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Cellular infiltration and cytokine mRNA expression in perennial allergic rhinitis

Key words: cytokines; eosinophils; perennial allergic rhinitis; T cells.

Background: Allergen challenge in allergic rhinitis patients leads to local eosinophilia and Th2-type cytokine expression. Natural exposure to grass pollen is additionally characterized by epithelial mast-cell infiltration. We hypothesized that perennial allergic rhinitis is also associated with T-cell and eosinophil infiltration of the nasal mucosa, local Th2-type cytokine expression, and increased numbers of nasal epithelial mast cells.

Methods: Nasal biopsies from perennial allergic rhinitis patients and controls were analysed by immunocytochemistry for different cell populations and *in situ* hybridization for cytokine mRNA-expressing cells.

Results: Perennial allergic rhinitis was associated with increased numbers of submucosal CD3+ T cells ($P=0.05$), EG2+ activated eosinophils ($P=0.01$), and CD68+ macrophages ($P=0.01$) compared to controls. Epithelial, but not submucosal, tryptase-positive mast cells were also elevated in rhinitis compared to controls ($P=0.01$). The numbers of cells expressing interleukin (IL)-5 were higher ($P=0.01$) and the numbers of cells expressing IL-2 were lower ($P=0.04$) in rhinitic patients than controls. There were no significant differences for either IL-4 or interferon-gamma between the groups.

Conclusions: Perennial allergic rhinitis is characterized by mast-cell migration into the epithelium; submucosal infiltration by T cells, eosinophils, and macrophages; and an imbalance in local T-cell cytokine production in favour of enhanced IL-5 and reduced IL-2 expression.

In patients with allergic rhinitis, allergen provocation results in early (0–1 h)- and late (2–24 h)-phase clinical responses, characterized by nasal discharge, sneeze, and/or blockage. While mast cells are presumed to play a role in the

Cells per mm² nasal submucosa

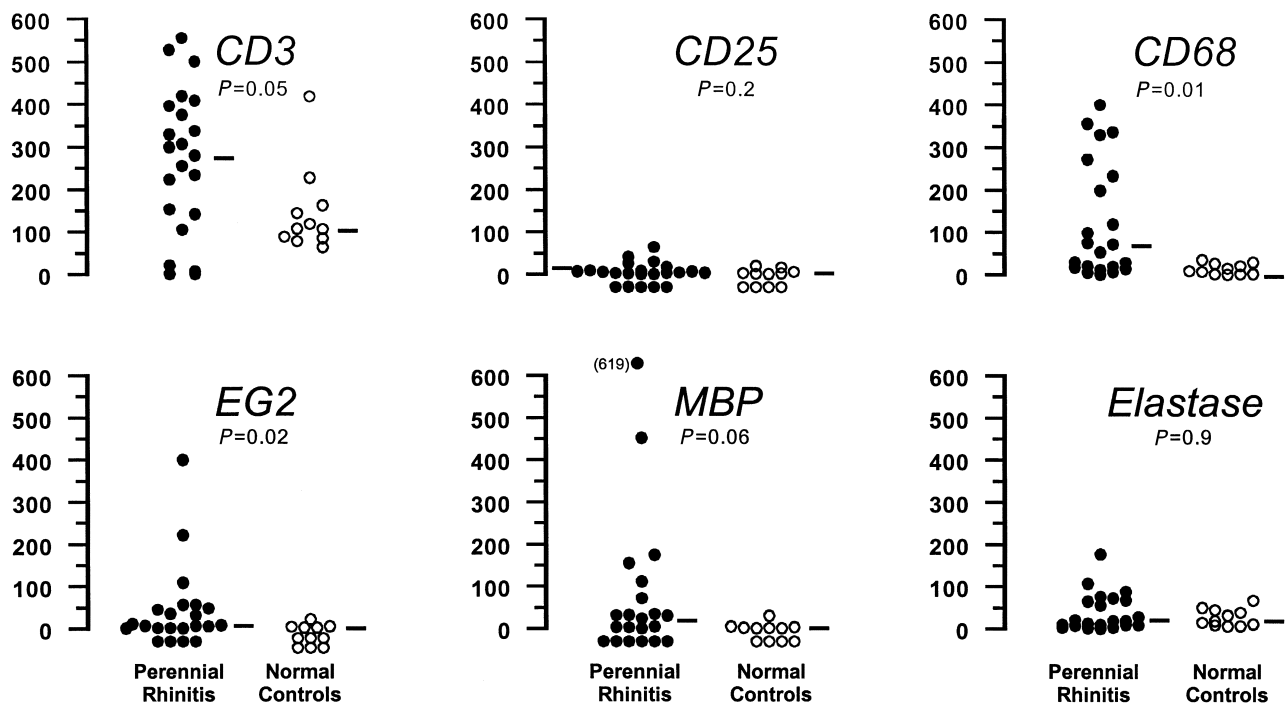


Figure 1. Numbers of nasal submucosal CD3+ T cells, CD25+ activated T cells, CD68+ macrophages, EG2+ activated eosinophils, MBP+ total eosinophils, and elastase-positive neutrophils in perennial allergic rhinitis patients and normal controls, as identified by immunohistochemistry. Bars represent median values ($\pm$ interquartile range). Between-group comparisons were performed by Mann-Whitney U test.

early nasal response and eosinophil recruitment and activation, release of lipid mediators, such as the cysteinyl leukotrienes, is thought to make a significant contribution to the late response.

Late-phase nasal responses induced by allergen challenge are accompanied by infiltration of the nasal mucosa by activated T cells, eosinophils, and neutrophils (1), and local expression of the "Th2-type" cytokines interleukin (IL)-4 and IL-5 (2). IL-4 is required for IgE synthesis by B cells and may play a role in IgE heavy-chain switching within the nasal mucosa (3) and upregulation of VCAM-1 expression on vascular endothelial cells (4). On the other hand, IL-5 has various specific effects on eosinophils, promoting maturation, endothelial adhesion, activation, survival (5–7), release from bone marrow (8), and responsiveness to chemoattractants such as RANTES (9). We have also previously shown that seasonal allergic rhinitis is characterized by nasal eosinophilia, increases in the numbers of epithelial mast cells (10), and increases in IL-4 (11) and IL-5 mRNA-expressing cells (12) in the nasal mucosa during the pollen season. Although cytokine immunoreactivity has been studied within the nasal mucosa in perennial allergic

rhinitis (13, 14), the patterns of cytokine mRNA expression have not yet been investigated.

In the present study, we have examined both cytokine mRNA expression and epithelial and submucosal cell counts in perennial allergic rhinitic patients and controls. We set out to test the following hypotheses: first, that the symptoms of perennial allergic rhinitis are associated with infiltration of the nasal mucosa by T cells and eosinophils, but not neutrophils; second, that macrophages are recruited to the nasal mucosa in perennial allergic rhinitis, thus potentially increasing local antigen presentation to T cells; and, third, that the numbers of mast cells are selectively increased in the nasal epithelium of patients with perennial allergic rhinitis, as we previously showed to be the case in seasonal allergic rhinitis. Finally, we hypothesized that the symptoms of perennial allergic rhinitis arise in part through expression of Th2-type cytokines in the nasal mucosa, with IL-4 and IL-5 driving IgE synthesis and tissue eosinophilia, respectively. Nasal biopsies were collected from patients with symptomatic perennial allergic rhinitis and a group of normal control subjects. Cell populations in the mucosa were quantified by immunocytochemistry, and the numbers

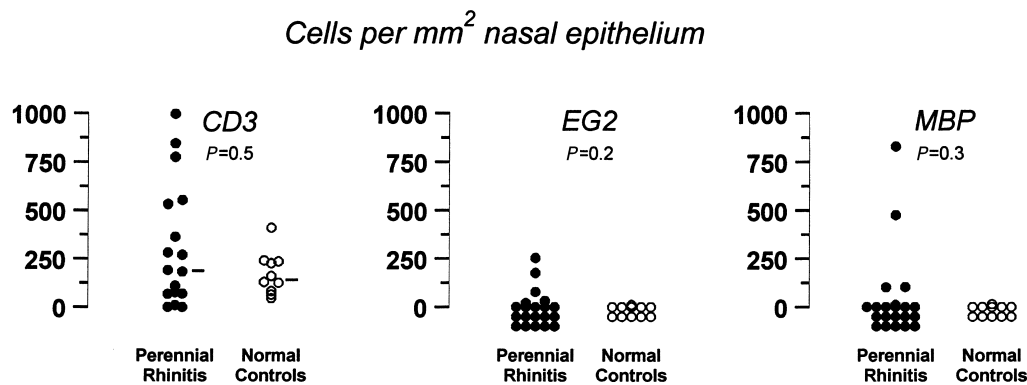


Figure 2. Numbers of nasal epithelial CD3+ T cells, EG2+ activated eosinophils, and MBP+ total eosinophils in perennial allergic rhinitis patients and normal controls, as identified by immunohistochemistry. Bars represent median values ($\pm$ interquartile range). Between-group comparisons were performed by Mann-Whitney U test.

of IL-5, IL-4, interferon-gamma (IFN- γ), and IL-2 mRNA-expressing cells were determined by *in situ* hybridization with specific riboprobes.

Material and methods

Inclusion criteria

Twenty-two nonsmoking patients with perennial allergic rhinitis were recruited on the basis of

- 1) a history of perennial nasal blockage, discharge, and/or sneeze for at least 2 years before the study
- 2) a positive skin prick test to house-dust-mite (HDM) extract (*Dermatophagoides pteronyssinus*, ALK, Hørsholm, Denmark) (>5 mm wheal diameter on skin testing, compared to a negative diluent control test)
- 3) nasal symptoms during activities such as bed-making, dusting, or vacuuming.

Table 1. Clinical details of study subjects

	Perennial allergic rhinitis	Normal controls
No. of patients	22	12
Male:female ratio	10:12	4:8
Age (years; mean $\pm$ SD)	31.9 $\pm$ 11.6	32.0 $\pm$ 7.2
Skin test positive	22/22	0/12
HDM RAST* class (mean $\pm$ SD)	3.0 $\pm$ 1.2	NT
Total IgE IU/ml (median)	370	NT
(interquartile ranges)	(208-813)	
* House-dust-mite radioallergosorbent test. NT: not tested.		

Venous blood was also collected for measurement of total and allergen-specific IgE concentrations. Eleven out of 22 patients had asthma associated with rhinitis. Patients were excluded if they had sinusitis or nasal polyps, gave a history of sensitivity to aspirin or other nonsteroidal anti-inflammatory drugs, had received immunotherapy within the last 5 years, gave a history of bleeding or clotting disorder, or had any other significant medical condition. For 1 week before biopsy, patients were required to record daily score symptoms of nasal blockage, discharge, sneeze, and itch. Each symptom was scored on a scale from 0 (no symptom) to 3 (severe symptoms), the maximum total score being 12. All medications (e.g., intranasal glucocorticoids or sodium cromoglycate and inhaled steroids) were withheld 2 weeks before nasal biopsy. The 12 nonallergic control subjects recruited for this study were required to have negative skin prick tests to a range of 12 common aeroallergens, and had no nasal symptoms or other symptoms indicative of allergic disease. The characteristics of the subjects participating in this study are shown in Table 1. The study was performed with the approval of the hospital ethics committee and the written, informed consent of all participants.

Nasal biopsy, immunohistochemistry, and *in situ* hybridization

Local anaesthesia of the inferior nasal turbinate was achieved with 3% cocaine and 0.025% adrenaline. A 2.5 mm biopsy was taken 10 min later with Gerritsma (15) forceps. Biopsy specimens were immediately cut in half and processed separately for subsequent *in situ* hybridization and immunohistology. Biopsy specimens were snap-frozen and stored at -80°C , pending analysis. Immunohistology was performed on 6- μm cryostat sections (fixed for 7 min in 60:40 acetone:methanol), by the modified alkaline

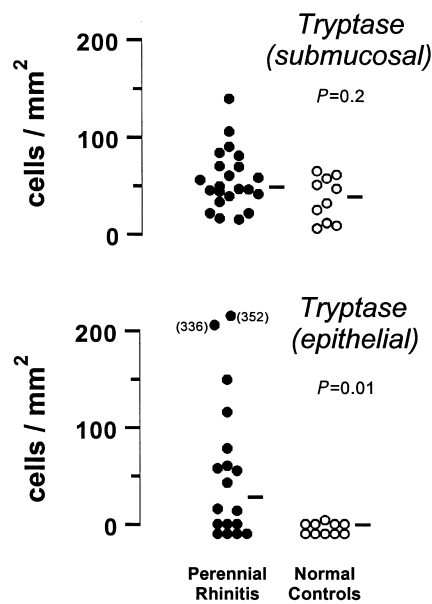


Figure 3. Numbers of nasal submucosal and epithelial tryptase+ mast cells in perennial allergic rhinitis patients and normal controls, as identified by immunohistochemistry. Bars represent median values ($\pm$ interquartile range). Between-group comparisons were performed by Mann-Whitney U test.

phosphatase-antialkaline phosphatase method, as previously described (1). Immunohistochemistry was performed with monoclonal antibodies (all Dako Ltd, High Wycombe, UK) recognizing T cells (CD3), activated T cells (CD25), macrophages (CD68), mast cells (AA1), and neutrophils (elastase). The BMK13 monoclonal antibody, used to quantify total eosinophils (MBP), was a kind gift of Ms Julia Barkans and Dr Redwan Moqbel. The EG2 monoclonal antibody (Kabia Pharmacia, Milton Keynes, UK) was used to quantify the numbers of activated eosinophils.

Riboprobes, both antisense (complementary to mRNA) and sense (identical sequence to mRNA), were prepared from cDNA encoding IL-5, IL-4, IFN- γ , and IL-2. cDNAs were inserted into different pGEM vectors and linearized with restriction enzymes before transcription. Transcription was performed in the presence of ^{35}S -labelled uridine triphosphate and the appropriate T7 or SP6 RNA polymerase. *In situ* hybridization on 10- μm cryostat sections was performed as previously described (2). To minimize non-specific binding of ^{35}S , preparations were treated with 10 mM iodoacetamide and 10 mM *N*-ethylmaleimide for 30 min at 37°C and then in 0.5% acetic anhydride in (0.1 M) triethanolamine for 10 min at 37°C. Positive controls for IL-5 and IL-4 mRNA expression were cytopins prepared with a peripheral blood T-cell clone propagated from a patient with hyper-IgE syndrome. Positive controls for IFN- γ and IL-2 mRNA expression were cytopins prepared with PHA-

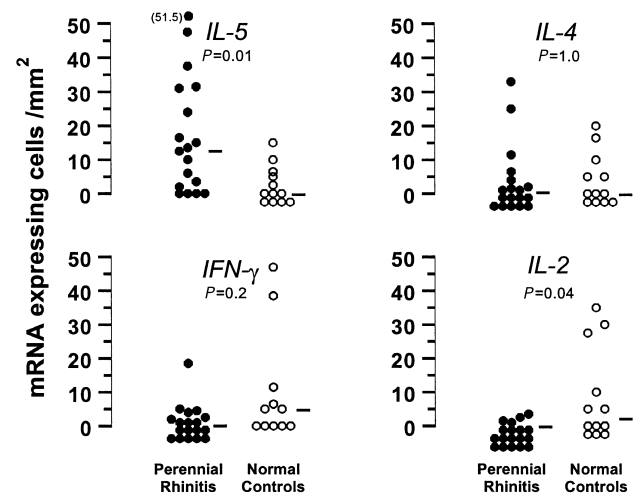


Figure 4. Numbers of cells expressing cytokine mRNA encoding IL-5, IL-4, IFN- γ , and IL-2 in perennial allergic rhinitis patients and normal controls, as identified by *in situ* hybridization with radiolabelled antisense riboprobes. Bars represent median values ($\pm$ interquartile range). Between-group comparisons were performed by Mann-Whitney U test.

stimulated peripheral blood mononuclear cells. For negative controls, nasal biopsy sections were

- 1) hybridized with sense riboprobes for IL-5, IL-4, IFN- γ , and IL-2
- 2) pretreated with RNAase A solution before hybridization with antisense riboprobes.

Specific hybridization was recognized as clear, dense deposits of silver grains in the photographic emulsion overlying tissue sections.

Sections were counted blind with an Olympus BH2 microscope (Olympus Optical Company Ltd, Tokyo, Japan) with an eyepiece grid (0.202 mm² at $\times 200$ magnification). At least two tissue sections (giving an average of eight grids) were counted per patient. Submucosal counts for the whole biopsy were made one grid depth (0.45 mm) beneath the basement membrane and were expressed as mean counts per square millimetre of submucosa. For epithelial counts, the cross-sectional area of the nasal epithelium was quantified with a computer-assisted graphics tablet (Summagraphics Corporation, Fairfield, CT, USA).

Statistical analysis

Between-group comparisons were made with the Mann-Whitney U test. Correlations were performed with Spearman's rank method. All analyses were performed with the aid of a commercial software package (Minitab, Inc., State College, PA, USA), and *P* values less than 0.05 were considered significant.

Results

Numbers of inflammatory cells in the nasal submucosa and epithelium

In patients with perennial allergic rhinitis, the numbers of submucosal CD3+ T cells were significantly increased compared to normal control subjects (in the rhinitic group, median=289.2 cells/mm² [IQ range=132.8–399.2], *n*=22; in the control group, median=107.5 cells/mm² [85–162.5], *n*=11; *P*=0.05) (Fig. 1). However, the numbers of CD25+ activated T cells were not statistically different between the two groups (in the rhinitic group, median=4.19 cells/mm² [0.95–11.1], *n*=22; in the control group, median=1.5 cells/mm² [0–6.0], *n*=11; *P*=0.17) (Fig. 1). The numbers of submucosal CD68+ macrophages were significantly elevated in the rhinitic group (median=62.7 cells/mm² [16.4–24.30], *n*=22) relative to controls (median=9.0 cells/mm² [1.5–26.5], *n*=11; *P*=0.01) (Fig. 1).

Submucosal EG2+ activated eosinophils were increased in rhinitics compared to normal controls (in the rhinitic group, median=7.4 cells/mm² [0.9–50.0], *n*=22; in the control group, median=0 cells/mm² [0–4.5], *n*=11; *P*=0.01) (Fig. 1), with a trend for an increase in MBP+ total eosinophils (in the rhinitic group, median=24 cells/mm² [0–91.7], *n*=21; in the control group, median=1.0 cells/mm² [0–3.5], *n*=11; *P*=0.06) (Fig. 1). Furthermore, the numbers of elastase-positive neutrophils were similar in rhinitics and controls (in the rhinitic group, median=19 cells/mm² [8.6–69.6], *n*=22; in the control group, median=19.0 cells/mm² [9.0–45.0], *n*=11; *P*=0.88) (Fig. 1).

There were no statistically significant differences between the groups in the numbers of epithelial CD3+ T cells (in the rhinitic group, median=190.8 cells/mm² [68.7–543.3], *n*=17; in the control group, median=145.3 cells/mm² [78.4–237.2], *n*=10; *P*=0.53) (Fig. 2), EG2+ activated eosinophils (in the rhinitic group, median=0 cells/mm² [0–15.4], *n*=20; in the control group, median=0 cells/mm² [0–0], *n*=11; *P*=0.24) (Fig. 2), or MBP+ total eosinophils (in the rhinitic group, median=0 cells/mm² [0–8.2], *n*=20; in the control group, median=0 cells/mm² [0–0], *n*=11; *P*=0.27) (Fig. 2).

Numbers of mast cells in the nasal submucosa and epithelium

Although the numbers of submucosal tryptase-positive cells stained by the AA1 monoclonal antibody were equivalent in the rhinitic and normal control groups (in the rhinitic group, median=48.1 cells/mm² [37.8–72.7], *n*=22; in the control group, median=39.5 cells/mm² [10.8–58.4], *n*=10; *P*=0.15)

(Fig. 3), there was evidence of significantly increased numbers of epithelial mast cells in patients with perennial allergic rhinitis (in the rhinitic group, median=29.5 cells/mm² [0–87.8], *n*=18; in the control group, median=0 cells/mm² [0–0], *n*=10; *P*=0.01) (Fig. 3).

Expression of mRNA encoding IL-5, IL-4, IFN- γ , and IL-2 in the nasal mucosa

The location of cytokine mRNA-expressing cells within nasal biopsies was predominantly submucosal. The numbers of IL-5 mRNA-expressing cells were significantly elevated in patients with perennial allergic rhinitis (median=13.0 cells/mm² [1.5–31.1], *n*=18) compared to normal controls (median=0 cells/mm² [0–6.12], *n*=12; *P*=0.01) (Fig. 4). In contrast, no statistically significant differences were observed between the groups in the numbers of either IL-4 mRNA-expressing cells (in the rhinitic group, median=0.5 cells/mm² [0–4.62], *n*=18; in the control group, median=0 cells/mm² [0–8.75], *n*=12; *P*=0.96) (Fig. 4) or IFN- γ mRNA-expressing cells (in the rhinitic group, median=0.5 cells/mm² [0–2.88], *n*=18; in the control group, median=5.0 cells/mm² [0–11.5], *n*=11; *P*=0.21) (Fig. 4). However, the numbers of IL-2 mRNA-expressing cells were found to be significantly lower in the perennial allergic rhinitis group than the normal control group (in the rhinitic group, median=0 cells/mm² [0–0.25], *n*=18; in the control group, median=2.5 cells/mm² [0–23.1], *n*=12; *P*=0.04) (Fig. 4).

The median nasal symptom score (interquartile ranges) for the perennial rhinitis group was 4.75 (3.44–7.99). No correlation was observed between nasal symptom scores and numbers of cell types and mRNA-expressing cells.

Discussion

This study represents the first systematic comparison of both cell populations and cytokine mRNA expression in perennial allergic rhinitis patients and normal controls. We report that perennial allergic rhinitis is associated with significantly increased numbers of submucosal CD3+ T cells, CD68+ macrophages, and EG2+ activated eosinophils compared to normal controls. Recruitment of T cells and activated eosinophils was restricted to the submucosa, since cell numbers within the epithelium were equivalent in rhinitic and control groups. For tryptase-positive mast cells, the opposite pattern was observed; i.e., higher numbers of epithelial, but not submucosal, mast cells were evident in rhinitics than normal controls. Finally, we report that

perennial allergic rhinitis is associated with increased numbers of nasal mucosal cells expressing mRNA encoding the proeosinophilic cytokine IL-5, although, surprisingly, we did not find evidence of increased IL-4 expression in the same patients. Although the numbers of IFN- γ -expressing cells were also statistically equivalent in both groups, the numbers of IL-2 mRNA-expressing cells were lower in the rhinitic group.

Evidence of T-cell recruitment to the nasal mucosa in allergic rhinitis is controversial. In nasal biopsies collected from seasonal rhinitis patients before and during the grass-pollen season, natural allergen exposure was not accompanied by a significant increase in numbers of either CD3+ or CD4+ T cells (10). Similarly, no increases were observed in numbers of submucosal CD3+ T cells after allergen challenge, although CD4+ numbers did increase (1). Nevertheless, our present data agree with those of Calderón et al. (16), who also found increased numbers of submucosal CD3+ T cells in perennial allergic rhinitis. These findings suggest that the extent of CD3+ T-cell infiltration in allergic rhinitis might depend on the chronicity of allergen exposure as well as the severity of disease.

Our demonstration of increased numbers of submucosal CD68+ macrophages in perennial allergic rhinitis is novel and is supported by studies performed on nasal biopsies (17) and nasal scrapings (18) from patients with seasonal allergic rhinitis. However, the exact role of macrophages in the allergic response is unknown. The recent demonstration of high-affinity IgE receptor alpha subunit (Fc ϵ RI α) expression by these cells (19) suggests that IgE-mediated antigen processing by nasal mucosal macrophages may play a role in driving local T-cell activation and cytokine production in response to inhaled allergen.

Local tissue eosinophilia and associated Th2-type cytokine expression are well established features of cutaneous (20) and nasal late-phase responses to allergen (2). Like the mast cell, the eosinophil is a source of proinflammatory lipid mediators and cytokines, and is considered to be an effector cell in allergic inflammation. The demonstration of eosinophil cationic protein in nasal lavage fluid after local allergen provocation is consistent with eosinophil activation and degranulation occurring within the nasal mucosa of rhinitics (21, 22). We have extended these observations to show that perennial allergic rhinitis caused by natural exposure to allergen, is also, like seasonal allergic rhinitis (10), characterized by significant recruitment of activated eosinophils into the nasal submucosa. However, activated eosinophil cell numbers in the nasal epithelium were not significantly different between perennial allergic rhinitics

and normal controls. Although we also observed a trend for increases in total eosinophil numbers in the nasal submucosa of perennial allergic rhinitics, it only approached statistical significance ($P=0.06$). In contrast to eosinophils, mast cells were selectively recruited to the nasal epithelium in perennial allergic rhinitis patients. These data are consistent with previous studies comparing normal controls and patients with seasonal (10) or perennial allergic rhinitis (13, 23) induced by natural allergen exposure, and suggest that during chronic allergen exposure, mucosal-type mast cells are induced either to migrate from the submucosa into the epithelium, or undergo proliferation (24) or maturation from precursors *in situ*. These findings are also consistent with a recent study suggesting that nasal mast cells undergo functional and phenotypic changes suggestive of activation in perennial allergic rhinitis (25).

The numbers of nasal mucosal cells expressing mRNA for the Th2-type cytokine IL-5 were significantly elevated in the perennial allergic rhinitics compared to normal controls. These data are consistent with previous reports of the upregulation of IL-5 mRNA expression in rhinitis induced by nasal allergen provocation (2) or seasonal exposure to grass pollen (12). Although we did not colocalize cytokine mRNA to different cell types, our previous studies in the nose (12, 26) and lung (27) suggest that most IL-5 mRNA-expressing cells in allergic tissue reactions are CD3+ T cells, with lesser contributions from mast cells and eosinophils. Previous studies have also demonstrated increased IL-4 mRNA expression after allergen challenge (2) or during the pollen season (11), although we were unable to demonstrate increased numbers of IL-4 mRNA-expressing cells in the nasal mucosa in perennial allergic rhinitis. One explanation for this may be that we selected patients with relatively mild disease, as reflected by a modest medium nasal symptom score of 4.75 out of the maximum score of 12. It is also possible that the pattern of cytokine expression in perennial allergic rhinitis differs from that observed after more acute exposure to allergen (such as an artificial challenge or seasonal exposure) with marked upregulation of IL-5, but not IL-4, mRNA expression. Thus, IL-4 mRNA expression could transiently increase after allergen exposure and play a role in inducing short-term symptoms.

Bradding et al. examined IL-4, IL-5, and IL-6 immunoreactivity in nasal biopsies and reported a significant increase in IL-4 immunostaining in perennial allergic rhinitis patients (13). Increases in IL-4, IL-5, and IL-6 immunoreactivity in nasal biopsies of perennial allergic rhinitics have also been reported by Saito et al. (14). However, immunostaining biopsy sections with monoclo-

nal antibodies does have the disadvantage of preferentially identifying preformed and stored cytokine protein within granular cells [28, 29].

In contrast, *in situ* hybridization identifies all cells actively expressing cytokine mRNA, including T cells, which secrete but do not store cytokines and are therefore not detectable by immunostaining techniques.

Cell numbers expressing the archetypal Th₁-cytokines IFN- γ and IL-2 were lower in the perennial allergic rhinitis group than in normal controls. For IL-2 mRNA expression, the difference between the two groups was significantly different. *In vitro* studies in human subjects previously showed reduced secretion of IL-2 by Th₂-type allergen-specific [30] T-cell clones, and *in vivo* studies demonstrated preferential expression of IL-2 mRNA in tuberculin-driven delayed-type hypersensitivity, but not allergen-driven late-phase reactions [31]. Therefore, these data support the

conclusion that in perennial allergic rhinitis there is an imbalance in T-cell cytokine production in the nasal mucosa, in favour of a Th₂-type response.

In summary, we have shown that perennial allergic rhinitis is associated with submucosal infiltration by T cells, activated eosinophils, and macrophages, and infiltration of the epithelium by mast cells. Mast cells represent an alternative source of preformed cytokines, including IL-4 and IL-5, which may be released immediately after allergen challenge. Production of Th₂-type cytokines by T helper cells within the nasal mucosa is likely to represent a more sustained source of chronic inflammatory changes in perennial allergic rhinitis.

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