigration across airway epithelium, which might be easily damaged by attached or migrating eosinophils during RV infection. Toxic granules, reactive oxygen species, and other mediators including leukotrienes, released from eosinophils during epithelial transmigration, may lead to exacerbations in patients with asthma.

It should be noted here that the *in vitro* model system used in the present study may not completely reflect the real functions of airway epithelium and SMGs *in vivo*. First, in

preparing the SMG cells, we employed enzymatic digestion and used hydrocortisone, which might affect the expression of surface receptors of these cells. Furthermore, since we infected human airway SMG cells with RV at 33 °C, it is uncertain whether the responses to RV infection are similar to cells *in vivo* at 37 °C. Second, in the eosinophil transepithelial migration assay, we did not recover intact eosinophils from the upper chamber, nor did we count the actual number of migrated eosinophils. However, we examined histologically the cultured airway epithelial cell layers after 4 h of incubation and confirmed large numbers of eosinophils migrating across the epithelial layers as described previously [16]. Finally, in preparing purified eosinophils, we used the NH₄Cl method for red cell lysis instead of hypotonic lysis, whereas we could not identify any significant differences between those two methods in the eosinophil transepithelial migration assay.

In conclusion, the present study indicates that RV infection induces the production of RANTES and GM-CSF from human airway SMG cells. These factors may be associated with an increase in eosinophil chemotaxis and an augmented transmigration across airway epithelium. The eosinophil activation by factors including RANTES and GM-CSF may contribute to exacerbations of asthma during RV infection.

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