Allergens, IgE, mediators, inflammatory mechanisms

Cultured nasal polyps from nonatopic and atopic patients release RANTES spontaneously and after stimulation with phytohemagglutinin

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Background: Eosinophil infiltration of tissue is a hallmark of nasal polyposis in both nonatopic and atopic patients. These cells are thought to play a key role in the nasal polyp inflammatory process.
Objective: The objective of this study was to investigate whether cultured nasal polyps derived from nonatopic and atopic patients release RANTES both spontaneously and after phytohemagglutinin (PHA) stimulation.
Methods: Nasal polyps were obtained from 12 subjects (6 nonatopic and 6 atopic), cut into 2 to 3 mm large specimens, and cultured for 48 hours with or without PHA. RANTES was measured in the culture supernatant by ELISA (R&D Systems, U.K.).
Results: Immunoreactive RANTES was found to be present in the culture supernatant of nasal polyps derived from both nonatopic and atopic patients with no difference between the two groups (median: 3.8 vs 2.9 pg/mg/ml). On incubation with PHA, nasal polyps from both nonatopic and atopic patients released sevenfold and 11-fold greater amounts of RANTES than unstimulated samples. As determined by immunohistochemistry, RANTES was localized to the vascular endothelium in nasal polyps from both groups of patients.
Conclusions: This study demonstrates that cultured nasal polyps derived from both nonatopic and atopic patients release RANTES spontaneously and after PHA stimulation. This observation and the finding that RANTES is present in nasal polyp endothelial cells suggest that this chemokine may be an important mediator of eosinophil and lymphocyte recruitment in both nonatopic and atopic nasal polyposis.
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Key words: Nasal polyps, RANTES, eosinophils, lymphocytes, endothelial cells

Leukocyte infiltration of tissue is a hallmark of diseases such as bronchial asthma, allergic rhinitis, and atopic dermatitis. Nasal polyps have been associated with allergic respiratory diseases; however, their relationship with atopy has recently been questioned.[1, 2]

Abbreviations used
GM-CSF: Granulocyte-macrophage colony-stimulating factor
mAb: Monoclonal antibody
PHA: Phytohemagglutinin

Nasal polyps are grapelike structures, which usually arise in the ethmoid sinus mucosa and cause nasal obstruction. A central feature of this condition is chronic inflammation with inflammatory cells of which the most prevalent cell type is the eosinophil. Interestingly, eosinophils infiltrate nasal polyp tissue of both nonatopic and atopic patients.[2] Although the role of these cells in nasal polyposis is not well understood, eosinophils have the potential to promote epithelial proliferation, matrix generation, and tissue remodeling through the release of cytokines such as transforming growth factor-α, transforming growth factor-β, and granulocyte-macrophage colony-stimulating factor (GM-CSF).[3-5] Activated eosinophils may also cause tissue damage through the release of reactive oxygen metabolites and cytotoxic granule-derived proteins such as major basic protein and eosinophil cationic protein.[6, 7]

Recruitment of cells into the inflammatory site involves a series of events including adhesion to endothelial cells, transendothelial migration, and subsequent chemotactic movement. These processes are regulated by the release of inflammatory mediators and cytokines. In the case of eosinophils, GM-CSF, IL-5, and RANTES have assumed a particular significance. RANTES is a member of the chemokine supergene family, which can be divided into CXC, CC, and C branches[8, 9] according to their amino acid sequences. RANTES belongs to the CC branch, and its in vitro effects on eosinophils include chemotaxis, transendothelial migration, induction and production of reactive oxygen species, and release of eosinophil cationic protein.[10-12] Beck et al.[13] have recently reported the presence of RANTES immunoreactivity in nasal polyp biopsy specimens. In this study we have investigated whether cultured nasal polyps derived from both nonatopic and atopic subjects spontaneously release RANTES, and we have examined the immuno-

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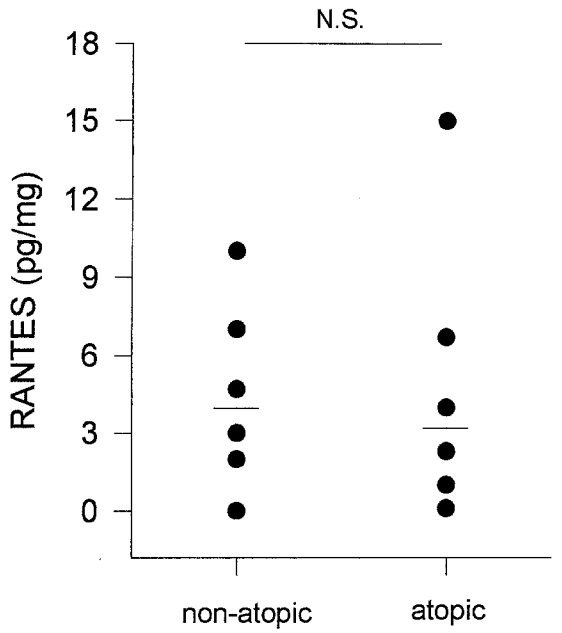

FIG. 1. Concentrations of RANTES immunoreactivity in culture supernatant of nonatopic and atopic nasal polyps. *Horizontal lines* represent median values.

localization and release of RANTES on stimulation with phytohemagglutinin (PHA), a specific T-cell activator.

METHODS

Twelve subjects (9 men; median age, 46 years; range, 26 to 65 years) were recruited for the study; their clinical characteristics are summarized in Table I. Six subjects were atopic, and six were nonatopic. Their atopic status was confirmed by skin prick testing with a series of common inhalant allergens (*Dermatophagoides pteronyssinus,* mixed grass pollen, dog, feathers, and cat dander; Hollister Stier, Elkhart, Ind.). Any resulting wheal that was more than 2 mm or larger than that produced by the histamine control was considered positive. None of the subjects were treated with corticosteroids or any other medication for at least 8 weeks before participation in the study. Three of the 12 subjects were sensitive to aspirin. The study was approved by the Southampton Hospitals and University Ethical Committee, and patients gave their written informed consent.

Sample processing

Culture of nasal polyp tissue. Nasal polyp tissues were obtained from subjects undergoing polypectomy for treatment of nasal obstruction. After washing with culture medium (RPMI-1640; Gibco, Paisley, U.K.), nasal polyps were cut into 2 to 3 mm large specimens and cultured in 0.5 ml of media, either with or without 2 μg/ml of PHA (this concentration was determined in control experiments [data not shown]), at 37° C in an incubator containing 0.5% CO_2. Culture medium consisted of RPMI-1640 with 5% human AB serum, 2 μmol/L mercaptoethanol, 1 mmol/L glutamine, 2 mmol/L sodium pyruvate, 100 U/ml streptomycin, and 0.5 μg/ml fungizone (Gibco). After 48 hours of incubation, supernatants were collected and kept at −80° C for measurement of the release of RANTES. Nasal polyp tissue was then placed on nitrocellulose paper to absorb excess fluid and weighed.

TABLE I. Clinical characteristics of subjects

Subject No.	Age (yr)	Sex	Atrophy	Aspirin sensitivity
1	56	F	Neg	Absent
2	62	M	Neg	Absent
3	28	M	Neg	Absent
4	65	M	Neg	Absent
5	45	M	Neg	Present
6	26	M	Neg	Absent
7	52	M	Pos	Absent
8	48	M	Pos	Absent
9	38	M	Pos	Absent
10	42	F	Pos	Present
11	35	M	Pos	Present
12	48	F	Pos	Absent

Processing of nasal polyp biopsy specimens for immunohistochemistry. Biopsy specimens from eight subjects (4 nonatopic and 4 atopic) were placed immediately into ice-cooled acetone containing the protease inhibitors iodoacetamide (20 mmol/L) and phenylmethylsulfonyl fluoride (2 mmol/L). After overnight fixation at −20° C, biopsy specimens were embedded in the water-soluble resin glycolmethacrylate (Park Scientific, Northampton, U.K.) as previously described.[15]

ELISA for RANTES

Measurement of immunoreactive RANTES in the supernatant of cultured nasal polyps was performed in duplicate samples (100 μl of nasal polyp culture supernatant) by using an ELISA kit according to the manufacturer's protocol (R&D Systems, Abingdon, U.K.). Concentrations of immunoreactive RANTES in culture supernatants were calculated from the standard curve. The lower limit of detection for immunoreactive RANTES was 5 pg/ml. Levels of RANTES are expressed in picograms per milligram of tissue.

Immunohistochemistry

The technique of immunohistochemistry was applied to 2 mm "semi-thin" sections of the glycolmethacrylate-embedded tissue as described by Britten et al.[14] Briefly, endogenous peroxidase activity was inhibited by incubating sections in 0.1% sodium azide and 0.3% hydrogen peroxide in Tris-buffered saline, pH 7.6, for 30 minutes. After washing three times with Tris-buffered saline, blocking medium (consisting of Dulbecco's modified eagle medium containing 10% [vol/vol] fetal bovine serum and 1% [wt/vol] bovine serum albumin) was applied for an additional 30 minutes. The primary monoclonal antibody (mAb) to RANTES (mAb 278, a gift from Dr. Harvey Garley, R&D systems, USA) at dilutions of 1:25 was then applied, and sections were incubated at room temperature overnight. This antibody does not cross-react with other chemokines including IL-8, gro-α, monocyte inhibitory peptide–1α, monocyte inhibitory peptide–1β, and monocyte chemotactic peptide–1. The next day, after washing, biotinylated rabbit anti-mouse IgG Fab (Dako Ltd., Wycombe, U.K.) at a dilution of 1:200 was applied to the sections for 2 hours and was then rinsed with streptavidin-biotin–horseradish peroxidase complex (Dako Ltd.) for an additional 2 hours. Finally, 0.02% 3-amino-9-ethylcarbazole in acetate buffer (pH 5.2) was used as a substrate to develop a peroxide-dependent red color reaction for 2 hours. Sections

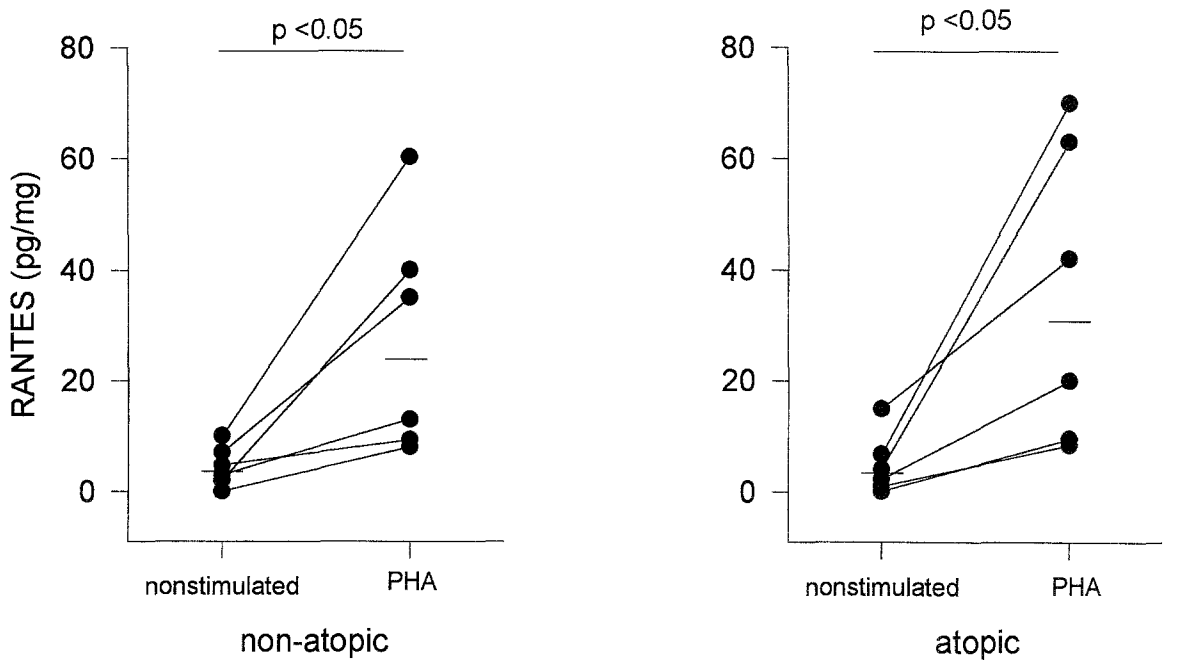

FIG. 2. Concentrations of RANTES immunoreactivity in culture supernatant of nonstimulated and PHA-stimulated nasal polyps from both nonatopic and atopic patients. *Horizontal lines* represent median values.

were counterstained with Mayer's hematoxylin and mounted in DPX.

Control slides were similarly treated either with the primary mAb omitted or in the presence of an unrelated antibody of the same isotype (M9269; Sigma, Poole, U.K.) at a concentration of 20 μg/ml.

Statistical analysis

Because levels of RANTES in nasal culture supernatants were not normally distributed, the Wilcoxon and Mann-Whitney U tests were used to analyze paired and unpaired data, respectively.

RESULTS

Nasal polyp tissue was obtained from all subjects taking part in this study. The median amounts of nonatopic nasal polyp tissue cultured with and without PHA were 11.2 mg (range, 2.0 to 67.4 mg) and 15.5 mg (range, 6.5 to 46.2 mg), respectively. Corresponding values for atopic nasal polyp tissue were 33.7 mg (range, 5.8 to 61.7 mg) and 18.8 mg (range, 8.4 to 37.1 mg).

Levels of immunoreactive RANTES

Measurements of immunoreactive RANTES showed that this cytokine was constitutively present in the 48-hour culture supernatant of nasal polyp cells derived from both nonatopic and atopic patients. However, there was no significant difference in the concentrations of this chemokine between these two groups (3.8 pg/mg; range, 0.5 to 10 pg/mg vs 2.94 pg/mg; range, 0.1 to 15 pg/mg) (Fig. 1).

To investigate whether PHA induces the release of RANTES, we stimulated nasal polyps with this mitogen. When compared with unstimulated samples, PHA-stimulated nasal polyps from both nonatopic and atopic patients released sevenfold and 11-fold greater amounts of RANTES, respectively. There was no significant difference in the levels of RANTES between these two groups of patients after PHA stimulation (26.5 pg/mg; range, 8.0 to 60.3 pg/mg vs 32.5 pg/mg; range, 8.4 to 70 pg/mg) (Fig. 2).

RANTES immunoreactivity in nasal polyp biopsy specimens

Immunoreactivity for RANTES was localized predominantly to the endothelium in nasal polyp biopsy specimens obtained from both nonatopic (n = 4) and atopic subjects (n = 4) (Fig. 3). In addition, there was sparse immunoreactivity localized to mononuclear cells present both in the blood vessels and free in the submucosa.

DISCUSSION

Tissue eosinophilia is a feature of nonatopic and atopic nasal polyposis. In this study we have demonstrated that nasal polyps derived from both atopic and nonatopic subjects release RANTES into the culture supernatant, most likely from the endothelium and T cells.

RANTES was initially identified as a gene of unknown function[15] but was subsequently shown to be chemotactic for lymphocytes, monocytes, and eosinophils.[10, 16] In addition to its ability to act as a soluble chemoattractant, recent attention has also been focused on the ability of RANTES to participate in the process of leukocyte recruitment by a haptotactic mechanism.[17, 18] It is pro-

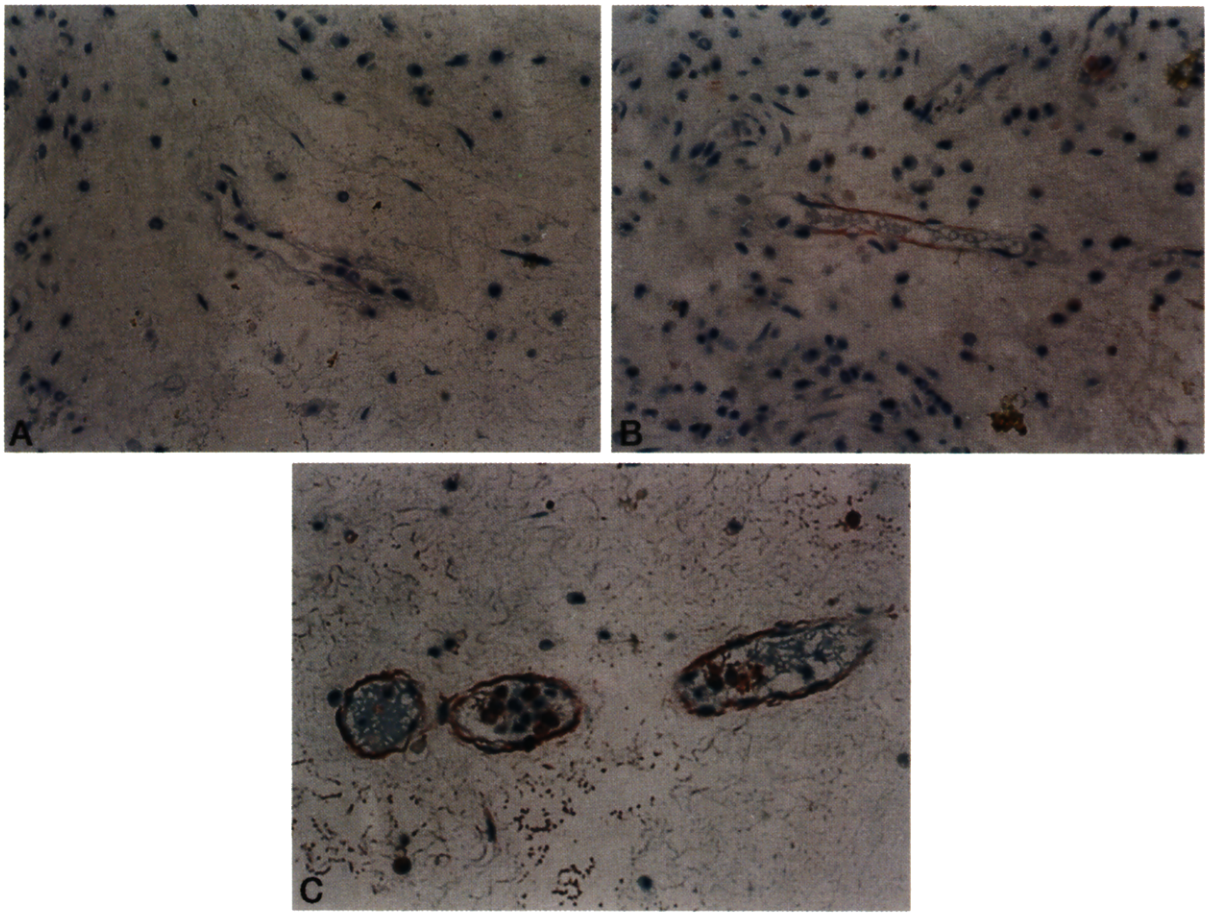

FIG. 3. A, Immunohistologic staining of negative control with an anti-IgG_1 mAb. **B and C,** Immunohistologic staining with antibody (mAb 278) against human RANTES. Biopsy specimens were obtained from a nonatopic subject **(A and B)** and an atopic subject **(C)**.

posed that RANTES is expressed on the lumenal surface of endothelial cells, probably bound to proteoglycan components such as heparan sulfate, allowing this cytokine to directly activate leukocyte integrins during the process of leukocyte-endothelial adhesion.[18]

We have demonstrated that RANTES is released into the supernatant of cultured nasal polyp tissue. This finding may be compared with that of Beck et al.[13] who have reported the presence of RANTES immunoreactivity in nasal polyp tissue. However, RANTES release by nasal polyps in relationship to atopy has not been previously investigated. This study has demonstrated that nasal polyps derived from both nonatopic and atopic patients release RANTES. Interestingly, levels of this cytokine between these two groups were not significantly different, suggesting that RANTES is locally produced by nasal polyps in both atopic and nonatopic patients.

PHA has previously been reported to induce RANTES messenger RNA expression in peripheral blood mononuclear lymphocytes and RANTES protein from bronchoalveolar lavage T lymphocytes.[15, 19] This study has demonstrated that on stimulation with PHA, nasal polyp cells derived from atopic and nonatopic patients also release a high concentration of RANTES. PHA specifically activates T lymphocytes through their CD3-lectin receptor,[20] which suggests that these cells may be an important source for the RANTES detected in the nasal polyp culture supernatant.

It is not known whether normal nasal tissue releases RANTES. However, in a preliminary report mRNA for RANTES has been shown to be expressed on nasal biopsy specimens derived from normal subjects.[21] Future studies may need to determine whether cultured nasal tissue releases this cytokine.

In this study we have not addressed the question of whether there was any difference in eosinophil and lymphocyte numbers in the nasal polyps of both atopic and nonatopic subjects. However, it has been previously shown that eosinophils infiltrate the nasal polyp tissue of these two groups of patients in a similar manner.[2] The finding that concentrations of RANTES were similar in nonatopic and atopic polyposis suggests that this cytokine may be involved in the recruitment of eosinophils

that characterizes the inflammatory reaction of this disease. In vivo evidence for the involvement of RANTES in the recruitment of eosinophils derives from a study showing that injection of RANTES into dog skin results in the recruitment of eosinophils and monocytes at the injection site.[22] Using in situ hybridization applied to the late-phase allergic response in human skin, Ying et al.[23] have demonstrated increased mRNA expression for RANTES on biopsy specimens obtained from atopic subjects 6 hours after allergen challenge. In this latter study the increased expression of mRNA for RANTES was associated with an elevated number of eosinophils and $CD4^+$ and $CD8^+$ T lymphocytes. We have recently performed endobronchial allergen challenge in patients with mild asthma and showed that bronchoalveolar lavage RANTES is biologically active on eosinophils.[24] Similarly, Sim et al.[25] have shown that nasal allergen challenge in atopic subjects leads to the release of RANTES into the nasal secretions 4 to 8 hours after challenge. Rajakulasingham et al.[21] have studied the expression of RANTES mRNA on nasal biopsy specimens of normal subjects and patients with atopic rhinitis. In this study they demonstrated an increase in RANTES mRNA expression on biopsy specimens derived from atopic subjects but not from normal subjects, 4 to 6 hours after allergen exposure.[21] RANTES mRNA has also been reported to be present on nasal polyp biopsy specimens.[26] Taken together, these observations suggest that RANTES is released in both nonatopic and atopic eosinophilic diseases such as nasal polyposis and bronchial asthma.

Other cytokines that have been associated with the presence of eosinophils in nasal polyps include IL-5, GM-CSF, and eotaxin.[2, 27, 29] Thus it is tempting to hypothesize that RANTES in concert with these other cytokines may orchestrate eosinophil recruitment into nasal polyp tissue.

To investigate the cellular source of the RANTES detected in the nasal culture supernatant, we have performed immunohistochemistry staining of nasal polyp biopsy specimens. RANTES can be produced by several cells potentially implicated in the inflammatory response in nasal polyposis including macrophages, lymphocytes, fibroblasts, and nasal polyp epithelial cells.[9, 13, 15] However, we have demonstrated that RANTES immunoreactivity was predominantly localized to the vascular endothelium. This finding is consistent with a previous report indicating that cytokine-stimulated vascular endothelial cells release RANTES.[30] The failure to localize RANTES immunoreactivity to lymphocytes may be explained by the small cytoplasmic content of these cells and by the observation that cytokines newly formed by T cells do not accumulate in the cytoplasm (rather, they are rapidly exported).[31]

In summary, this study has demonstrated that cultured nasal polyps derived from both nonatopic and atopic patients spontaneously release RANTES. Moreover, on stimulation with PHA, nasal polyps release greater concentrations of RANTES. In this study RANTES immunoreactivity was localized to endothelial cells, suggesting that this cytokine may directly stimulate transendothelial eosinophil migration. These observations together suggest that RANTES may be involved in the pathogenesis of both nonatopic and atopic nasal polyposis.

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