

Increase in IL-8, IL-10, IL-13, and RANTES mRNA levels (in situ hybridization) in the nasal mucosa after nasal allergen provocation

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Background: Allergic inflammation is regulated by the local production and release of several cytokines.

Objectives: This study was designed to assess the changes in mRNA cytokine-positive cells after allergen provocation and to compare these cytokines with tissue eosinophilia as a marker of allergic inflammation.

Methods: A grass pollen allergen provocation study was conducted in autumn, out of the hay fever season. Nasal mucosal biopsy specimens were taken before provocation and 1 hour, 24 hours, and 1 week after allergen provocation. Eosinophils and mRNA-positive cells (in situ hybridization for IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IFN- γ , RANTES, and TNF- α) were assessed in the biopsy specimens.

Results: After allergen provocation, an increase in cell number was found for eosinophils and cells expressing mRNA for the chemokines IL-8 and RANTES and for the T_{H2} cytokines IL-10 and IL-13. Significant correlations were found between eosinophils and RANTES and eosinophils and IFN- γ in the early phase and between eosinophils and IL-5 and eosinophils and RANTES in the late phase. The increase in eosinophils and IL-10 and IL-13 mRNA-positive cells could still be observed 1 week after allergen provocation.

Conclusions: Nasal allergen provocation induced significant tissue eosinophilia and a significant increase in IL-8, IL-13, and RANTES mRNA-positive cells. A significant increase in eosinophils and IL-10 and IL-13 mRNA-positive cells compared with baseline can still be observed 1 week after a single allergen provocation. (*J Allergy Clin Immunol* 1999;103:441-50.)

Key words: Allergen provocation, hay fever, cytokines, eosinophils, nasal mucosa, biopsy, in situ hybridization, mRNA

The pathophysiology of allergic rhinitis is based on the presence of inflammatory cells in the nasal mucosa.¹ Allergen-induced rhinitis is accompanied by a cellular infiltrate in which eosinophils, mast cells, T cells, and Langerhans cells are prominent.²⁻⁸ Allergic mucosal inflammation is regulated by the local production and

Abbreviations used

BU: Biologic unit

SSC: Sodium sodium citrate

release of several cytokines.⁹⁻¹³ A major role was originally assigned to the cytokines produced by T_{H2} lymphocytes, such as IL-3, IL-4, IL-5, and IL-13.^{9,12,14-17} In addition to the well-known T_{H2} cytokines, chemokines (IL-8 and RANTES)^{11,18-20} and perhaps TNF- α are also thought to play a role in allergic inflammation.^{13,21,22} Accumulating data now points to a large network of interacting cytokines produced by a number of cells that regulate mucosal allergic inflammation. However, the existing data about cytokines is partially contradictory and sometimes confusing. This is the result of several factors, including different study designs, the limited numbers of cytokines evaluated, and observations on various levels, such as the protein and mRNA levels.

Allergen challenge studies have found increases in protein and mRNA for IL-4 and IL-5 and an increase in IL-6 protein.^{9,16,23} However, in natural disease no differences were found between season and preseason IL-4, IL-5, and IL-6 protein levels.¹² Rises in numbers of TNF- α mRNA-positive cells were observed after filter-disk provocation.²¹ However, in a study comparing nasal polyps of allergic and nonallergic patients, more TNF- α -positive cells were found in the nonallergic group.²²

To our knowledge, no in vivo data is available about the dynamics of a number of cells expressing IL-6, IL-8, and IL-10 mRNA in the upper airway mucosa after allergen provocation. For other cytokines, details on time-related changes after allergen provocation are incomplete.

In this out-of-season biopsy study, we investigated, on several occasions after the provocation, the effect of a single provocation with grass pollen in patients with isolated grass pollen allergy. Eosinophils and a wide range of T_{H1} and T_{H2} cytokine- and chemokine-expressing cells (in situ hybridization for IL-2, IL-3, IL-4, IL-5, IL-6, IL-8, IL-10, IL-13, IFN- γ , RANTES, and TNF- α) were determined in mucosal biopsy specimens before and at various times after allergen provocation. Correlations between eosinophils (as a marker of allergic inflammation) and cytokines were evaluated.

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Received for publication July 27, 1998; revised Nov 30, 1998; accepted for publication Nov 30, 1998.

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0091-6749/99 \$8.00 + 0 1/1/96177

TABLE I. Primer sequence for each cytokine used for making digoxigenin dUTP DNA probes

	Sequence (5'-3') primers	Fragment(bp)
IL-2 sense	AAGAATCCCAAACTCACCAGGATGC	(exon 2) 200 bp
IL-2 α -sense	CCCTTTAGTTCCAGAACTATTACGT	(exon 3-4) ⁵¹
IL-3 sense	GCCTTTGCTGGACTTCAACA	(exon 1-2) 194 bp
IL-3 α -sense	TTGGATGTCGCGTGGGTGCG	(exon 4-5) ⁵²
IL-4 sense	ACTCTGTGCACCGAGTTGACCGTAA	(exon 2) 300 bp
IL-4 α -sense	TCTCATGATCGTCTTTAGCCTTTCC	(exon 4) ⁴⁷
IL-5 sense	AGCCAATGAGACTCTGAGGA	(exon 1-2) 319 bp
IL-5 α -sense	GGGAATAGGCACACTGGAGAGTCAA	(exon 4) ⁵³
IL-6 sense	ATGAATCCTTCTCCACAAGC	(exon 1) 610 bp
IL-6 α -sense	TGGACTGCAGGAACCTCCTT	(exon 5) ⁵⁴
IL-8 sense	CTGTGTGAAGGTGCAGTTTGGCC	(exon 1-2) 237 bp
IL-8 α -sense	CTCAGCCCTCTTCAAAAACCTTCTCC	(exon 3-4) ⁵⁵
IL-10 sense	ATGCCCAAGCTGAGAACCAAGACCCA	(exon ?) 352 bp
IL-10 α -sense	TCTCAAGGGGCTGGGTGCTATCCCA	(exon ?) ⁵⁶
IL-13 sense	CCCAGAACCAAGAGGCTCCGC	(exon 1-2) 185 bp
IL-13 α -sense	GCTGGAAACTGCCCAGCTGAG	(exon 3-4) ⁵⁷
IFN- γ sense	TTTAATGCAGGTCATTAGATG	(exon 1-2) 388 bp
IFN- γ α -sense	CAGGGATGCTTCTTCGACCTCGAAAC	(exon 4) ⁴⁷
RANTES sense	CGCTGTGTCCTCATTTGCTA	(exon ?) 197 bp
RANTES α -sense	CACACACTTGGCGGTTCTT	(exon ?) ⁵⁸
TNF- α sense	AGAGGGAAGAGTCCCCAGGGAC	(exon 1-2) 443 bp
TNF- α α -sense	TGAGTCGGTCACCTTCTCCAG	(exon 4) ⁵⁹

METHODS

Patients

Nine patients (5 women and 4 men; median age, 22 years [range, 19 to 51 years]) participated in this study. All patients had a history of seasonal allergic rhinitis for grass pollen of at least 2 years duration. Allergy to grass pollen was confirmed by a positive skin prick test response (3+) with Alutard Soluprick extract (1 SQ/mL), with no skin prick reactions to other relevant allergens. All patients were asymptomatic at the start of the study. None of the patients were using any medication during the study or had undergone immunotherapy in the 3 years before the study. No relevant abnormalities were found on ear, nose, and throat examination. All patients gave their written informed consent, and the study was approved by the medical ethics committee.

Study design

This allergen provocation study was conducted between October and December 1995, well out of the hay fever season. Biopsy specimens were taken from the inferior turbinate by using a Gerritsma forceps.²⁴ Each patient underwent 4 nasal biopsies: the first at baseline, the second 1 hour after provocation with grass pollen, the third 24 hours after provocation, and the fourth 1 week after provocation. Biopsy specimens were coded, and therefore the analyses were done in a blinded fashion.

Provocation

The patients were acclimatized to the room for at least 15 minutes. After that, PBS, an inert water-rich solution, was administered to rule out nonspecific hyperreactivity. After 10 minutes, patients were provoked with 50 μ L of an aqueous nasal spray containing grass pollen (10,000 biologic units [BU]/mL; ALK, Groningen, The Netherlands) and 10 minutes later with another 100,000 BU/mL. After both provocations, symptoms such as sneezing, rhinorrhea, itching, and nasal blockage were recorded on a 4-point (range, 0 to 3) scale. Patients were also asked to rate their symptoms during the day after provocation after 3, 6, 9, 12, and 24 hours.

Immunohistochemical staining of eosinophils

Eosinophil staining was performed by using an alkaline-phosphatase procedure with an mAb against major basic protein (BMK-13) (Sanbio, Uden, Netherlands), as previously described by Godthelp et al.³

Digoxigenin probe manufactured by PCR

The digoxigenin probe was manufactured by PCR with a modification of the method described by Klein.^{25,26} Total RNA was isolated from stimulated (concanavalin A or LPS) blood mononuclear cells or in vivo allergen-stimulated nasal mucosal cells by the phenol extraction method, a modification of the protocol described by Chomczynski and Sacchi.²⁷ One microgram of RNA was then reverse transcribed to cDNA (AMV Boehringer Mannheim Biochemica). The reverse-transcribed mix, a total volume of 20 μ L, contains 50 mmol/L Tris-HCL (pH 8.3), 10 mmol/L MgCl₂, 50 mmol/L KCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 10 μ g/mL BSA (Boehringer Mannheim), 1 mmol/L dNTP mix (Pharmacia), 10 μ g/mL oligo(dT)12-18 (Pharmacia), 2.5 OD/mL hexanucleotide pd(N)6 (Pharmacia), and 1 mmol/L spermidine-HCl. This mixture was incubated at 42°C for 60 minutes. After this reaction, the final volume was topped up to a total volume of 200 μ L with ultrapure water.

Ten microliters of the cDNA was directly subjected to PCR in the presence of a master mix containing PCR buffer, 1.5 mmol/L MgCl₂, 50 mmol/L KCl, 10 mmol/L TRIS-HCL (pH 8.3), gelatin (0.1%), 1 U AmpliTaq polymerase, 0.025 mmol/L dNTP, and 1 μ L from each cytokine primer for a total volume of 50 μ L (Table I).

The reaction tubes were placed for 3 minutes in the PCR block (MJ Research), which was preheated to 94°C to avoid cold oligodeoxyribonucleic acid fusion. After preparing the PCR mixture on ice, PCR was carried out for 35 cycles under the following conditions: denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. Final-cycle extension was at 72°C for 10 minutes.

The specificity of amplification was checked by assessing whether a fragment of the expected size had been obtained by gel (1.5%) electrophoresis or by southern blot hybridization with an internal 32P- γ ATP (Amersham)-labeled probe. The membranes were exposed to Kodak XAR films.

PCR reaction products were run on gel, isolated from gel, and cleaned up with the Wizard DNA Clean-Up System (Promega). The isolated DNA was used in a repeated PCR with the same cytokine-specific primers under the following conditions: 5 μ L of isolated PCR product, 10 μ L of 10 $\times$ Taq polymerase buffer, 3 μ L of 10 OD/mL sense primer and 3 μ L of 10 OD/mL antisense primer (Table I), 5 U Taq polymerase (Ampli Taq; Perkin Elmer Cetus, Norwalk, Conn), and 5 μ L of dNTP (2 mmol/L each of dATP, dCTP, dGTP, 1.3 mmol/L dTTP, and 0.7 mmol/L DIG-11-dUTP; Boehringer Mannheim) in a total volume of 100 μ L. The PCR protocol started with an adaptation of the hot start to avoid cold oligodeoxynucleic acid fusion. It was followed by 30 seconds at 94°C, 45 seconds at 55°C, and 1.5 minutes at 72°C during 200 cycles. The resulting digoxigenin-labeled DNA (probe) was controlled on 1.5% agarose gel. The specificity of amplification was checked by assessing whether a fragment of the expected size (approximately 25% larger in base pairs than the normal PCR product) had been obtained.

In situ hybridization

All reactions were carried out with RNase-free materials and solutions except for the RNase treatment of the negative controls. Each tissue specimen was cut into serial 6- μ m thick sections on a Reichert-Jung 2800e frigocut cryostat and transferred to poly-L-lysine (Sigma)-coated microscope slides, dried, and stored at -80°C. The slides were used within 3 months of storage, heated to room temperature, dried, and fixed in buffered 4% formalin for 15 minutes. The slides were rinsed twice with PBS for 5 minutes. Proteinase K (1 μ g/mL; Boehringer Mannheim) treatment was applied for 5 minutes at 37°C, followed by washing with PBS and fixing in buffered 4% formalin for 5 minutes to stop the proteinase K reaction. There then followed 2 more rinses in PBS for 5 minutes, permeabilization with 0.01% triton-X100, and a repeat washing with PBS. This was followed by dehydration by incubation, with increasing amounts of ethanol (70% for 2 minutes, 96% for 2 minutes, and 100% for 5 minutes) air drying. The mixture for hybridization was pipetted onto ice and contained 30 μ L 100% deionized formamide, 20 μ L 20 $\times$ sodium sodium citrate (SSC), 40 μ L denatured salmon sperm DNA (10 μ g/mL Tris-EDTA; Boehringer Mannheim), 1 μ L t-RNA (100 mg/mL), and 9 μ L diluted probe. The hybridization mix was incubated at 100°C for 1 minute to make the DNA single stranded and then cooled immediately on ice for 5 minutes. The sections were incubated with the hybridization mix at 42°C for 1 hour and then for 16 hours (overnight) at 37°C in a humidity room. After hybridization, the cells were washed with 30% formamide/2 $\times$ SSC at room temperature, 30% formamide/0.2 $\times$ SSC, at 42°C for 15 minutes, and PBS for 5 minutes. The slides were then placed in a semiautomatic stainer (Sequenza, Shandon). After this, the sections were incubated for 10 minutes with BSA 0.5% to 1% in PBS incubated with normal sheep serum (CLB, Netherlands) for 10 minutes and subsequently incubated with 1:500 alkaline phosphate-conjugated sheep-digoxigenin F(ab)2 fragments diluted in PBS-BSA 0.5% to 1% and normal human serum 10%. They were rinsed again with PBS for 5 minutes, rinsed with TRIS buffer (0.1 mol/L, pH 9.5) for 5 minutes, and incubated with NBT-BCIP substrate (Sigma). The incubation time is probe dependent. The substrate development was controlled with the microscope. Finally, the sections were rinsed in distilled water and mounted in glycerin-gelatin. Optimal probe concentration was obtained by dilution titration.

Controlling the method

The controls used were those previously described by Bloch et al.²⁸ To control the mRNA in situ hybridization, cryostat sections were incubated with RNase A (Boehringer Mannheim). This resulted in a significant signal reduction. Hybridization without a probe or unrelated probes gave no signal. Different cytokine probes resulted in different staining patterns.^{25,26}

Quantification

The biopsy specimens were coded, and 2 sections of each specimen were counted in a blinded fashion at a magnification of 400 $\times$. The surface area of the epithelium and the lamina propria of 2 sections were determined by computer image analysis (Kontron Image Analysis System Videoplan). The number of eosinophils and the number of mRNA-positive cells per square millimeter of the section area in the lamina propria was calculated.

Statistical analysis

The statistical analysis was performed with SPSS 7.5 for Windows 95. Friedman 2-way ANOVA was used for statistical analysis. The Wilcoxon signed-rank test was used for a comparison between baseline and several points after allergen provocation (Fig 2).

Correlation coefficients were obtained by using Spearman's rank method. Biopsy specimens taken at baseline represent the baseline level of symptoms and saline provocation (no symptoms were observed at these moments), and the biopsy specimens taken 1 hour after allergen provocation were compared with the early-phase symptomatology between 30 minutes and 1 hour; the specimens taken 24 hours after provocation were compared with the symptomatology described during 3 to 24 hours after provocation. The data of these 3 time points were correlated with each other ($n = 27$). Correlation between eosinophils and cytokine-expressing cells were made at the time of each biopsy ($n = 9$). Because of multiple testing, a P value of less than .025 was considered to indicate a significant correlation.

RESULTS

Nasal symptoms

No symptoms were observed before provocation or after provocation with saline. Nasal provocation with the allergen resulted in all patients having a significant increase in immediate nasal symptoms (eg, sneezes, itching, nasal blockage, and rhinorrhea). Between 6 and 12 hours after the allergen provocation, late-phase nasal symptoms, especially blockage and itching, were observed in all patients.

Microscopic evaluation

The cryostat sections of the nasal mucosa had an average surface area of 2 mm² and an intact epithelium. Staining with mAb for eosinophil major basic protein (BMK-13) could easily be identified by the red cytoplasmic granules and dark violet nucleus (Fig 1, *a-d*). Eosinophil numbers were determined in the lamina propria. The mRNA in situ hybridization staining patterns for cytokines varied from a dark purple circle to a large dark purple dot as shown in Fig 1, *e-h*. Fig 1 shows representative sections from nasal mucosal biopsy specimens expressing mRNA. mRNA-positive cells were located among the inflammatory cell infiltrates within the lamina propria. The majority of cells showing hybridization signals for cytokines were present in the

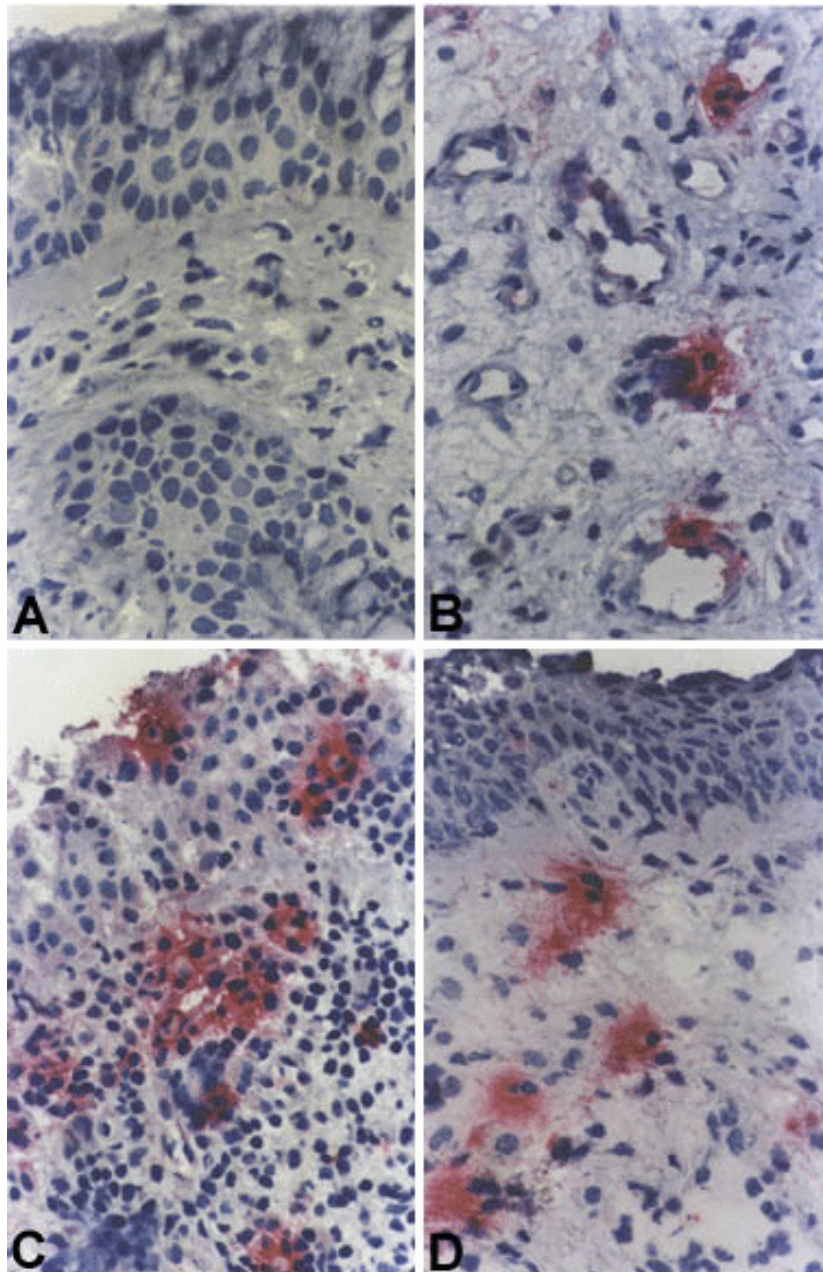


FIG 1. Photomicrographs of 4 nasal mucosal biopsy sections immunohistochemically stained with BMK-13 antibodies against major basic protein. Before allergen provocation, almost no eosinophils were present (**a**); 1 hour after allergen provocation, eosinophil extravasation into the nasal tissue was observed (**b**); and 24 hours after allergen provocation in the epithelium, as well as in the lamina propria, eosinophils were present (**c**). **d**, One week after allergen provocation, the most eosinophils were present in the lamina propria (magnification $\times 550$).

subepithelial layer. The number of positive cells was only determined in the lamina propria, because the number of positive cells in the epithelium was too small for statistical analysis.

Changes after allergen provocation

Changes after allergen provocation are shown in Fig 2. Eosinophils increased after allergen provocation ($P <$

.002). Before provocation, very small numbers of eosinophils were found, mainly in the deep layers of the lamina propria. Within 1 hour after provocation, a remarkable increase in eosinophils was observed. Eosinophils were still present 1 week after the provocation. Significantly higher eosinophil numbers were observed compared with baseline 1 week after provocation ($P = .008$).

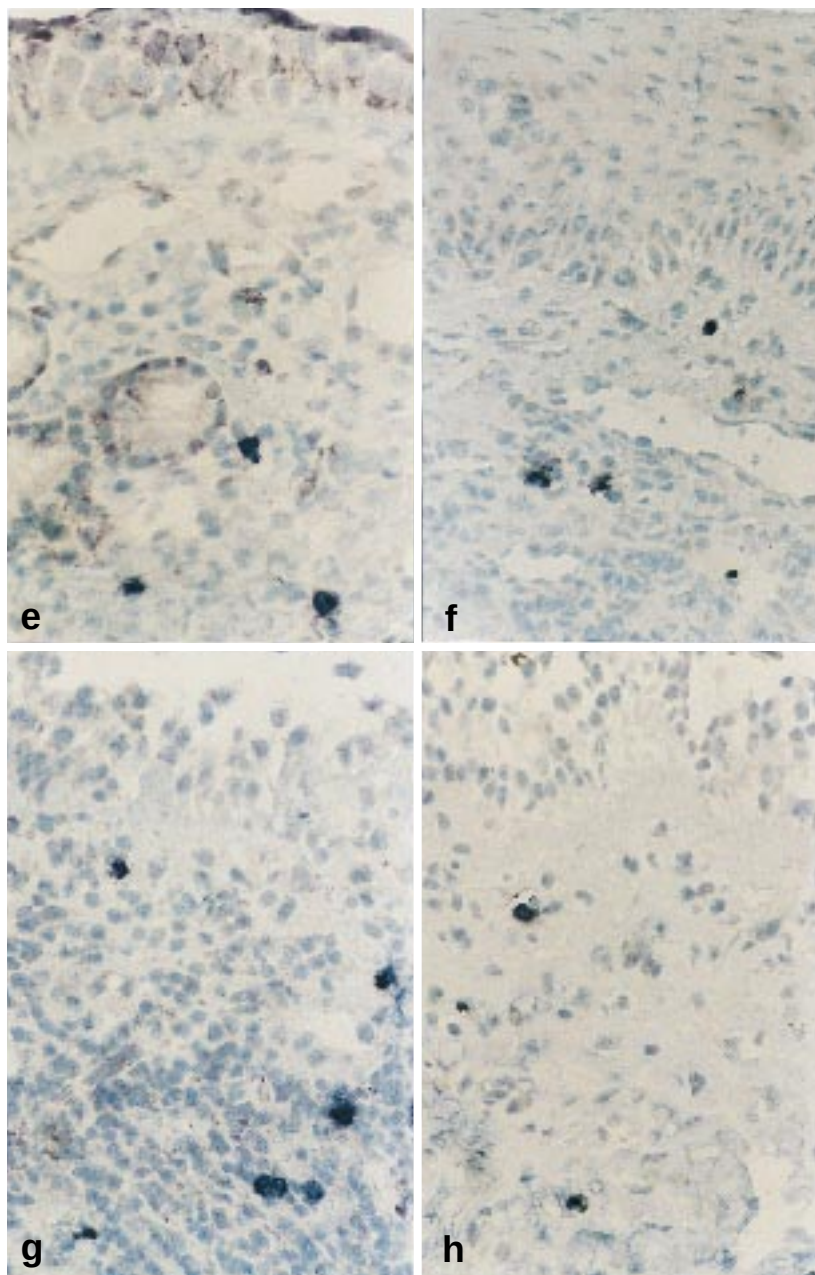


FIG 1. CONT. **e-h**, Representative photomicrographs of in situ hybridization of nasal biopsy sections from patients with allergic rhinitis after allergen challenge with digoxigenin-labeled DNA probes coding for IL-3 (**e**), IL-10 (**f**), IL-13 (**g**), and RANTES (**h**). mRNA-positive cells exhibited a dark purple staining and displayed optimal cellular and subcellular resolution (magnification $\times 550$).

IL-2, IL-3, IL-4, IL-5, IL-6, IFN- γ , and TNF- α mRNA-expressing cells showed no significant changes after allergen provocation. Numbers of TNF- α -expressing cells decreased 24 hours after allergen provocation, but not to a significant extent. Numbers of chemokine IL-8 ($P = .02$) and RANTES ($P < .01$) mRNA-positive cells increased significantly after allergen provocation. The dynamics for both IL-8 (Fig 2, *g*) and RANTES (Fig 2, *k*) were the same after allergen provocation (ie,

an increase only 1 hour after allergen provocation, which remained relatively stable at 24 hours and 1 week). The T_H2 cytokine IL-13 ($P < .04$) increased significantly, and this effect remained significant until 1 week after provocation. There was a tendency toward an increase in IL-10 mRNA-positive cells ($P < .06$) after allergen provocation. Here also, a significant increase was found 1 week after allergen provocation compared with baseline ($P = .04$).

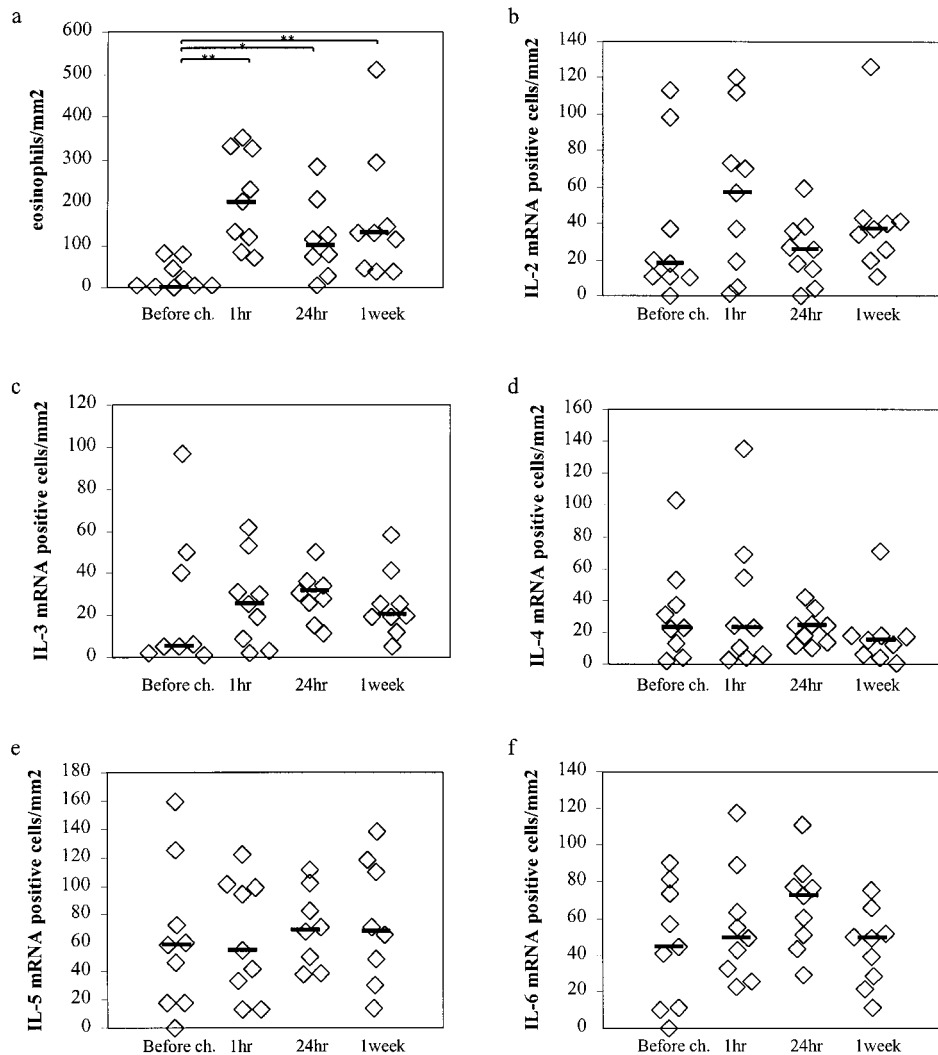


FIG 2. Number of eosinophils (**a**) and mRNA-positive cells (**b-f**) in nasal mucosa biopsy specimens. Nasal mucosa biopsy specimens were taken before provocation, 1 hour after provocation, 24 hours after provocation, and 1 week after provocation. Allergen challenge (*ch*) was performed with 1000 and 10,000 BU grass pollen.

TABLE II. Eosinophil correlations with cytokines

	Eosinophils correlated (1 h after provocation)	Eosinophils correlated (24 h after provocation)
IL-2	0.6 (.1)	0.2 (.7)
IL-3	0.7 (.04)	0.2 (.7)
IL-4	0.7 (.5)	0.5 (.2)
IL-5	0.3 (.5)	0.8 (.009)
IL-6	0.2 (.6)	0.2 (.7)
IL-8	0.6 (.1)	-0.3 (.5)
IL-10	0.2 (.5)	0.3 (.5)
IL-13	0.3 (.4)	0.04 (.9)
RANTES	0.8 (.025)	0.7 (.007)
TNF- α	0.7 (.2)	0.8 (.8)
IFN- γ	0.4 (.02)	0.1 (.007)

Correlations were between the number of BMK-13-positive cells compared with the number of cells expressing positive hybridization signals at 1 hour and 24 hours after provocation, and all values are regression (*P*) values.

Cellular relationship to nasal symptoms

Correlations were found between numbers of eosinophils in biopsy sections, and nasal symptom scores were as follows: itching, 0.654 ($P < .001$); discharge, 0.439 ($P = .025$); sneezing, 0.543 ($P < .001$); and total symptom score, 0.445 ($P = .02$).

Correlations were found between numbers of IL-8 mRNA-expressing cells in biopsy sections, and nasal symptom scores were as follows: blockage, 0.468 ($P = .02$); discharge, 0.639 ($P = .001$); and total symptom score, 0.539 ($P < .001$). Correlations were observed between RANTES in biopsy sections, and the nasal symptom score for itching was 0.580 ($P = .002$). Correlations were also observed for IL-10 in biopsy sections, and the nasal symptom score for discharge was 0.512 ($P = .007$).

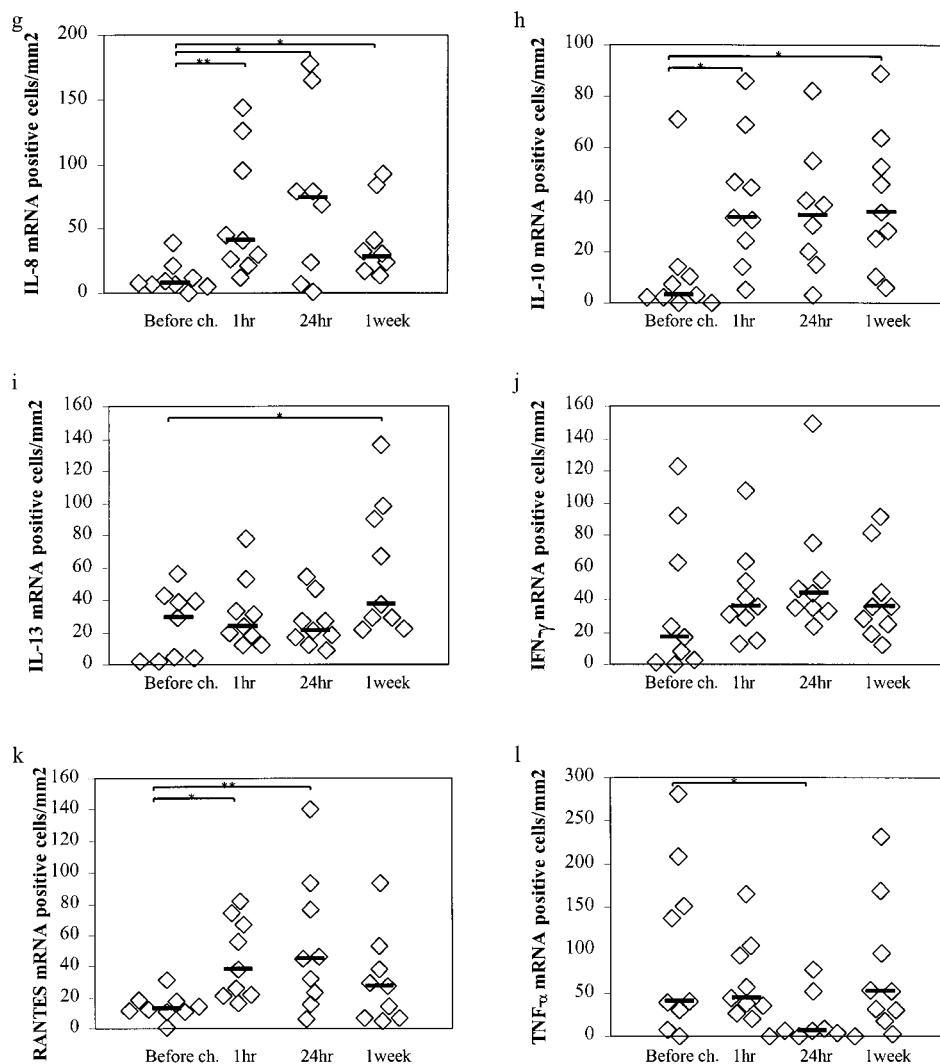


FIG 2. CONT.

Cytokine mRNA relationship to tissue eosinophilia

Before allergen provocation, no significant correlations were observed between the number of cells expressing mRNA for cytokines and the number of eosinophils. In the early phase a significant correlation was observed between the number of eosinophils and the number of cells expressing mRNA for RANTES. Twenty-four hours after allergen provocation, correlations were observed between eosinophils and IL-5, RANTES, and IFN- γ mRNA-positive cell numbers. A correlation between eosinophils and IL-6 ($r = 0.8$, $P = .007$) was observed 1 week after allergen provocation (for r [regression] and P values, see Table II).

DISCUSSION

The effect of a single allergen provocation with grass pollen on cytokine mRNA-expressing cells in nasal

mucosal biopsy specimens was investigated and compared with tissue eosinophilia and symptomatology. Digoxigenin-labeled probes were used because they have almost unlimited shelf life and provide optimal cellular and subcellular resolution compared with radioactive probes.²⁸ DNA probes were used because it is easier to avoid DNase than RNase when manufacturing these digoxigenin probes.

Single allergen provocation was used as a model for studying allergic inflammation in the upper airways. In this study a 50- μ L allergen solution was sprayed into each nostril (11,000 BU in total), as described above.^{4,5,29}

To rule out the chance of nonspecific changes caused by provocation with PBS or the biopsy specimen, a second study was performed. In this study patients were sprayed with a PBS solution containing benzalkonium chloride, which was similar to the diluent of the allergen solution. Biopsy specimens were taken before and 24 hours after the last PBS provocation. Eosinophils, RANTES, and IL-8 were evaluated at both time points.

TABLE III. Cytokine mRNA-positive cell fluctuation after allergen provocation in nasal mucosal biopsy specimens from patients with hay fever

Cytokine	Suspected cell targets in allergic inflammation	Allergen provocation	Correlation with eosinophilia
IL-2	T cells and B cells (proliferation, activation, and differentiation)	—	—
IL-3	Mast cells and eosinophils (colony-stimulating factor)	—	—
IL-4	B cells (IgE-isotype switch), endothelium	—	—
IL-5	Eosinophils (differentiation, activation, and survival)	—	+
IL-6	T cells, B cells, (proliferation and differentiation) and fibroblasts	—	—
IL-8	Lymphocytes, neutrophils, basophils, and eosinophils (chemotaxis)	↑	—
IL-10	Macrophages and T cells (inhibitor of IFN- γ functions)	↑	—
IL-13	B cells (IgE-isotype switch)	↑	—
IFN- γ	Macrophages (activation) and IL-4 antagonist (IgE isotype switch)	—	±
RANTES	Eosinophils and monocytes (chemotaxis)	↑	+
TNF- α	Fibroblasts and endothelium (production of other cytokines and adhesion molecules [ELAM-1, ICAM-1])	—	—

ICAM-1, Intracellular adhesion molecule-1; ELAM-1, endothelial leucocyte adhesion molecule-1.

No significant differences (Wilcoxon signed-rank test) were found by comparing specimens taken before and after PBS provocation.

IL-2, a T_{H1} cytokine, plays a role in the proliferation, activation, and differentiation of T cells and B cells. No changes were observed in the numbers of IL-2 mRNA-positive cells. Once again, it was shown that this T_{H1} cytokine was not involved in allergic inflammation during allergen provocation. These findings confirm other reports that did not find a change in IL-2 (on protein and mRNA level) in the nose,^{16,30} lung,^{31,32} and skin³³ after allergen challenge.

IL-4 and IL-13 have similar functions in B-cell isotype switching to IgE and also have a pivotal role in the upregulation of intracellular adhesion molecule-1 and vascular cell adhesion molecule-1 expression on endothelial cells.³⁴ We failed to find an increase in IL-4 during allergen provocation. This confirms data from the lower airways, in which no differences were found between IL-4 protein-positive bronchoalveolar lavage fluid T cells in asthmatic subjects compared with nonallergic patients with asthma or control subjects,³¹ and also no IL-4 production/upregulation was found after allergen challenge.³² Bradding et al¹² also found no upregulation of IL-4 protein in patients with seasonal allergic rhinitis in the hay fever season compared with those patients out of the hay fever season. Those results contradict the findings of Durham et al^{9,16,35} who used filter-disk nasal allergen provocation, which might be a stronger stimulus. The local allergen concentration in this filter-disk provocation is higher, and the filter disk is held in position for 10 minutes, implying that mucociliary clearance cannot remove allergens. By contrast with IL-4 cells, we found a significant increase in the number of IL-13 mRNA-positive cells. These findings confirm data from Ghaffar¹⁷ for the nasal mucosa and Ying³⁶ for the skin.

IL-3 and IL-5 are T_{H2} cytokines that stimulate the growth of mast cells and eosinophils. They are found to be more numerous in allergic patients compared with nonallergic control subjects and are found to be increased in allergic patients after allergen provocation.^{9,33,37-39}

This study found no significant increase for IL-5 mRNA-positive cells after allergen provocation. This can probably be explained by the large range and high baseline levels in IL-5 mRNA-positive cell numbers. A clear correlation was observed between eosinophils and IL-5 24 hours after allergen provocation.

The chemokines RANTES and IL-8 are proinflammatory cytokines that are important in eosinophil chemotaxis. A significant increase of RANTES and IL-8 mRNA-expressing cells was observed after allergen challenge. Similar observations were made for protein level in nasal lavage fluid,^{11,40-42} bronchoalveolar lavage fluid,³² and for RANTES mRNA and RANTES protein-positive cells in nasal biopsy specimens of allergic patients after local allergen challenge.²⁰ The number of TNF- α mRNA-positive cells was unchanged after provocation, indicating that TNF- α is not involved in the allergen-induced reaction. This data confirms that TNF- α plays a role in bacterial infections but not in allergic inflammation, as is indicated by studies in nasal polyps²² and bacterial infections.^{43,44} This is despite the fact that one study found an increase in TNF- α mRNA-positive cells after filter-disk allergen provocation²¹ and once an increase was found, 2 hours after allergen provocation in nasal lavage fluid on the protein level.¹³

There was no significant increase in IL-6 after allergen provocation. On the protein level (protein-positive cells) in a number of studies, no differences were observed in the upper airways between allergic patients and control subjects,⁴⁵ either before or after allergen provocation.¹² However, in nasal lavage fluids, IL-6 protein levels were found to be increased during the late-phase reaction.^{13,41}

Numbers of IL-10 mRNA-positive cells were increased after allergen provocation. IL-10, which is considered to be a T_{H2} cytokine, inhibits the release of IFN- γ ⁴⁶ and generally inhibits cytokine synthesis by human monocytes.⁴⁷ IFN- γ , which is considered to be a T_{H1} cytokine, did not show a significant increase after allergen provocation, thereby confirming Durham's data.⁹

The number of eosinophils correlated with nasal symp-

toms was previously observed by Pipkorn et al.⁴⁸ Furthermore, correlation between the chemokines (IL-8 and RANTES) and nasal symptoms confirms the data from Douglass et al,⁴⁹ who observed an increase in rhinitis symptomatology after IL-8 challenge in the nasal mucosa.

We used eosinophils as an indication of the amount of allergic inflammation. In the early phase only the numbers of RANTES and IFN- γ mRNA-positive cells were significantly correlated with eosinophil numbers. The symptomatology of patients in the early phase after allergen provocation is mainly caused by the release of histamine and other inflammatory mediators by mast cells.⁵⁰ However, in the late phase significant correlations were found between eosinophils and IL-5 and eosinophils and RANTES. One week after a single allergen provocation, there was still significant tissue eosinophilia and a significant increase in IL-13 and IL-10 mRNA-positive cells.

The correlation of RANTES with eosinophils in the early and late phases indicate that RANTES may be as important as IL-5 for eosinophil recruitment. In conclusion, nasal allergen provocation in patients with allergic rhinitis induced significant tissue eosinophilia and a significant increase in IL-8, IL-13, and RANTES mRNA-positive cells (Table III). This increase in eosinophils and IL-10 and IL-13 mRNA-positive cells could still be observed 1 week after allergen provocation.

We thank Dr S. P. Thomas for his textual advice.

REFERENCES

- Mygind N. Pathophysiology of allergic rhinitis. *Eur Respir Rev* 1994;4:247-7.
- Fokkens WJ, Godthelp T, Holm AF, Blom H, Mulder PG, Vroom TM, et al. Dynamics of mast cells in the nasal mucosa of patients with allergic rhinitis and non-allergic controls: a biopsy study. *Clin Exp Allergy* 1992;22:701-10.
- Godthelp T, Holm AF, Fokkens WJ, Doornenbal P, Mulder PG, Hoefsmid EC, et al. Dynamics of nasal eosinophils in response to a nonnatural allergen challenge in patients with allergic rhinitis and control subjects: a biopsy and brush study. *J Allergy Clin Immunol* 1996;97:800-11.
- Godthelp T, Fokkens WJ, Kleinjan A, Holm AF, Mulder PG, Prens EP, et al. Antigen presenting cells in the nasal mucosa of patients with allergic rhinitis during allergen provocation. *Clin Exp Allergy* 1996;26:677-88.
- Varney VA, Jacobson MR, Sudderick RM, Robinson DS, Irani AM, Schwartz LB, et al. Immunohistology of the nasal mucosa following allergen-induced rhinitis. Identification of activated T lymphocytes, eosinophils, and neutrophils. *Am Rev Respir Dis* 1992;146:170-6.
- Bentley AM, Jacobson MR, Cumberworth V, Barkans JR, Moqbel R, Schwartz LB, et al. Immunohistology of the nasal mucosa in seasonal allergic rhinitis: increases in activated eosinophils and epithelial mast cells. *J Allergy Clin Immunol* 1992;89:877-83.
- Karlsson MG, Davidsson A, Hellquist HB. Increase in CD4+ and CD45RO+ memory T cells in the nasal mucosa of allergic patients. *APMIS* 1994;102:753-8.
- Igarashi Y, Goldrich MS, Kaliner MA, Irani AM, Schwartz LB, White MV. Quantitation of inflammatory cells in the nasal mucosa of patients with allergic rhinitis and normal subjects. *J Allergy Clin Immunol* 1995;95:716-25.
- Durham SR, Ying S, Varney VA, Jacobson MR, Sudderick RM, Mackay IS, et al. Cytokine messenger RNA expression for IL-3, IL-4, IL-5, and granulocyte/macrophage-colony-stimulating factor in the nasal mucosa after local allergen provocation: relationship to tissue eosinophilia. *J Immunol* 1992;148:2390-4.
- Bachert C, Hauser U, Prem B, Rudack C, Ganzer U. Proinflammatory cytokines in allergic rhinitis. *Eur Arch Otorhinolaryngol Suppl* 1995;1:S44-9.
- Sim TC, Reece LM, Hilsmeier KA, Grant JA, Alam R. Secretion of chemokines and other cytokines in allergen-induced nasal responses: inhibition by topical steroid treatment. *Am J Respir Crit Care Med* 1995;152:927-33.
- Bradding P, Feather IH, Wilson S, Holgate ST, Howarth PH. Cytokine immunoreactivity in seasonal rhinitis: regulation by a topical corticosteroid. *Am J Respir Crit Care Med* 1995;151:1900-6.
- Bachert C, Wagenmann M, Holtappels G. Cytokines and adhesion molecules in allergic rhinitis. *Am J Rhinol* 1998;12:1-8.
- Romagnani S. Human TH1 and TH2 subsets: doubt no more. *Immunol Today* 1991;12:256-7.
- Hamilos DL, Leung DY, Wood R, Cunningham L, Bean DK, Yasruel Z, et al. Evidence for distinct cytokine expression in allergic versus nonallergic chronic sinusitis. *J Allergy Clin Immunol* 1995;96:537-44.
- Masuyama K, Jacobson MR, Rak S, Meng Q, Sudderick RM, Kay AB, et al. Topical glucocorticosteroid (fluticasone propionate) inhibits cells expressing cytokine mRNA for interleukin-4 in the nasal mucosa in allergen-induced rhinitis. *Immunology* 1994;82:192-9.
- Ghaffar O, Laberge S, Jacobson MR, Lowhagen O, Rak S, Durham SR, et al. IL-13 mRNA and immunoreactivity in allergen-induced rhinitis: comparison with IL-4 expression and modulation by topical glucocorticoid therapy. *Am J Respir Cell Mol Biol* 1997;17:17-24.
- Baggiolini M, Dahinden CA. CC chemokines in allergic inflammation. *Immunol Today* 1994;15:127-33.
- Alam R, Stafford S, Forsythe P, Harrison R, Faubion D, Lett-Brown MA, et al. RANTES is a chemotactic and activating factor for human eosinophils. *J Immunol* 1993;150:3442-8.
- Rajakulasingham K, Hamid Q, O'Brien F, Shotman E, Jose PJ, Williams TJ, et al. RANTES in human allergen-induced rhinitis: cellular source and relation to tissue eosinophilia. *Am J Respir Crit Care Med* 1997;155:696-703.
- Ying S, Robinson DS, Varney V, Meng Q, Tsicopoulos A, Moqbel R, et al. TNF- α mRNA expression in allergic inflammation. *Clin Exp Allergy* 1991;21:745-50.
- Hamilos DL, Leung DY, Wood R, Bean DK, Song YL, Schotman E, et al. Eosinophil infiltration in nonallergic chronic hyperplastic sinusitis with nasal polyposis (CHS/NP) is associated with endothelial VCAM-1 upregulation and expression of TNF- α . *Am J Respir Cell Mol Biol* 1996;15:443-50.
- Saito H, Asakura K, Ogasawara H, Watanabe M, Kataura A. Topical antigen provocation increases the number of immunoreactive IL-4-, IL-5- and IL-6-positive cells in the nasal mucosa of patients with perennial allergic rhinitis. *Int Arch Allergy Immunol* 1997;114:81-5.
- Fokkens WJ, Vroom TM, Gerritsma V, Rijntjes E. A biopsy method to obtain high quality specimens of nasal mucosa. *Rhinology* 1988;26:293-5.
- Klein SC, van Wichen DF, Golverdingen JG, Jacobse KC, Broekhuizen R, de Weger RA. An improved, sensitive, non-radioactive in situ hybridization method for the detection of cytokine mRNAs. *APMIS* 1995;103:345-53.
- Van Hoffen E, Van Wichen D, Stuij I, De Jonge N, Klopping C, Lahpor J, et al. In situ expression of cytokines in human heart allografts. *Am J Pathol* 1996;149:1991-2003.
- Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 1987;162:156-9.
- Bloch B. Biotinylated probes for in situ hybridization histochemistry: use for mRNA detection. *J Histochem Cytochem* 1993;41:1751-4.
- Rajakulasingham K, Durham SR, O'Brien F, Humbert M, Barata LT, Reece L, et al. Enhanced expression of high-affinity IgE receptor (Fc ϵ RI) α chain in human allergen-induced rhinitis with co-localization to mast cells, macrophages, eosinophils, and dendritic cells. *J Allergy Clin Immunol* 1997;100:78-86.
- Moss RB, Nagata H, Goff J, Okubo K, Hausefeld J, Kaliner MA. Constitutive mRNA and immunoreactivity for IL-2 in human nasal mucosa. *Clin Exp Allergy* 1997;27:406-12.
- Krug N, Madden J, Redington AE, Lackie P, Djukanovic R, Schauer U, et al. T-cell cytokine profile evaluated at the single cell level in BAL and blood in allergic asthma. *Am J Respir Cell Mol Biol* 1996;14:319-26.
- Virchow JC Jr, Walker C, Hafner D, Kortsik C, Werner P, Matthys H, et al. T cells and cytokines in bronchoalveolar lavage fluid after segmental allergen provocation in atopic asthma. *Am J Respir Crit Care Med* 1995;151:960-8.

33. Kay AB, Ying S, Varney V, Gaga M, Durham SR, Moqbel R, et al. Messenger RNA expression of the cytokine gene cluster, interleukin 3 (IL-3), IL-4, IL-5, and granulocyte/macrophage colony-stimulating factor, in allergen-induced late-phase cutaneous reactions in atopic subjects. *J Exp Med* 1991;173:775-8.
34. Jahnsen FL, Brandtzaeg P, Haye R, Haraldsen G. Expression of functional VCAM-1 by cultured nasal polyp-derived microvascular endothelium. *Am J Pathol* 1997;150:2113-23.
35. Ying S, Durham SR, Jacobson MR, Rak S, Masuyama K, Lowhagen O, et al. T lymphocytes and mast cells express messenger RNA for interleukin-4 in the nasal mucosa in allergen-induced rhinitis. *Immunology* 1994;82:200-6.
36. Ying S, Humbert M, Barkans J, Corrigan CJ, Pfister R, Menz G, et al. Expression of IL-4 and IL-5 mRNA and protein product by CD4+ and CD8+ T cells, eosinophils, and mast cells in bronchial biopsies obtained from atopic and nonatopic (intrinsic) asthmatics. *J Immunol* 1997;158:3539-44.
37. Ying S, Durham SR, Barkans J, Masuyama K, Jacobson M, Rak S, et al. T cells are the principal source of interleukin-5 mRNA in allergen-induced rhinitis. *Am J Respir Cell Mol Biol* 1993;9:356-60.
38. Terada N, Konno A, Fukuda S, Yamashita T, Shirotori K, Okamoto Y, et al. Interleukin-5 gene expression in nasal mucosa and changes in amount of interleukin-5 in nasal lavage fluid after antigen challenge. *Acta Otolaryngol (Stockh)* 1994;114:203-8.
39. Humbert M, Durham SR, Ying S, Kimmitt P, Barkans J, Assoufi B, et al. IL-4 and IL-5 mRNA and protein in bronchial biopsies from patients with atopic and nonatopic asthma: evidence against "intrinsic" asthma being a distinct immunopathologic entity. *Am J Respir Crit Care Med* 1996;154:1497-504.
40. Gosset P, Tillie-Leblond I, Malaquin F, Durieu J, Wallaert B, Tonnel AB. Interleukin-8 secretion in patients with allergic rhinitis after an allergen challenge: interleukin-8 is not the main chemotactic factor present in nasal lavages. *Clin Exp Allergy* 1997;27:379-88.
41. Weido AJ, Reece LM, Alam R, Cook CK, Sim TC. Intranasal fluticasone propionate inhibits recovery of chemokines and other cytokines in nasal secretions in allergen-induced rhinitis. *Ann Allergy Asthma Immunol* 1996;77:407-15.
42. Bachert C, Wagenmann M, Hauser U. Proinflammatory cytokines: measurement in nasal secretion and induction of adhesion receptor expression. *Int Arch Allergy Immunol* 1995;107:106-8.
43. Fong Y, Lowry SF. Tumor necrosis factor in the pathophysiology of infection and sepsis. *Clin Immunol Immunopathol* 1990;55:157-70.
44. Cybulsky MI, Chan MK, Movat HZ. Acute inflammation and microthrombosis induced by endotoxin, interleukin-1, and tumor necrosis factor and their implication in gram-negative infection. *Lab Invest* 1988;58:365-78.
45. Noah TL, Henderson FW, Henry MM, Peden DB, Devlin RB. Nasal lavage cytokines in normal, allergic, and asthmatic school-age children. *Am J Respir Crit Care Med* 1995;152:1290-6.
46. Pene J, Rousset F, Briere F, Chretien I, Bonnefoy JY, Spits H, et al. IgE production by normal human lymphocytes is induced by interleukin 4 and suppressed by interferons γ and α and prostaglandin E2. *Proc Natl Acad Sci U S A* 1988;85:6880-4.
47. de Waal Malefijt T. Molecular aspects of human T cell activation and lymphokine production [thesis]. Leiden: University of Leiden; 1991.
48. Pipkorn U, Karlsson G, Enerback L. Nasal mucosal response to repeated challenges with pollen allergen. *Am Rev Respir Dis* 1989;140:729-36.
49. Douglass JA, Dhami D, Gurr CE, Bulpitt M, Shute JK, Howarth PH, et al. Influence of interleukin-8 challenge in the nasal mucosa in atopic and nonatopic subjects. *Am J Respir Crit Care Med* 1994;150:1108-13.
50. Busse WW, Holgate ST, editors. *Asthma and rhinitis*. Boston: Blackwell Scientific Publications; 1995.
51. Taniguchi T, Matsui H, Fujita T, Takaoka C, Kashima N, Yoshimoto R, et al. Structure and expression of a cloned cDNA for human interleukin-2. *Nature* 1983;302:305-10.
52. Yang YC, Ciarletta AB, Temple PA, Chung MP, Kovacic S, Witek-Gianotti JS, et al. Human IL-3 (multi-CSF): identification by expression cloning of a novel hematopoietic growth factor related to murine IL-3. *Cell* 1986;47:3-10.
53. Tanabe T, Konishi M, Mizuta T, Noma T, Honjo T. Molecular cloning and structure of the human interleukin-5 gene. *J Biol Chem* 1987;262:16580-4.
54. Taga T, Kishimoto T. Interleukin-6. In: Aggarwal BB, Puri RK, editors. *Human cytokines: their role in disease and therapy*. Cambridge: Blackwell Scientific; 1995. p. 142-53.
55. Mukaida N, Shiroo M, Matsushima K. Genomic structure of the human monocyte-derived neutrophil chemotactic factor IL-8. *J Immunol* 1989;143:1366-71.
56. Vieira P, de Waal-Malefijt R, Dang MN, Johnson KE, Kastelein R, Fiorentino DF, et al. Isolation and expression of human cytokine synthesis inhibitory factor cDNA clones: homology to Epstein-Barr virus open reading frame BCRF1. *Proc Natl Acad Sci U S A* 1991;88:1172-6.
57. McKenzie AN, Li X, Largaespada DA, Sato A, Kaneda A, Zurawski SM, et al. Structural comparison and chromosomal localization of the human and mouse IL-13 genes. *J Immunol* 1993;150:5436-44.
58. Schall TJ, Jongstra J, Dyer BJ, Jorgensen J, Clayberger C, Davis MM, et al. A human T cell-specific molecule is a member of a new gene family. *J Immunol* 1988;141:1018-25.
59. Wang AM, Creasey AA, Ladner MB, Lin LS, Strickler J, Van Arsdel JN, et al. Molecular cloning of the complementary DNA for human tumor necrosis factor. *Science* 1985;228:149-54.