

RANTES production in nasal epithelial cells and endothelial cells

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Background: It is well documented that the chemokine that is regulated upon activation, normal T expressed and presumably secreted, RANTES, is produced by macrophages, platelets, fibroblasts, and renal tubular epithelial cells. Recently, however, production of RANTES by vascular endothelium and airway epithelial cells was demonstrated in human umbilical vein endothelial cells (HUVECs) and epithelial cell lines.

Objective: This investigation was aimed at determining whether human nasal epithelial cells (HNECs) and human mucosal microvascular endothelial cells (HMMECs) produce RANTES when they are stimulated by several cytokines.

Methods: HNECs and HMMECs were isolated from nasal mucosa and subsequent continuous subcultures and were stimulated either by IL-1 β or by the combination of tumor necrosis factor alpha (TNF- α) and interferon gamma (IFN- γ).

Results: After the combined stimulation by TNF- α and IFN- γ , HNECs and HMMECs dramatically produced RANTES, as previously observed in HUVECs and bronchial epithelial cell line BEAS-2B. IL-1 β also increased RANTES production to a lesser extent. We also demonstrated that the amount of RANTES induced by TNF- α and IFN- γ was higher in HNECs and HMMECs obtained from patients with nasal allergy than in those from patients without allergy.

Conclusion: RANTES from HNECs and HMMECs likely plays a critical role in eosinophil infiltration of the nasal mucosa in subjects with nasal allergy. (*J Allergy Clin Immunol* 1996; 98:S230-7.)

Key words: RANTES, nasal epithelial cells, endothelial cells, RT-PCR, nasal allergy

Activated eosinophils are believed to be involved in nasal swelling and hyperreactivity to nonspecific stimuli in the nasal mucosa of patients with nasal allergy.¹⁻³ The precise accumulation mechanism of eosinophils, however, is not fully understood.

The cytokine RANTES (regulated upon activation, normal T expressed and presumably secreted) is a member of the proinflammatory chemokine

Abbreviations used

HNECs:	Human nasal epithelial cells
HMMECs:	Human mucosal microvascular endothelial cells
IFN- γ :	interferon gamma
PBS:	phosphate-buffered saline solution
RANTES:	regulated upon activation, normal T expressed and presumably secreted
TNF- α :	tumor necrosis factor alpha

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superfamily of cytokines (low molecular weight cytokines with chemotaxis for leukocytes).⁴⁻⁹ RANTES, besides being a potent chemoattractant for eosinophils, basophils, monocytes, and CD45RO/CD4+ memory T lymphocytes,⁹⁻¹¹ also exhibits

TABLE I. Clinical background of patients with and without allergy

Pt. No	Group	Age/Sex	Antigen	Eosinophilia in nasal smear
1	Nasal allergy	32M	HD, M, JC	+
2	Nasal allergy	19M	HD, R	2+
3	Nasal allergy	41F	HD, M	+
4	Nasal allergy	26F	HD, M, Mu	2+
5	Nasal allergy	40M	HD, M, JC, R, Mu	3+
6	Nasal allergy	16F	HD, M, JC, R, Mu	3+
7	Nasal allergy	35F	HD, M, JC, R, Mu	3+
1	Non-allergy	17M	—	—
2	Non-allergy	28F	—	—
3	Non-allergy	46F	—	—
4	Non-allergy	15M	—	—
5	Non-allergy	29M	—	—
6	Non-allergy	23F	—	—
7	Non-allergy	33F	—	—

HD, house dust; M, mites; JC, Japanese cedar pollen; R, ragweed pollen; Mu, mugwort pollen.

pleiotropic effects on a variety of target cell types, including activation of eosinophils^{11, 12} and T lymphocytes,¹³ induction of eosinophil transendothelial migration in the extracellular spaces,¹⁴ and histamine release from basophils.¹⁵ Thus RANTES may be involved in both acute and chronic stages of allergic inflammation. It is well documented that RANTES is produced by macrophages, platelets, fibroblasts, and renal tubular epithelial cells.^{10, 16-19} Recently, however, the production of RANTES by vascular endothelium and airway epithelial cells was detected in both human umbilical vein endothelial cells (HUVEC)²⁰ and epithelial cell lines.²¹ Platelet activating factor (PAF), leukotriene B₄ (LTB₄), C5a, and IL-5 play important roles as eosinophil migrating factors,²²⁻²⁶ whereas they are known to be produced mainly in inflammatory cells. Furthermore, RANTES from epithelium and vascular endothelial cells in mucous membrane probably also plays a critical role in eosinophil infiltration of the nasal mucosa and the lower respiratory tract.

In the current study, we revealed RANTES production in human nasal epithelial cells (HNECs) and human mucosal microvascular endothelial cells (HMMECs), obtained by isolation from nasal mucosa and subsequent continuous subcultures. We also demonstrated that the amount of RANTES production induced by TNF- α and IFN- γ was higher in HNECs and HMMECs obtained from patients with nasal allergy than in those from patients without allergy.

METHODS

mAB and cytokines

TNF- α , IFN- γ , and IL-1 β were purchased from Genzyme, Boston, Mass.

Collection of inferior nasal mucosa

Turbinal mucosal samples were collected from seven patients with perennial allergy who were sensitized to house dust or mites (three male and four female patients, mean age 29.9 ± 3.7 years) and seven nonallergic patients with hypertrophic rhinitis (three male and four female patients, mean age $27. \pm 4.0$ years). The patients in both groups had moderate to severe nasal obstruction, judged by Okuda's criteria.²⁷ Diagnosis of nasal allergy was made on the basis of a typical history of perennial nasal allergy symptoms, eosinophilia in nasal smears, and a positive RAST result for house dust (HD) or mites (Table I). The patients without allergy were selected from a patient population that met the following criteria: absence of eosinophilia in nasal smears; RAST result negative for HD, mites, Japanese cedar pollen, ragweed pollen, and mugwort pollen; IgE level not higher than 300 U/ml; and symptoms of nasal hyperreactivity, such as sneezing and watery nasal discharge. Inferior nasal mucosal samples were obtained at the time of surgery for submucosal resection.

Purification of epithelial cells and endothelial cells

The HNECs and HMMECs were isolated from human nasal mucosa by using a method of Altman et al.²⁸ and Fukuda et al.,²⁹ respectively. The HNECs were cultured in MCDB153 (GIBCO RBL, Life Technologies, N.Y.) medium containing 10 ng/ml epithelial cell growth factor and 1% bovine pituitary body extract. HMMECs were cultured in MCDB107 medium

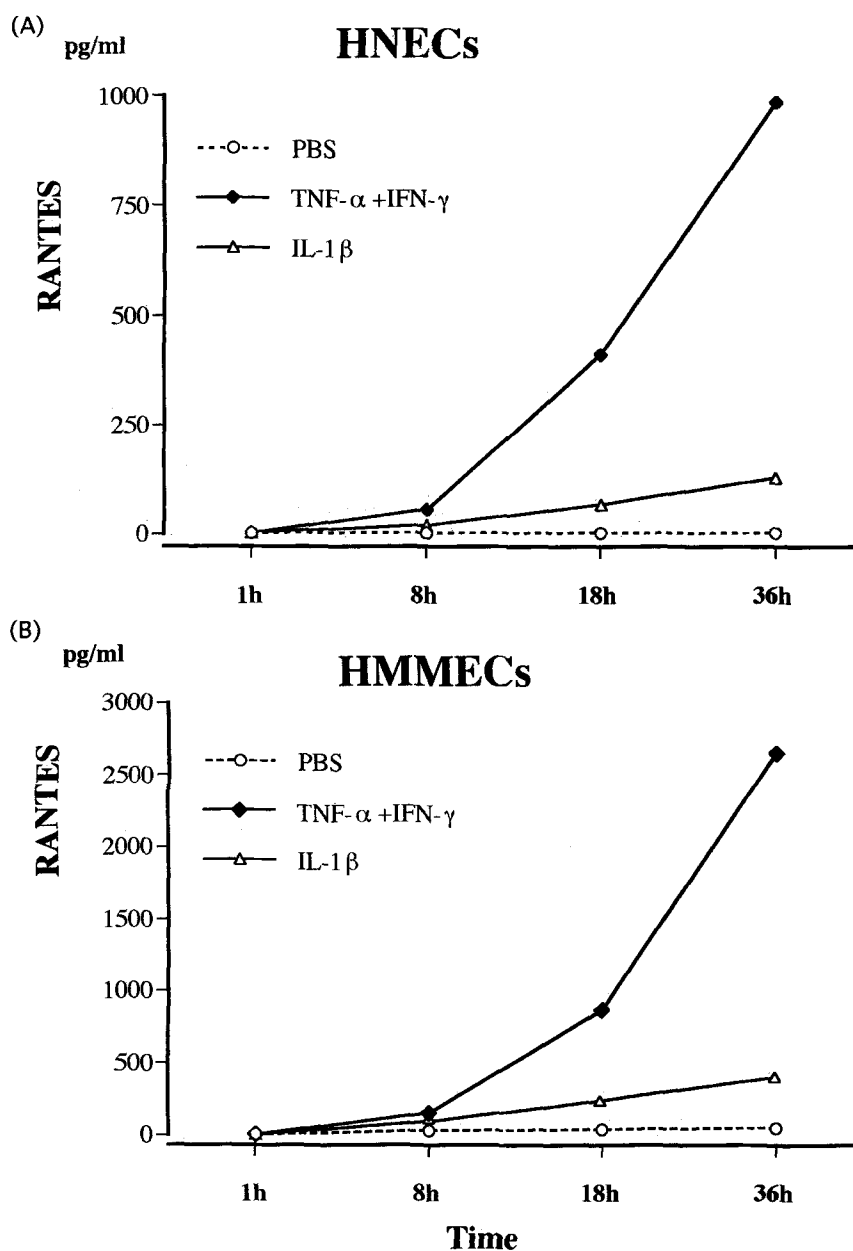


FIG. 1. Kinetics of RANTES release on the combined stimulation by TNF- α and IFN- γ , or the stimulation by IL-1 β . Cells were grown to confluence in 24-well culture wells coated with collagen type I and were stimulated for 1 to 36 hours by a combination of TNF- α (10 ng/ml) and IFN- γ (100 U/ml), or by IL-1 β (10 ng/ml). The results are representative of three different experiments.

(GIBCO) containing 12.5% fetal calf serum (FCS), 75 mg/ml endothelial cell growth supplement (Collaborative Research Inc, Bedford, Mass.). After the third passage, the cells were seeded ($10^5/\text{cm}^2$) in a collagen type I coated 24-well culture plate.

Cell culture

The HNEC and HMMEC monolayers were checked using phase-contrast microscopy. When at confluence,

the HNECs and HMMECs were washed three times with phosphate-buffered saline solution (PBS) containing 10 $\mu\text{g}/\text{ml}$ gentamicin (Sigma, St. Louis, Mo.) and were stimulated either by IL-1 β or by the combination of TNF- α and IFN- γ for 1 to 36 hours at 37° C in 5% carbon dioxide. The supernatants were centrifuged and stored frozen at -80° C until use. The cells were harvested by trypsinization and immediately frozen in liquid nitrogen until use.

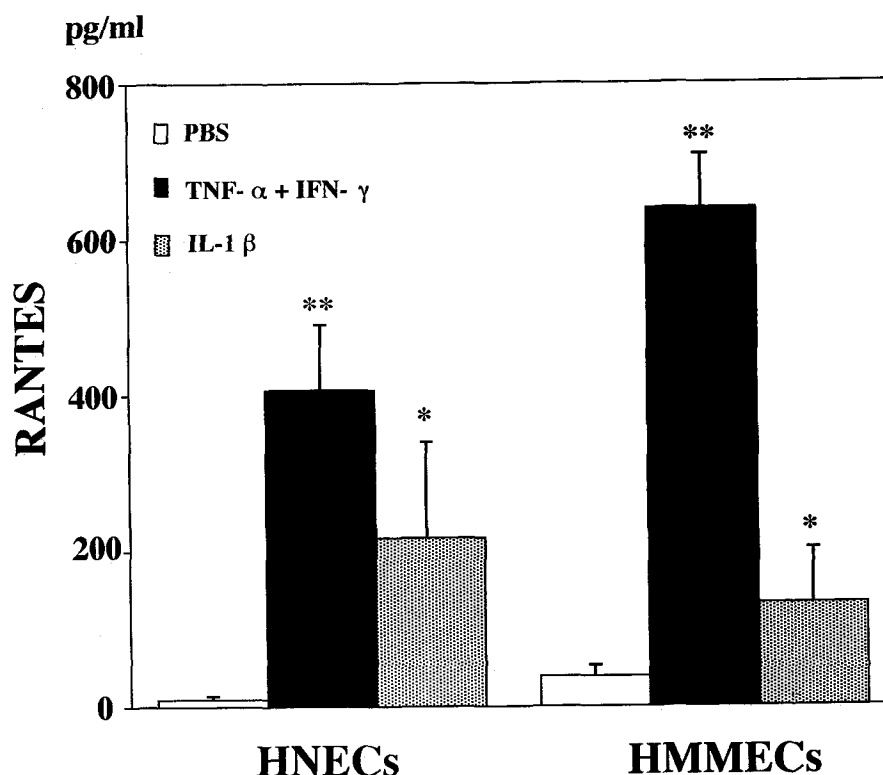


FIG. 2. Comparison of the RANTES release from HNECs and from HMMECs induced by the combined stimulus TNF- α and IFN- γ , or by IL-1 β . Bars represent means $\pm$ SE (n=14). * p < 0.05; ** p < 0.01 versus PBS.

The expression of RANTES messenger RNA (mRNA) in HNECs and HMMECs was determined after the addition of cytokines by using a reverse transcription-polymerase chain reaction (RT-PCR) and Southern blot analysis.

Detection of cytokine protein

Concentrations of RANTES in culture media were measured by using a RANTES sandwich ELISA assay purchased from Amersham, Arlington Heights, Ill. The sensitivity of the test was 2.5 pg/ml.

Extraction of total RNA and RT-PCR

RNA extraction, complementary DNA (cDNA) synthesis, and the PCR reaction were carried out as described elsewhere.^{30,31} Briefly, total RNA was extracted using the guanidium isothiocyanate cesium chloride method, and synthesis of cDNA was performed using a cDNA Synthesis Kit (Boehringer Mannheim, Mannheim, Germany). A cDNA equivalent of 500 ng total RNA was amplified in a 50 μ L reaction volume containing 200 mM each of d-nucleoside triphosphates, 3 units of Amplitaq DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn.), 25

pmole of 5' primer and 3' primer, and a $\times 10$ PCR reaction buffer (1.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, 100 mg of gelatin, pH 8.3). The PCR was performed in a DNA thermal cycler (Quick Thermo Systems, Nippon Genetics, Tokyo, Japan) for 29 cycles under the following conditions: (1) predenaturation at 94° C for 5 minutes, (2) denaturation at 94° C for 2 minutes, (3) annealing at 55° C for 2 minutes, (4) extension at 72° C for 2 minutes, and (5) denaturation at 94° C for 2 minutes. Steps (3) to (5) were repeated for 29 cycles, then reextension was made at 72° C for 5 minutes.

The sequences of the 5' sense primers and the 3' antisense primers, respectively, used in this study were as follows³²: RANTES, 5'-ATATTCCTCGGACACCA-CAC-3' and 5'-CACGTCCAGCCTGGGGAAGG-3' (product size 370 base pairs [bp]); β -actin, 5'-CTTCTA-CAATGAGCTGCGTG-3' and 5'-TCATGAGGTAG-TCAGTCAGG-3' (product size 305 bp).

Southern blot analysis

The specificity of the PCR product for RANTES was determined by Southern blot analysis.³³ A portion (10 μ L) of the PCR product was electrophoresed with 3%

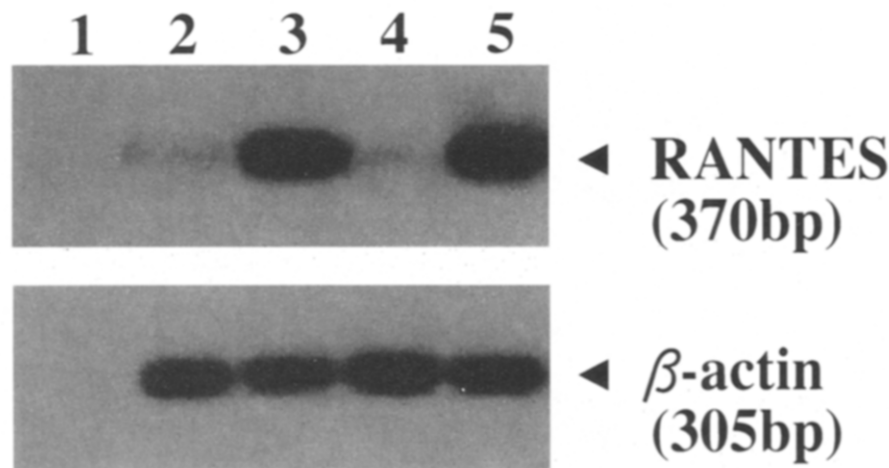


FIG. 3. Effect of $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ on the expression of RANTES mRNA in HNECs or HMMECs. This figure is from one experiment, which is representative of three others performed with similar results. Total RNA was isolated from HNECs or HMMECs after incubation for 6 hours with the combined stimulation by $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$. PCR-assisted Southern blot analysis was performed as described in text and its expression was determined with a densitometer. Lane 1, Sterilized water was used as a negative control; Lane 2, HNECs unstimulated; Lane 3, HNECs stimulated with $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$; Lane 4, HMMECs unstimulated; Lane 5, HMMECs stimulated with $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$. Amounts of β -actin were adjusted to be almost equal between samples.

agarose gel, and after denaturation with alkaline solution, was transfected to a nylon membrane filter for Southern blotting. After transfer, the DNA was fixed by UV irradiation, and hybridization was performed using a human RANTES probe that was labeled with ^{32}P using random primed DNA labeling. The conditions for hybridization were prehybridization at 42°C for 2 hours and hybridization at 42°C for 1 day. The wash conditions were $2 \times$ saline sodium citrate ($1 \times \text{SSC} = 150 \text{ mM NaCl}$ and $15 \text{ mM trisodium citrate}$, $\text{pH} = 7.0$) and 0.1% sodium dodecyl sulfate (SDS) at room temperature for 20 minutes, performed twice, and $0.2 \times \text{SSC}$ and 0.1% SDS at 52°C for 15 minutes, performed three times. X-ray film was then exposed to the product for 1 hour. The sequences of the internal probes used in this study were as follows: RANTES, 5'-ACACCAGTG-GCAAGTGCTCCAACCCAGCAG-3'; β -actin; cDNA.

Statistical assessment

The results were expressed as mean $\pm$ standard error of mean. Statistical significance was determined by the paired Wilcoxon test, with $p < 0.05$ considered to be statistically significant.

RESULTS

Cultured HNECs and HMMECs express RANTES mRNA and produce RANTES protein when stimulated with a combination of $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$, or with $\text{IL-1}\beta$

Unstimulated HNECs and HMMECs produced small amounts of RANTES. In time-course experi-

ments, RANTES production continued to increase at least 36 hours after the $\text{IL-1}\beta$ stimulation or the combined stimulation by $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$ (Fig. 1).

After the combined stimulation by $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$, the HNECs and HMMECs dramatically produced RANTES, as previously observed in HUVEC²⁰ and bronchial epithelial cell line BEAS-2B.²¹ The amounts of RANTES in the culture medium of unstimulated HNECs and HMMECs after the 18-hour incubation were 9.6 ± 4.3 and $38.7 \pm 13.8 \text{ pg/ml}$, respectively (Fig. 2). These amounts increased to 407 ± 83.7 and $640.2 \pm 65.8 \text{ pg/ml}$ after the combined stimulation by $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$. The differences were statistically significant ($p < 0.01$).

In HNECs and HMMECs, $\text{IL-1}\beta$ also increased RANTES production to a lesser extent (216.6 ± 159.5 and $132.3 \pm 70.2 \text{ pg/ml}$, respectively) compared with the combined stimulation by $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$.

To evaluate whether the expression of RANTES mRNA was accompanied by RANTES secretion, we tried to detect RANTES mRNA by using RT-PCR and Southern blot analysis, as shown in Figure 3. Unstimulated HNECs and HMMECs expressed a small amount of RANTES mRNA. After 6 hours of combined stimulation by $\text{TNF-}\alpha$ and $\text{IFN-}\gamma$, the HNECs and HMMECs showed enhancement of RANTES mRNA levels. The RANTES mRNA levels reached their peak within

the first 6 hours of stimulation and gradually returned to base levels within the following 18 hours (data not shown).

Comparison of the TNF- α and IFN- γ induced RANTES release in HNECs and HMMECs obtained from patients with nasal allergy with those from patients without allergy

The amounts of RANTES production induced by TNF- α and IFN- γ in HNECs and HMMECs obtained from patients with allergy were 605.3 ± 130.9 pg/ml and 734.0 ± 49.9 pg/ml, respectively (Fig. 4). These amounts were significantly higher than the amounts of RANTES produced in HNECs and HMMECs obtained from patients without allergy (268.7 ± 58.9 pg/ml, 546.0 ± 39.7 pg/ml, respectively).

DISCUSSION

Production of RANTES initially was confirmed in T cells⁴ but was also demonstrated in various kinds of other cells.^{10, 16-19} Recently its production by vascular endothelium and epithelial cells was revealed, and these investigations were performed using HUVEC or cell lines.^{20, 21} In the current study, we found that HNECs and HMMECs that had been isolated from human nasal mucosa and subsequent continuous subcultures produced RANTES on stimulation with the combination of TNF- α and IFN- γ , or on stimulation with IL-1 β . It was reported that migration and activation of eosinophils were induced by RANTES,^{11, 12} whereas it appeared that enhancement of transendothelial migration in the extracellular spaces¹⁴ may indirectly have fostered eosinophil infiltration.

Among various chemical mediators released from eosinophils, PAF and leukotrienes were found to cause dilation of capacitance vessels and induce leakage of plasma components from post-capillary veins, resulting in swelling of the nasal mucosa.¹⁻³ Furthermore, PAF stimulated histamine-induced swelling of nasal mucosa whereas leukotriene D₄ and eosinophil cationic protein (ECP) augmented histamine-mediated secretion of nasal discharge.^{1, 2} Recently, however, it was revealed in some experiments on the mucous membrane of the hamster cheek pouch that among the eosinophil granular proteins, major basic protein and eosinophil cationic protein directly caused vascular leakage.³⁴ As in the hamster, granular proteins in humans may be directly responsible for swelling of the nasal mucosa. Eosinophils migrate from capillary vessels in the superficial layer of the nasal mucous basement membrane to its inherent

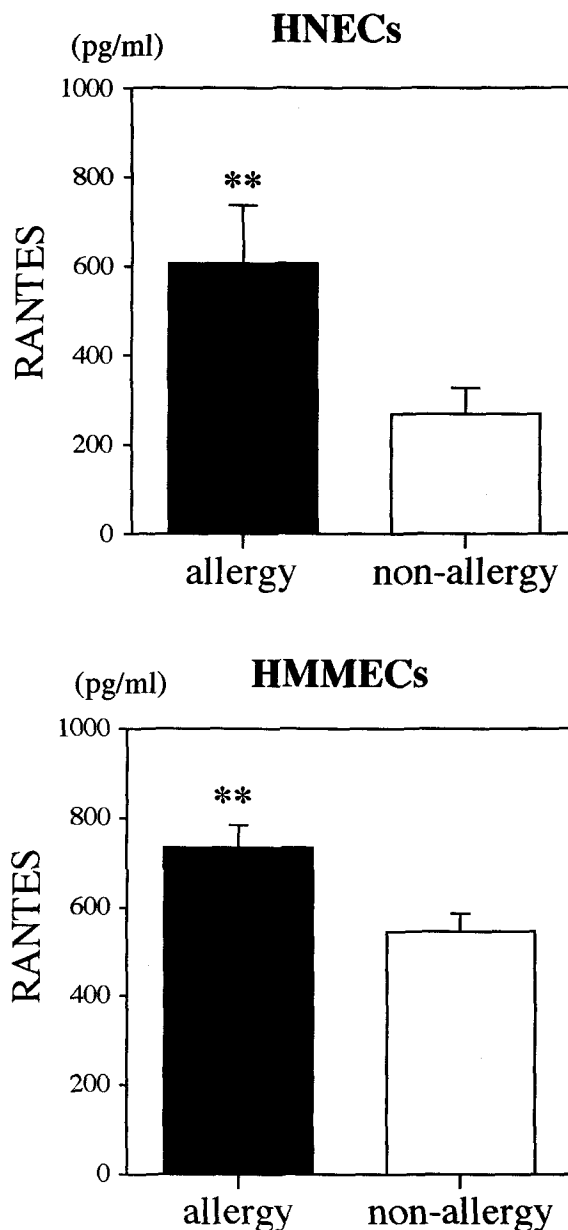


FIG. 4. Comparison of RANTES production induced by TNF- α and IFN- γ in HNECs and HMMECs obtained from patients with nasal allergy (n=7) and patients without allergy (n=7). Bars represent means $\pm$ standard error. ** $p < 0.01$ versus patients without allergy.

layer; during these migration processes, RANTES derived from vascular endothelial cells coordinates with PAF, LTB₄, C5a, and IL-5 to play a role as the eosinophil migrating factor.^{10, 11, 22-26} Coupled with this effect, RANTES also induces transendothelial migration in the extracellular spaces,¹⁴ leading to enhancement of eosinophil infiltration as a whole. After extravascular migration, eosinophils are further recruited to epithelial cells. During such serial

migration, epithelium-derived RANTES probably plays a key role as the eosinophil migrating factor.

Both TNF- α and IFN- γ , which cause RANTES production in the epithelium, might be generated mainly in T lymphocytes. The autoregulatory role of eosinophils, however, also may be involved in the production of these cytokines.

In recent years, production of cytokines has been successively substantiated in eosinophils through their autocrine mechanisms; production of granulocyte-macrophage colony-stimulating factor, IL-3, TNF- α , transforming growth factor beta, IL-5, IL-6, and IL-8 have been reported so far.³⁵⁻⁴² These cytokines are extensively associated with autoregulatory roles inherent to eosinophils, including differentiation, proliferation, migration, activation, and degranulation. These findings indicate that eosinophils exert regulatory effects on allergic inflammation besides playing their own autoregulatory role through cytokine production and their release. In coordination with T lymphocytes, eosinophils induce elevation of the tissue levels of both TNF- α and IFN- γ and stimulation of RANTES production from epithelial cells, eventually fostering further eosinophil migration.

At least in the peripheral blood of patients with nasal allergy, the possibility of autoregulatory production and release of RANTES by eosinophils is lower. Even after the costimulation of eosinophils by TNF- α and IFN- γ , RANTES production by eosinophils was not observed. The same was true with IL-1 β stimulation (data not shown).

In this study, we also demonstrated that the TNF- α and IFN- γ induced production of RANTES in HMMECs and HNECs obtained from patients with nasal allergy was higher than in HMMECs and HNECs obtained from patients without allergy. It is noteworthy that the response to TNF- α and IFN- γ differed between the nasal mucosa of patients with nasal allergy and the mucosa of patients without allergy. This fact might explain, in part, the predominant eosinophil accumulation in nasal mucosa of patients with nasal allergy. The nasal mucosa of such patients is characterized by hyperreactivity to various forms of stimulation. Hyperreactivity is induced by various factors, such as an increase in the number of effector receptors, increased affinity, and exposure of sensory nerves caused by epithelial decudation. For instance, nasal secretion is increased to a certain extent in patients with nasal allergy when the nasal mucosa is stimulated with methacholine. This phenomenon can be explained by the fact that patients with nasal allergy exhibit an increased number of muscarinic receptors in nasal mucosa.⁴³ It thus re-

mains to be determined whether the number and the affinity of RANTES receptors in HMMECs and HNECs of nasal mucosa obtained from patients with nasal allergy differs from that of patients without allergy.

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