

RANTES Induces Nasal Mucosal Inflammation Rich in Eosinophils, Basophils, and Lymphocytes *In Vivo*

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RANTES is a CC chemokine that causes chemotaxis of eosinophils, basophils, and lymphocytes *in vitro*. The objective of this study was to investigate the effect of RANTES on the influx of inflammatory cells into the nasal mucosa of 12 allergic patients. In the first phase, each patient was challenged with RANTES or diluent on two subsequent days. RANTES caused a significant ($p < 0.05$) influx of eosinophils as compared with the diluent. The number of eosinophils were $5,548 \pm 1,532/\text{ml}$ and $462 \pm 206/\text{ml}$ after RANTES and diluent challenge, respectively, at the peak of the response at 2 h. There was also a significant influx of metachromatic cells and lymphocytes, but not monocytes, neutrophils, or epithelial cells after RANTES challenge. In the second phase, the patients were first challenged with an allergen and 24 h later, challenged with RANTES or diluent. In the allergen-primed mucosa RANTES induced a significantly higher influx of eosinophils, basophils, and lymphocytes. Further, RANTES caused migration of monocytes and neutrophils, and shedding of epithelial cells. The influx of the inflammatory cells was associated with symptoms of rhinitis. We conclude that RANTES induces a clinically symptomatic inflammatory response *in vivo* by causing chemotaxis of eosinophils, basophils, and mononuclear cells. Kuna P, Alam R, Ruta U, Gorski P. RANTES induces nasal mucosal inflammation rich in eosinophils, basophils, and lymphocytes *in vivo*.

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Chemokines represent a family of low-molecular-weight (8 to 10 kD) cytokines that are predominantly chemotactic for inflammatory cells (1, 2). This family is divided into four subfamilies based upon the configuration of the N-terminal conserved cystein residues: (1) The CXC subfamily is characterized by the presence of two N-terminal conserved cysteine residues separated by a single amino acid. (2) The CC subfamily contains two conserved cysteines in juxtaposition. (3) The C subfamily has only one cysteine in the conserved region and is designated as the C chemokine subfamily (3). (4) The members of the CX3C subfamily are membrane-anchored glycoproteins with a C-terminal lectin-like sequence and an N-terminal chemokine-like structure. The chemokine has two cysteine residues separated by three nonconserved residues (4).

RANTES is produced by T cells (5). Subsequently platelets have been shown to contain RANTES, and release the chemokine upon stimulation with thrombin (6). Other important sources of RANTES include airway epithelial cells (7, 8), fibroblasts (9), endothelial cells (10), and eosinophils (11, 12). RANTES induces directed migration of CD4, CD45 RO+

T cells and monocytes (13), chemotaxis and activation of eosinophils (6, 14, 15), and transendothelial migration of eosinophils *in vitro* (16). RANTES causes an eosinophilic influx into the skin of dogs (17) and humans (18) when injected *in vivo*. RANTES also activates basophils and induces histamine release (19). Further, RANTES and macrophage inflammatory protein-1 α (MIP-1 α) seem to stimulate IgE+ tonsillar B cells for IgE production (20). The foregoing activity puts RANTES in a central position to promote IgE-driven eosinophil-rich inflammation. The activity of RANTES in human airways is unknown. The objective of this study was to investigate whether RANTES induces an eosinophil-rich inflammation in the nasal mucosa of allergic patients.

METHODS

Twelve allergic patients (5 female, 7 male, 27 to 51 yr old, average 35.33 ± 3.35 yr) were recruited from the outpatient clinic of the Department of Occupational Medicine and the Division of Pneumonology and Allergology, Lodz, Poland. Allergic status was evaluated by skin prick testing with a panel of 12 allergens including grass pollen, rye pollen, tree pollen, *Dermatophagoides pteronyssinus*, feathers, cat dander, dog dander, and the molds *Cladosporium*, *Aspergillus fumigatus*, *Alternaria alternata*, flour, and *Penicillium* (Allergopharma Joachim Ganzer KG, Hamburg, Germany). Total IgE level was determined in all patients and was 518 ± 114 IU. Ten patients were allergic to *D. pteronyssinus*, eight patients to grass pollen, six patients to tree pollen, five to flour, and two to *Penicillium*. Sensitive patients had a history of allergic rhinitis occurring during the grass and tree pollen season. Patients, allergic to flour and penicillinum, had a history of rhinitis after exposure to these allergens although none were symptomatic at the time of the study. Patients were prescreened for development of aller-

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gen-induced rhinitis symptoms (sneezing, rhinorrhea, and nasal congestion). All patients refrained from taking oral antihistamines and topical nasal medication for a period of 14 d before and during the study. Because of the long half-life of astemizole, volunteers receiving this drug were excluded from the study. Patients with concurrent asthma, pregnancy, severe systemic illness, and recurrent nasal bleeding were excluded. RANTES was purchased from Pepro Tech Inc., Rocky Hill, NJ.

Study Design

The study was performed in two phases as shown in Figure 1. In the first phase, patients were challenged with RANTES or diluent for RANTES (phosphate-buffered saline [PBS]/0.3% human serum albumin [HSA]) on two different days. In the second phase, patients were first challenged with a skin test–positive allergen, and 24 h later, challenged with diluent or RANTES in a random manner. One week later the allergen challenge was repeated and the protocol was completed with diluent or RANTES challenge.

Study Protocol

On the study day, patients underwent a baseline nasal lavage with saline. The lavage fluid was centrifuged and the total and differential counts of the cell pellet were obtained. After 10 min, 50 μ l of diluent or RANTES (5 μ g or otherwise mentioned) were instilled in the lower part of the right inferior turbinate. Patients were asked to record symptom scores according to the following protocol. Sneezing was scored as follows: none—0 points; 1–3/10 min—1 point; 3–6/10 min—2 points; more than 6/10 min—3 points. Rhinorrhea, nasal congestion, postnasal drip, and frontal headache were scored by the subjective assessment as follows: none—0 points; mild—1 point; moderate—2 points; severe—3 points. A positive clinical challenge was defined as a total of more than 3 points. Allergen challenge was performed with a predetermined dose previously shown to induce an immediate allergic response (sneezing, rhinorrhea, and nasal congestion) in the study patients. The study protocol was reviewed and approved by the regional ethics committee. An informed consent was obtained from each patient.

Allergen extracts were purchased from Allergopharma Joachim Ganzer KG (Hamburg, Germany). The technique of allergen challenge has been described elsewhere (21). In brief, 0.1 ml of an allergen extract was instilled from a tuberculin syringe. Nasal lavage with saline was performed by the “nasal pool” technique described by Greiff and coworkers (22) 10 min before, and 30 min, 2 h, 4 h, and 24 h after the challenge. The “nasal pool” device (10 ml syringe with closely fitting nostril), filled with 6 ml saline, was inserted in the nasal cavity for 5 min and then recovered. The volume of nasal fluid recovered was 3.84 ± 0.09 ml (mean \pm SEM). A centrifugation (10 min at $400 \times g$) of the saline washing separated the cell pellet and the supernatant. The obtained sediment was washed in sterile PBS (Sigma) and then suspended in 1.0 ml of RPMI 1640 (Sigma). After staining the cells with Hansel’s stain the total number of eosinophils and basophils (metachromatic cells) was counted with the use of a Fuchs–Rosenthal chamber allowing determination of the number of cells in 1.0 ml of recovered fluid. The sample was further centrifuged at $200 \times g$, and transferred onto a slide stained by the Giemsa method. A minimum of 200 cells was counted per smear to obtain a differential cell count. Cells were classified as epithelial cells, eosinophils, neutrophils, metachromatic cells (mostly basophils), lymphocytes, and monocytes.

Statistical Analyses

Paired data were analyzed by Wilcoxon’s signed rank test using the statistical program StatWorks (Cricket Software, Philadelphia, PA). The results are presented as mean \pm SEM. All statistical analyses are considered significant at $p < 0.05$.

RESULTS

Analyses of Symptom Scores

The challenge of unprimed mucosa with RANTES did not significantly induce symptoms of rhinitis as compared with diluent (Figure 2). However, we observed mild symptoms of rhinitis in three subjects after RANTES challenge. The result of RANTES challenge of the allergen-primed nasal mucosa was

STUDY DESIGN

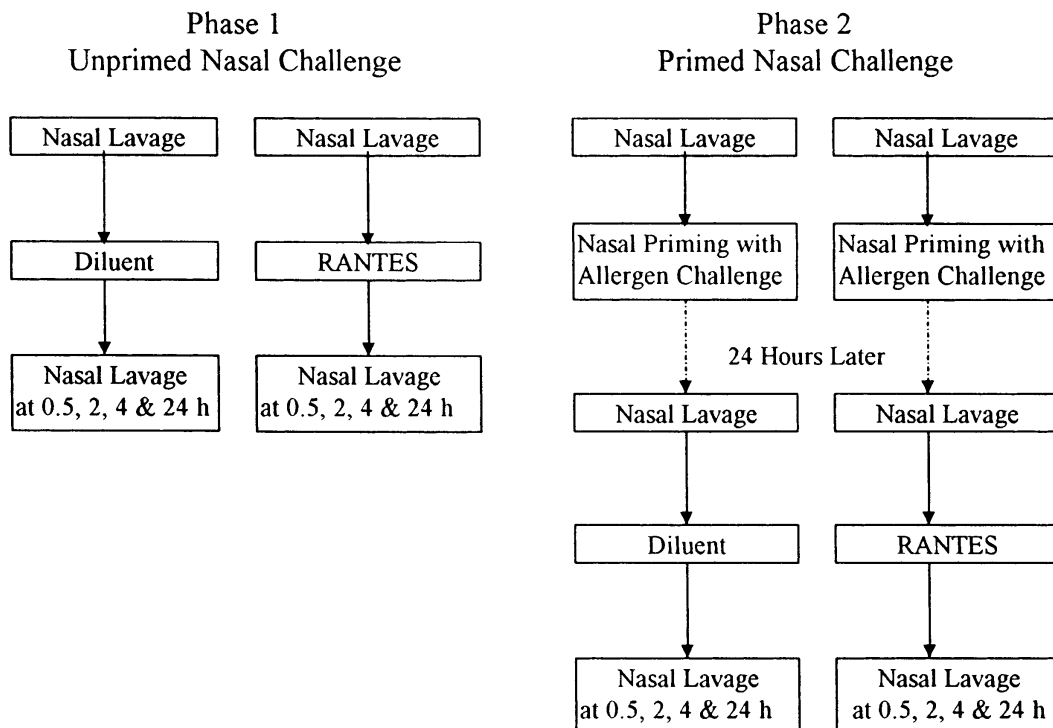


Figure 1. RANTES challenge of unprimed and allergen-primed nasal mucosa of 12 allergic patients.

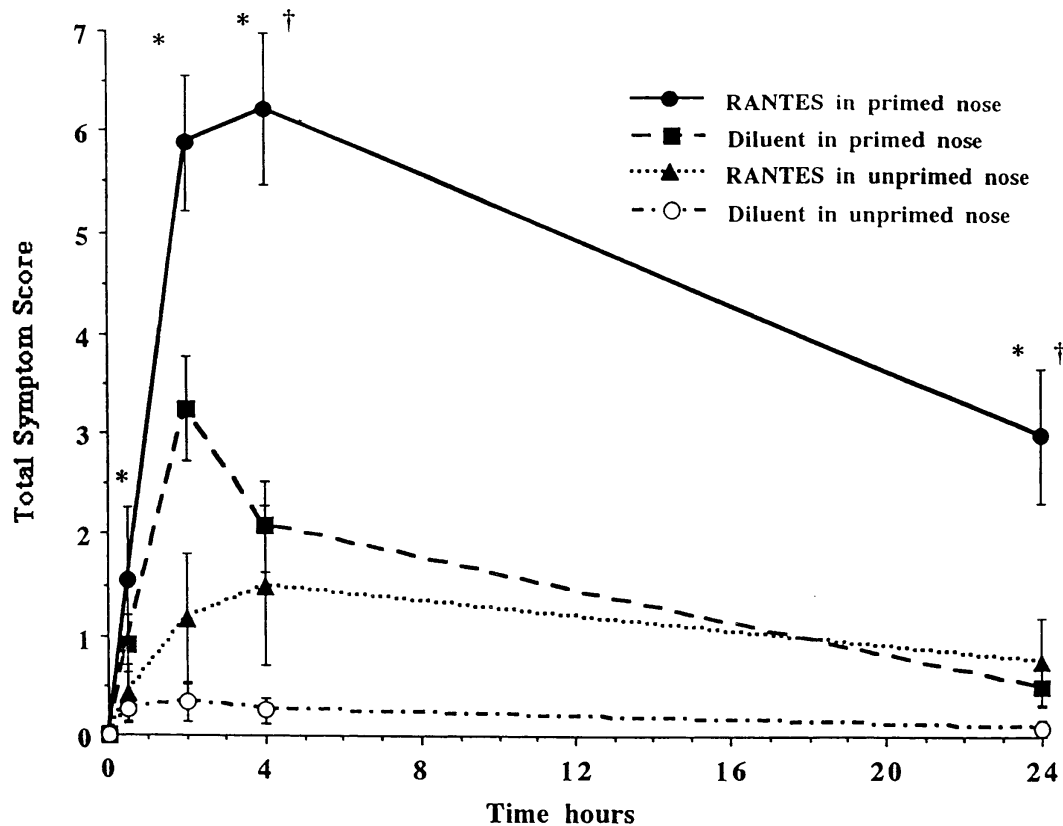


Figure 2. Composite symptom scores after RANTES and diluent (PBS/HSA) challenge of the primed (post-allergen provocation) and unprimed nasal mucosa. The results are mean \pm SEM ($n = 12$) at each time point. Mean symptom scores after RANTES treatment of primed nose were significantly ($*p < 0.05$) different from those of unprimed nose at 0.5, 2, 4, and 24 h. The symptom scores between RANTES and diluent challenge of primed nose differed significantly ($†p < 0.05$) at 4 and 24 h.

completely different. RANTES elicited a significant ($p < 0.05$) increase in rhinitis symptoms as compared with diluent. Eight of 12 donors reported symptoms of rhinitis. Three subjects also reported symptoms of conjunctivitis. The difference in RANTES-induced symptom scores between primed and unprimed mucosa was also significant ($p < 0.05$).

Analyses of Cellular Influx in Unprimed Nasal Mucosa

The diluent challenge did not significantly increase eosinophil count at any time point, rather a significant decrease occurred at 0.5, 2, and 4 h (Figure 3A). The number of metachromatic cells and lymphocytes were also lower at 0.5, 2, and 4 h after the diluent challenge as compared with the baseline. This decrease is likely a washout effect of repeated saline lavage of the nasal mucosa. RANTES significantly increased the number of eosinophils from $1,876 \pm 317$ cells/ml at the baseline to $2,203 \pm 234$, $5,548 \pm 1,532$, and $3,446 \pm 754$ cells/ml, at 0.5, 2, and 4 h, respectively (Figure 3A). The difference between RANTES and diluent challenge in regard to eosinophil influx was significant at 0.5, 2, and 4 h ($p < 0.05$). The number of metachromatic cells and lymphocytes also increased over the baseline at 2, 4, and 24 h (Figure 3A). The difference between RANTES and diluent challenge was statistically significant ($p < 0.05$) at 0.5, 2, 4, and 24 h for both cell types. Although we did not definitively characterize metachromatic cells, light microscopic examination revealed cells with bilobed nucleus indicating that most of these cells were basophils. RANTES also caused a significant ($p < 0.05$) increase in monocyte in-

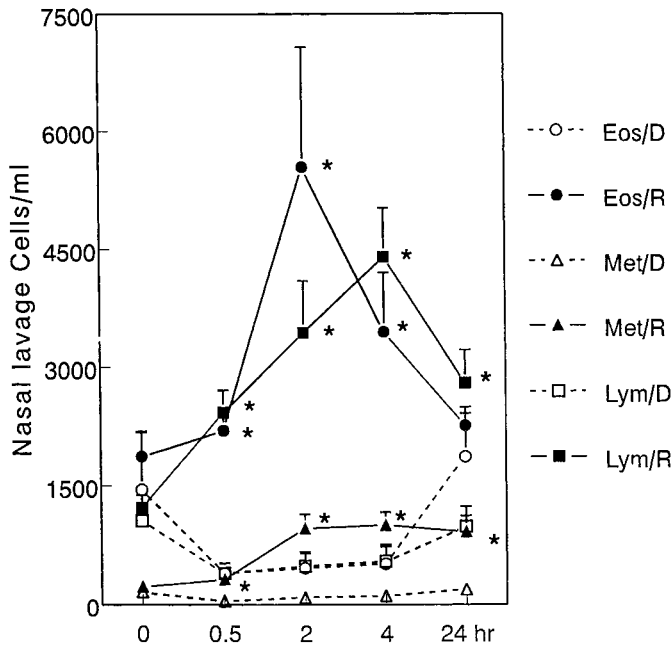
flux at 2 and 4 h as compared with diluent (Figure 3B). In contrast, there was no increase in neutrophils and epithelial cells at any time points (Figure 3B).

Cellular Influx in Allergen-primed Nasal Mucosa

In the second phase of the study patients were first challenged with the sensitizing allergen. The symptoms of rhinitis due to allergen challenge subsided in 24 h (Figure 2, baseline symptoms in primed mucosa). Then, the patients were challenged with RANTES or diluent. The diluent caused a significant increase in lymphocytes but not other cells over the baseline in the allergen-primed mucosa at 2, 4, and 24 h. RANTES increased the influx of eosinophils, metachromatic cells, lymphocytes, and monocytes significantly over the baseline ($p < 0.05$) at each time point except for monocytes at 24 h (Figure 4A and B). In contrast to unprimed mucosa, RANTES challenge of the allergen-primed mucosa caused a significant increase in epithelial cells and neutrophils (Figure 4B).

When compared with diluent, RANTES caused a significant ($p < 0.05$) increase in eosinophils, metachromatic cells, lymphocytes, monocytes, and epithelial cells at all time points studied except eosinophils at 0.5 h and monocytes at 24 h (Figure 4A and B). The peak influx of eosinophils, metachromatic cells, lymphocytes, and epithelial cells occurred at 4, 4, 2, and 24 h, respectively. The number of eosinophils at the peak of inflammatory response at 4 h were $19,200 \pm 9,288$ /ml and $1,134 \pm 388$ /ml after RANTES and diluent, respectively. RANTES caused a statistically significant influx of neutrophils only at 4 h.

A RANTES Challenge Without Priming



B RANTES Challenge Without Priming

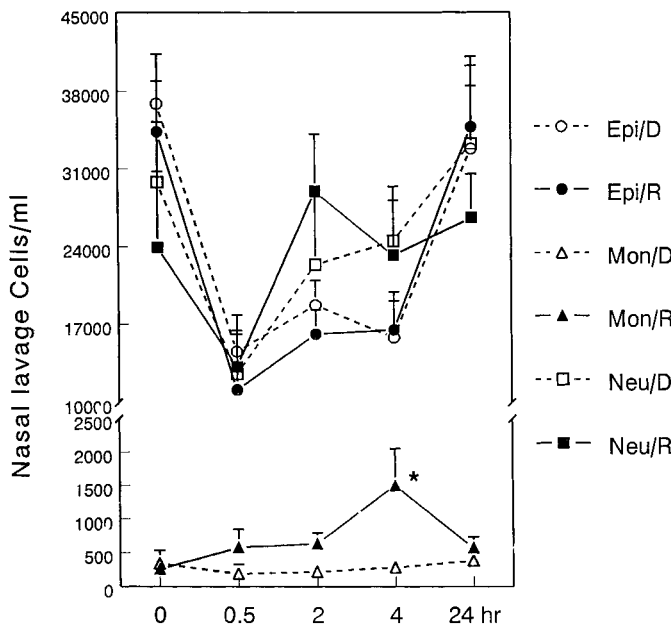
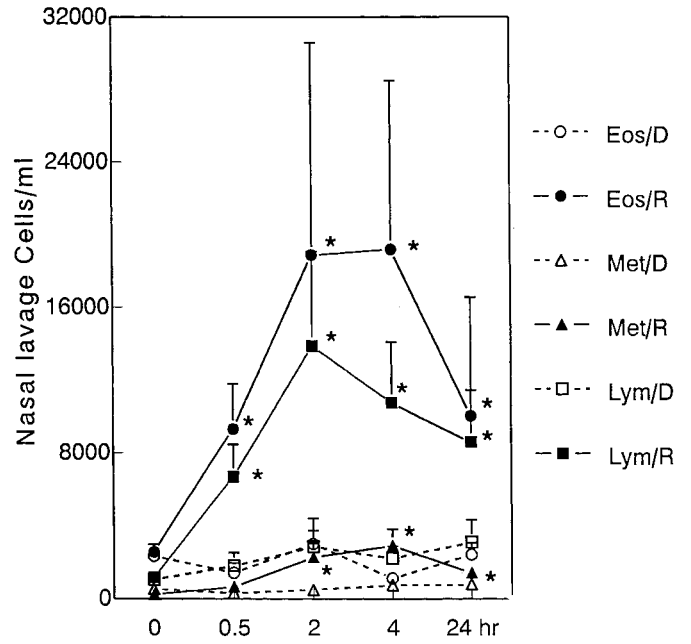


Figure 3. The effect of RANTES on the influx of inflammatory cells in the nasal mucosa. (A) The effect on eosinophils, metachromatic cells, and lymphocytes. Twelve allergic subjects were challenged with RANTES or diluent. Nasal lavage fluid was obtained at baseline, 0.5, 2, 4, and 24 h. An asterisk indicates a statistical difference between RANTES and diluent. The difference between RANTES and diluent challenge was significant ($p < 0.05$) at 0.5, 2, and 4 h for eosinophils. The counts for metachromatic cells and lymphocytes were significantly ($p < 0.05$) higher at all time points. Eos = eosinophils; Met = metachromatic cells; Lym = lymphocytes; D = diluent challenge; R = RANTES challenge. (B) The effect of RANTES on the recovery of monocytes, neutrophils, and epithelial cells from nasal lavage fluid. RANTES induced a significant increase in monocyte count at 4 h postchallenge. There was no increase in neutrophil or epithelial cell counts. Epi = epithelial cells; Mon = monocytes; Neu = neutrophils.

A RANTES Challenge After Priming



B RANTES Challenge After Priming

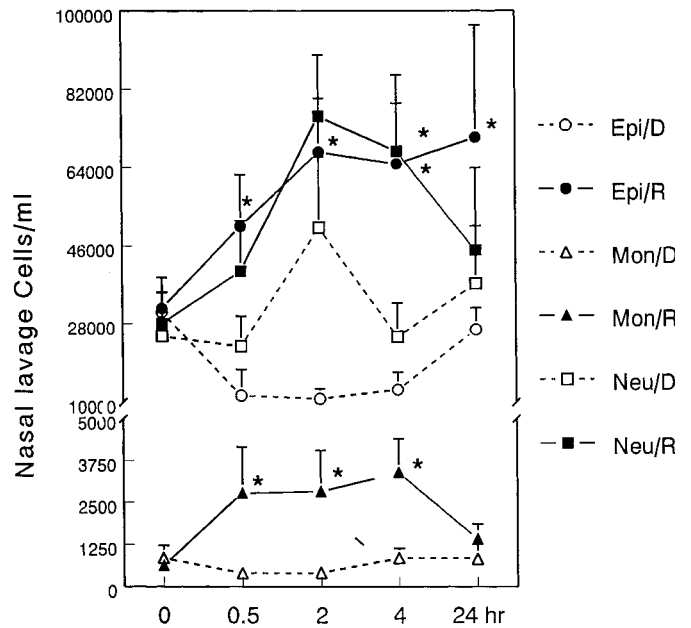


Figure 4. The effect of allergen priming on RANTES-induced influx of inflammatory cells in the nasal mucosa. (A) The effect on chemotaxis of eosinophils, metachromatic cells, and lymphocytes. Twelve patients were first challenged with allergens, and 24 h later they were challenged with RANTES or diluent. An asterisk indicates a statistical difference between RANTES and diluent. RANTES induced a significantly ($p < 0.05$) higher influx of eosinophils and lymphocytes at all time points. The increase in metachromatic cells was significantly higher ($p < 0.05$) at 2, 4, and 24 h. Eos = eosinophils; Met = metachromatic cells; Lym = lymphocytes; D = diluent challenge; R = RANTES challenge. (B) The response of monocytes, neutrophils, and epithelial cells to RANTES after allergen priming. RANTES induced a significant increase in monocyte count at 0.5, 2, and 4 h. In contrast, neutrophils were elevated at 4 h only. Epithelial cells were increased at all time points. Epi = epithelial cells; Mon = monocytes; Neu = neutrophils.

Comparison of Cellular Influx between Primed and Unprimed Mucosa

We compared the cell counts of primed and unprimed nasal lavage after RANTES challenge. The number of eosinophils in the primed nasal lavage was significantly ($p < 0.05$) higher at all time points. The number of metachromatic cells and epithelial cells were significantly ($p < 0.05$) higher at 0.5, 2, and 4 h, whereas the lymphocyte and monocyte counts were increased significantly ($p < 0.05$) at 0.5 and 2 h. The number of neutrophils was significantly ($p < 0.05$) elevated at 4 h.

Dose-dependent Inflammatory Response

In a separate set of experiments, four patients underwent serial challenge with increasing concentrations (1, 5, and 10 μg) of RANTES. The subjects were initially challenged with sensitizing allergens or diluent. Twenty-four hours later they were challenged with RANTES. The experiment was repeated twice within a period of 2 wk. The influx of eosinophils and lymphocytes is shown in Figure 5. RANTES induced a dose-dependent increase in eosinophils and lymphocytes in the nasal mucosa. Similar results were observed with metachromatic cells and monocytes (data not shown).

DISCUSSION

We demonstrated that RANTES caused an influx of inflammatory cells into nasal mucosa *in vivo*. The RANTES-induced inflammation was characterized by the presence of eosinophils, metachromatic cells, lymphocytes, and monocytes. The

peak influx of major inflammatory cells was early and occurred in 2 to 4 h. The inflammatory response to RANTES was much more pronounced in allergen-primed mucosa than that in unprimed mucosa. The influx of neutrophils and shedding of epithelial cells were noted only in primed mucosa. The latter finding confirms that neutrophils are not the primary target of RANTES. The influx of inflammatory cells following allergen priming was associated with clinical symptoms of allergic rhinitis. This is the first demonstration of induction of eosinophilic inflammation by RANTES in human upper airways.

In recent years a number of CC chemokines have been identified that cause eosinophil chemotaxis. They include RANTES, eotaxin (23), monocyte chemoattractant protein-3 (MCP-3) (24), MCP-4 (25), and MIP-1 α (26). Neutralizing antibodies against RANTES (27), MIP-1 α (27), and MCP-3 (28) have been shown to block eosinophilic inflammation in a mouse model of asthma. Further, the knockout mice for eotaxin have a reduced capability to mount an eosinophilic inflammation in the early period (e.g., at 18 h) after allergen challenge (29). However, the eosinophilic response is normal at 48 h indicating that other CC chemokines are also important. Eosinophils predominantly express the CC chemokine receptor CCR-3 which binds eotaxin, RANTES, MCP-3, and MCP-4 (30, 31). CCR-3 is also expressed on basophils. It is likely that CCR-3 plays a critical role in chemotaxis and activation of eosinophils in allergic inflammation. In addition to attracting eosinophils, RANTES induces chemotaxis of lymphocytes, monocytes, and basophils *in vitro*. One of the most common eosinophilic inflammatory diseases is allergic rhinitis. The mechanism of influx of eosinophils in this disorder is not clearly established. RANTES is a potential mediator of allergic inflammation. For this reason it is important to determine the inflammatory activity of RANTES in the nasal mucosa. The results of our study clearly establish that RANTES is capable of eliciting eosinophilic inflammation in the upper airways. Because there are development and pathophysiological similarities between upper and lower airways, our results may also be relevant to eosinophilic inflammation of the pulmonary airways.

We have instilled 5 μg of RANTES into one nostril of the study subjects. The tissue level of RANTES in acute inflammation is unknown at this time. RANTES is active at 10^{-9} to 10^{-7} M (~ 0.9 $\mu\text{g}/\text{ml}$) concentrations *in vitro*. For this reason a concentration of 5 μg was considered reasonable for an *in vivo* organ challenge. As mentioned previously, intradermal injections of RANTES were performed in dogs (17) and humans (18). Meurer and coworkers used 5 μg of RANTES (17) whereas Beck and coworkers used 4 μg of RANTES for human studies (18). Recently, the effect of intranasally applied interleukin-8 (IL-8) was studied by Douglas and coworkers (32). They used 2.5 μg of IL-8 in this study. When applied alone, IL-8 failed to elicit an inflammatory response. However, a pretreatment of nasal mucosa with histamine followed by IL-8 instillation induced a significant neutrophilic inflammation. A less impressive but significant eosinophilic influx was noted in atopic subjects.

We have shown that RANTES has a significant but moderate activity on unprimed nasal mucosa and causes influx of eosinophils, basophils, and lymphocytes. A more profound inflammatory activity of RANTES is observed in the allergen-primed mucosa. This is not a surprising observation. The degree of penetration of RANTES into the nasal mucosa is unknown. For an inflammatory response of leukocytes, a chemotactic gradient must be established between epithelium and mucosal post-capillary venules. The allergen-challenged mucosa may have increased permeability for RANTES owing to the action of his-

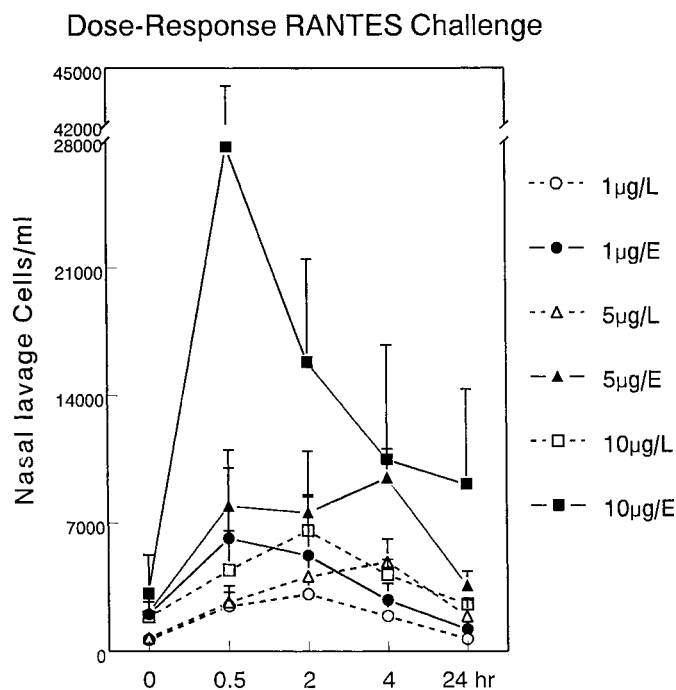


Figure 5. The dose-response relationship of RANTES challenge and the influx of leukocytes. Four allergic subjects were initially challenged with allergens, and 24 h later with RANTES. The experiment was repeated twice within 2 wk in order to perform challenges with three different concentrations of RANTES (1, 5, and 10 μg). RANTES caused a dose-dependent increase in eosinophils and lymphocytes in the nasal lavage. L = lymphocytes; E = eosinophils.

tamine or leukotrienes. The study of Douglas and coworkers with IL-8 (32) clearly demonstrated that the permeability of mucosa is important for chemokine action. Another explanation for the enhanced accumulation of leukocytes after allergen challenge is that the cells have already been mobilized into nasal mucosa as a result of allergen stimulation, and the action of RANTES is to attract these leukocytes into the airway lumen.

Other factors in the primed mucosa may have played a potentiating role in RANTES action. There is evidence of increased production of proeosinophilic (e.g., IL-5 and granulocyte-macrophage colony-stimulating factor [GM-CSF]) and proinflammatory (e.g., IL-1, tumor necrosis factor- α [TNF- α]) cytokines in the allergen-challenged mucosa (33, 34). IL-5 and GM-CSF prime inflammatory cells for increased chemotactic response to RANTES. IL-1 and TNF- α upregulate adhesion molecules on endothelium. The importance of the sequential action of various cytokines has recently been well documented. For example, the injection of eotaxin into the guinea pig skin does not cause an eosinophilic inflammation (35). However, if the animals first receive IL-5 intravenously followed by eotaxin locally, there is a significant eosinophilic infiltration at the site of the injection. We speculate that allergens stimulated the production of IL-5 and other cytokines in the nasal mucosa of our patients and the action of these priming cytokines facilitated the inflammatory response to RANTES. Interestingly, RANTES on its own appears to be capable of inducing eosinophilic inflammation in atopic patients as well as in other animals as mentioned previously (17, 18).

The allergic response of the human airways to allergens is usually biphasic. The immediate response is followed by a late-phase response that is characterized by the presence of eosinophils, basophils, and lymphocytes. The mechanism of influx of the foregoing inflammatory cells in the late-phase reaction is unclear. The challenge with allergens causes the secretion of cytokines into the nasal secretion of allergic patients. For example, elevated concentrations of IL-1, GM-CSF, IL-8, RANTES, and MIP-1 α have been detected in the allergen-challenged nasal lavage (33, 34). The peak level of most cytokines is coincidental with the peak of the late-phase reaction. Interestingly, the peak appearance of RANTES occurs at 3 h postchallenge which precedes the development of the late-phase allergic reaction. Because the chemotactic response of leukocytes requires a lag period, the appearance of RANTES 2 to 3 h before the late-phase reaction may imply a causal relationship. The concentration of RANTES is also elevated in the bronchoalveolar lavage fluid from patients with asthma (36–39). The eosinophil chemotactic activity of the lavage fluid is inhibited by an antibody against RANTES. In this study we show that RANTES induces nasal inflammation that is comprised of eosinophils, basophils, and lymphocytes. On the basis of these data we postulate that RANTES plays an important role in the pathogenesis of allergic inflammation.

RANTES is not necessarily specific for allergic inflammation. It has been detected in tissue specimens from other types of inflammatory diseases such as rheumatoid arthritis (9) and sarcoidosis (10). It is possible that a combination of various cytokines acting together or in sequence may determine the nature of inflammation. For example, the presence of IL-5 or interferon- γ may affect the nature of the inflammatory response to RANTES. The presence of IL-5 may promote the differentiation of eosinophils and prime them for chemotactic response (40) whereas interferon- γ may inhibit this process by blocking the production of IL-5 (41). In the latter instance RANTES may induce an inflammation that is dominated by lymphocytes and monocytes.

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