

The effect of short-term immunotherapy with molecular standardized grass and rye allergens on eosinophil cationic protein and tryptase in nasal secretions

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Background: Activation of mast cells and eosinophils under pollen exposure can be inhibited by specific immunotherapy. **Objective:** The effect of short-term immunotherapy with 7 preseasonal injections of molecular standardized allergens from grass and rye pollen on eosinophil cationic protein (ECP) and tryptase levels in nasal secretions has been compared with symptomatic drug treatment in an open, randomized study with 48 patients.

Methods: Nasal reactivity and mediator levels in nasal secretions were measured at baseline, before season, in season, and after season.

Results: Symptom scores in the immunotherapy group were 134.5 (95% CI, 65 to 336) versus 386.0 (95% CI, 185 to 563), significantly lower as in the drug-treated group. ECP and tryptase levels increased significantly during natural allergen exposition. The seasonal levels in the immunotherapy group were significantly lower than in the drug-treated group with 272.1 ng/mL (252.0 to 293.9 ng/mL; immunotherapy) versus 470.4 ng/mL (SEM, 435.6 to 508.0 ng/mL; drugs) for ECP and with 8.73 ng/mL (SEM, 8.20 to 9.29 ng/mL) versus 17.47 ng/mL (16.42 to 18.60 ng/mL) for tryptase (all, $P < .001$). The ECP level induced by nasal provocation was 105.6 ng/mL (99.0 to 112.6 ng/mL) versus 180.4 ng/mL (169.2 to 192.4 ng/mL), significantly lower ($P < .001$) in the immunotherapy group, as was the tryptase level with 12.12 ng/mL (11.53 to 12.75 ng/mL) versus 8.19 ng/mL (7.79 to 8.62 ng/mL; $P < .001$) at the after-season visit.

Conclusion: Short-term immunotherapy is able to reduce tryptase and ECP in nasal secretions more effectively than drug treatment in patients with allergic rhinitis. (*J Allergy Clin Immunol* 1999;103:47-53.)

Key words: Short-term immunotherapy, specific immunotherapy, allergic rhinitis, grass pollen, rye pollen, eosinophil cationic protein, tryptase

Abbreviations used

ANCOVA:	Analysis of covariance
BU:	Biologic units
DSCG:	Disodium cromoglycate
HEP:	Histamine equivalent in skin prick test
NPT:	Nasal provocation test
SE:	Specific units of short-term immunotherapy
SPT:	Skin prick test
STI:	Short-term immunotherapy
T0:	Control investigation at baseline
T1:	Control investigation before season
T2:	Control investigation in season
T3:	Control investigation after season

The clinical efficacy of specific immunotherapy in pollen-induced allergic rhinitis was demonstrated in several controlled clinical trials.¹⁻⁵ Moreover, immunologic changes induced by successful immunotherapy provide evidence that immunotherapy interferes with pathophysiologic mechanisms responsible for mediator release and subsequent inflammatory changes in allergic diseases.⁴⁻¹⁰ These mechanisms are to a great extent related to mast cells and eosinophils, and an effect on these cells is postulated in successful immunotherapy.^{4,9-13}

Immunotherapy should be preferably applied as a perennial treatment; patient compliance, however, is asserted to be higher during a short preseasonal treatment.⁶ The efficacy and safety of short-term immunotherapy (STI) with 7 injections of molecular standardized grass pollen allergens has been demonstrated in a previous placebo-controlled, double-blind study.¹⁴ To investigate the effect of STI on cell activation markers for mast cells and eosinophils, we determined tryptase and eosinophil cationic protein (ECP) levels in the course of natural pollen exposure and with an allergen challenge model in an open randomized study, with drug-treated patients as the control group.

METHODS

Patients

Forty-eight patients in the age range of 15 to 50 years who were allergic to grass and/or rye pollen, confirmed by a wheal size of 5 mm or greater in the skin prick test (SPT), were enrolled into the study. Patients had typical rhinoconjunctivitis symptoms in the grass pollen season from May to August (Table I). Patients with a

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Received for publication Dec 23, 1997; revised Aug 3, 1998; accepted for publication Aug 4, 1998.

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0091-6749/99 \$8.00 + 0 1/1/93580

TABLE I. Clinical data of the patients

	Treatment	
	STI	Symptomatic
Patients (no)	24	24
Sex		
Male	15	16
Female	9	8
Mean age (range; y)	30.5 (21-49)	30.7 (15-50)
Median duration of allergy (range; y)	13.0 (3-39)	12.5 (1-30)
Median score of symptoms (range)		
Eyes (score; range, 0-9)	7.0 (3-8)	5.0 (1-8)
Nose (score; range, 0-12)	8.5 (5-10)	8.0 (5-10)
Bronchial symptoms		
None (0)	14	16
Mild (1)	5	2
Moderate (2)	5	6
Severe (3)	—	—

need for the treatment of allergic asthma, perennial rhinitis, and/or acute infected nasal mucosa; patients treated chronically with systemic glucocorticosteroids; and patients treated by specific immunotherapy in the past 3 years were excluded. Other exclusion criteria were the known contraindications of immunotherapy according to the European Academy of Allergology and Clinical Immunology Immunotherapy Position Paper.⁶

Symptoms were recorded by the patients in diaries for symptoms at the eyes (itching, running, reddening), the nose (congestion, itching, rhinorrhea, sneezing), and bronchi by a score 0 to 3 from April to September 1995. Symptoms were compared by cumulated scores for the period of exposure with grass pollen. The study was performed according to European community good clinical practice guidelines¹⁵ and the Declaration of Helsinki.¹⁶ The study was approved by the ethics committee of the Landesärztekammer Rheinland-Pfalz, Mainz, Germany.

Allergen extracts

The grass and rye allergen extracts used for STI were partially purified and standardized extracts¹⁷ composed of equal parts of the 6 grasses (*Dactylis glomerata*, *Lolium perenne*, *Avena elatior*, *Phleum pratense*, *Poa pratensis*, *Festuca pratensis*) and rye (*Secale cereale*). Extracts for STI, nasal provocation test (NPT), and SPT were manufactured by ALK A/S, Hørsholm, Denmark. The allergen extracts are standardized relative to an internal standard by qualitative and quantitative immunoelectrophoresis for characterization and quantitation of the major allergens and by luminescence immunoassay inhibition for total allergenic activity. The biologic activity of the extracts used was determined by SPT, as previously described.^{18,19} The potency was expressed in arbitrary specific units of STI (SE) units; 1000 SE contains approximately 1.5 µg (between 1.0 and 2.0 µg for individual grasses) grass group 5 major allergen.

For STI, a depot extract adsorbed to aluminium hydroxide (0.5% phenol) was used (ALK7 Gräsermischung und Roggen; ALK-SCHERAX, Hamburg, Germany) and an identically manufactured, partly purified extract of equal components of the 6 grasses without rye for SPT (ALK prick SQ/soluprick SQ, 10 histamine equivalent in SPT, 10,000 biologic units (BU)/mL, dissolved in 50% glycerol) and for NPT (Aquagen SQ/ALK lyophilisiert SQ, 10 to 100,000 SQ

units/mL). STI was applied out of 2 vials with the concentrations 30 and 1000 SE/mL. All extracts were stored at 4°C until used; the stability assigned by the manufacturer was much longer than the total duration of the study.

STI

Patients treated by STI received 7 injections in weekly intervals before the expected beginning of the grass pollen season with ascending concentrations (3, 9, 30, 100, 300, 600, 1000 SE). Dose modifications were allowed for medical indications according to the routine procedure of specific immunotherapy.⁶ Patients with a modified dosage could be treated by additional injections to reach the maximum dose, but all injections had to be applied before grass pollen exposure. Concomitant symptomatic treatment was allowed as described later.

Pharmacotherapy

For patients in both groups, a drug treatment with disodium cromoglycate (DSCG) eyedrops and nasal sprays, local or systemic antihistamines, sympathomimetics, and local glucocorticosteroids was prescribed according to the stepwise approach recommended by the International Consensus Report on the Diagnosis and Management of Rhinitis²⁰; simultaneous use of different drug types was allowed. Drugs were given on an on-demand basis so that patients were able to adjust the drug intake according to their individual needs after thorough explanation.

Study design

Baseline levels of ECP and tryptase in serum and nasal secretions were determined at study entry out of season (T0, week 9) and during the 3 following control investigations before season (T1, week 20), in season (T2, weeks 26 to 28), and after season (T3, week 44). Nasal secretions were analyzed for ECP and tryptase after nasal allergen provocation at T0, T1, and T3.

Random assignment

Patients were randomly assigned either to STI or to symptomatic treatment with a block size of 4 (2 STI-treated and 2 drug-treated patients).

NPT

Nasal reactivity of the patients was analyzed by specific NPT. After an adaptation period to the test environment of at least 30 minutes, the basal value was determined 15 minutes after the application of the suspension buffer without allergens (ALK-diluent) to the inferior and middle turbinate by a nasal spray applicator. The reactivity to allergen was then measured 15 minutes after application of the allergen in a concentration range from 10 to 100,000 SQ units. The test was assessed as positive either by a decrease in nasal flow of more than 40% or by reaching a symptom score of more than 4 score points for rhinorrhea (no secretion, 0; little secretion, 1; much secretion, 2), irritation (0 to 2 sneezings, 0; 3 to 5 sneezings, 1; more than 5 sneezings, 2), itching (no, 0; mild, 1; severe, 2), and distant symptoms (no, 0; tear flow and/or palatal itching and/or itching of the ears, 1; conjunctivitis and/or chemosis and/or urticaria and/or coughing and/or shortness of breath, 2).

Nasal secretions sampling

Nasal secretions were collected with absorbent foam rubber samplers by a technique well established for the determination of ECP in nasal secretions.²¹ Patients were advised to blow their nose before insertion. Samplers were placed with bayonet pincers under anterior rhinoscopic control between the septum and the head of the inferior turbinate on both sides and were left for 10 minutes. They

TABLE II. Drug use in the parallel groups

Type of drug	Patients using drugs				P value χ^2 -test	Frequency of use			
	STI		Drugs			STI		Drugs	
	N*	%	N	%		Days	%†	days	%‡
DSCG, herbal	3	12.5	6	25.0	.267	25	1.3	121	7.3
Topical antihistamine	9	37.5	17	70.8	.020	71	3.6	546	32.8
Topical steroids	4	16.7	5	20.8	.712	164	8.3	342	20.6
Sympathomimetics	5	20.8	5	20.8	1.000	72	3.7	67	4.0
Oral antihistamine	11	45.8	17	70.8	.079	159	8.1	480	28.8
Oral steroids	1	4.2	—	—	.312	33	1.7	—	—

*Some patients used multiple drugs.

†1971 Total days, based on 20 evaluable diaries.

‡1664 Total days, based on 17 evaluable diaries.

were then removed and transferred into special centrifuge tubes with a sieve placed 1.5 cm above the bottom of the tube. The tubes were centrifuged at 1650g for 10 minutes. The nasal secretions were squeezed out, and the clear sol phase was separated from mucus and cells and transferred to an Eppendorf tube. The amount of nasal secretions gained was documented for all samples. Nasal secretions were immediately shock frozen in liquid nitrogen and stored at -80°C until used for laboratory investigations.

ECP and tryptase

Samples of 50 μL nasal fluid were measured in duplicate. ECP was determined by a competitive radioimmunoassay research kit (Pharmacia, Uppsala, Sweden) as described previously.^{22,23} Samples were incubated with ^{125}I -labeled human ECP and rabbit anti-human ECP antiserum for 3 hours and were then incubated with sheep anti-rabbit serum and decanted. Subsequently, radioactivity was measured in a gamma counter over a 1-minute period, and the sample values were calculated by spline interpolation. Samples containing over 200 ng/mL were diluted with zero standard. The detection limit of the assay is 2.0 ng/mL.²⁴

Tryptase was measured by a radioimmunoassay (Pharmacia) as described previously.²⁵ Fifty microliters of native nasal fluid were tested. Samples were incubated for 16 hours in tubes coated with monoclonal mouse anti-tryptase antibodies, and then ^{125}I -anti-tryptase monoclonal mouse antibodies were added. After being washed 3 times, radioactivity was measured in a gamma counter. Values were calculated by a spline interpolation from standards. Tryptase levels are listed in nanograms per milliliter equal to units per liter. The detection limit of the assay is 0.5 ng/mL.²⁶

Statistics

Data were statistically analyzed by minimum, maximum, median, arithmetic, and geometric mean, respectively, and SEM. The baseline characteristics and the symptom and medication scores of the STI and drug-treated groups were compared by the *t* test and the Mann-Whitney U test for continuous variables and by the χ^2 -test for categoric variables; the symptom score variables were analyzed nonparametrically by means of the U test.

The ECP and tryptase levels in nasal secretion were evaluated by means of analysis of covariance (ANCOVA) for the repeated measurement model with baseline data as a covariate. *T* tests for the least squares means were used to compare the trial groups at T1, T2, and T3 separately. Paired comparison *t* tests were performed within the trial group to analyze pre- and post-NPT levels at T0, T1, and T3 and to compare the seasonal T2 level with post-NPT levels at T0, T1, and T3. All tests concerning ECP and tryptase were carried out parametrically assuming log normal distributions (ie, with logarithmically transformed data).

RESULTS

Patients

Data obtained with all 48 patients could be evaluated (STI, *n* = 24; drugs, *n* = 24). Clinical data of the patients at inclusion are shown in Table I. The patient groups were comparable with respect to sex, age, and duration of allergic disease. The severity of symptoms in the previous year was comparable between the 2 groups, except that conjunctival symptoms were more severe in the STI group compared with the drug-treated group.

Symptoms and pharmacotherapy

Median overall symptom scores were 134.5 (95% confidence interval of the median [CI], 65 to 336; STI) versus 386.0 (CI, 185 to 563; drugs), which were significantly lower in the STI group during the period with maximum grass pollen exposure (weeks 21 to 33; *P* = .022) as were median nasal symptom scores with 106.5 (CI, 60 to 279; STI) versus 264.0 (CI, 164 to 434; drugs; *P* = .020). Low median score levels without statistical intergroup differences were recorded for conjunctival symptoms with 21.0 (STI) versus 40.0 (drugs; *P* = .099) and for bronchial symptoms with 0.0 (STI) and 1.0 (drugs; *P* = .290).

Drug intake is described in detail in Table II. The numbers of patients using drugs and the frequency of drug use were significantly lower in the STI group for oral and topical antihistamines and DSCG or herbal drugs. Topical steroids were administered by equal numbers of patients, but the patients in the STI group who chose to take topical steroids used them significantly less frequent. Sympathomimetics and oral steroids were taken equally in both groups.

Safety of STI

Large local reactions (>5 cm) were observed after 16 of the 175 total injections (9.1%). Weak systemic allergic reactions were documented after 5 injections (2.9%) as nasal and conjunctival symptoms. Itching of the palms and feet together with paleness 1 hour after the injection in one case disappeared without treatment. Paleness and anxiety in another patient 4 minutes after the injection were reversible after treatment with an antihistamine and diazepam.

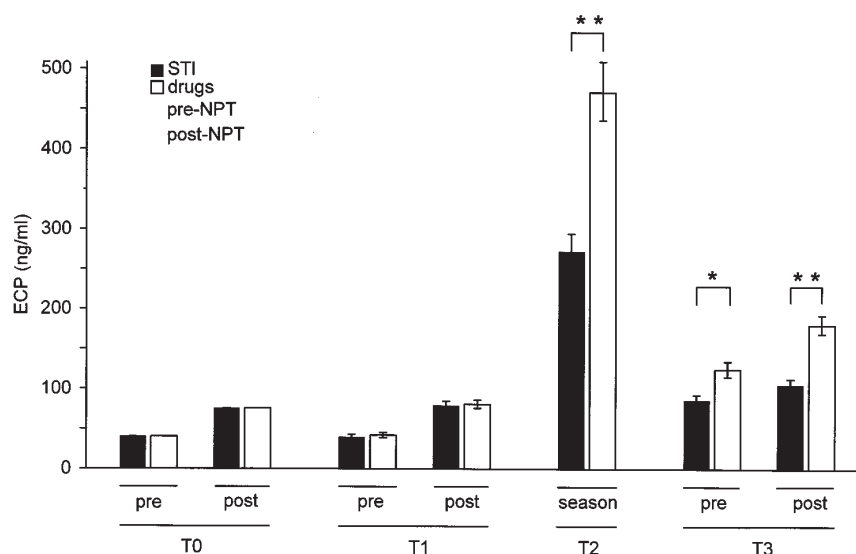


FIG 1. Geometric means ($\pm$ SEM) of ECP levels in nasal secretions before (*pre*) and after (*post*) NPT for the STI-treated group and the drug-treated group at the control investigations at baseline (T0), before season (T1), and after season (T3) and of levels in season (T2) without NPT. Values for the STI and the drug-treated group were adjusted to T0 levels. Statistical analysis was performed by means of ANCOVA for the repeated measurement model. * $P = .001$; ** $P < .001$.

The dosage schedule was modified in 9 of 24 patients. Twenty-two patients were treated with the maximum dose of 1000 SE. The therapy was discontinued in 2 patients, in one patient because of a systemic reaction, and in one patient because of beginning symptoms to grass pollen.

ECP

No changes in the ECP levels in nasal secretions were observed during the control investigations before the grass pollen season. ECP, however, increased significantly in season (Fig 1). This increase was significantly lower in the STI group than in the drug-treated group ($P < .001$). After season, we measured increased ECP levels compared with the preseasonal values, which were still lower in the STI group than in the drug-treated group ($P = .001$).

Post-NPT levels of ECP were significantly higher than levels without NPT at the investigations at baseline, before season, and after season in both groups (all $P < .001$). Post-NPT levels of ECP were significantly lower in the STI group than in the drug-treated group after season ($P < .001$ at T3).

Significantly higher levels of ECP were induced by seasonal exposure (T2) compared with the levels of ECP induced by NPT (post-NPT at T0, T1, T3; all $P < .001$ by paired comparison *t* test within both groups).

Tryptase

Preseasonal levels of tryptase were mostly below the limit of detection. Tryptase increased significantly in season (T2). This increase was significantly lower in the STI group than in the drug-treated group ($P < .001$; Fig 2).

Post-NPT levels of tryptase were significantly higher than levels without NPT at baseline, before season, and after season in both groups (all $P < .001$). Post-NPT lev-

els of tryptase were significantly lower in the STI group than in the drug-treated group at T1 and T3 ($P < .001$).

Significantly higher levels of tryptase by seasonal exposure (T2) compared with the tryptase levels induced by NPT were induced only in the drug-treated group and not in the STI group ($P < .001$ at T0, T3; $P = .002$ at T1; paired comparison *t* test within drug-treated group).

DISCUSSION

Immunotherapy represents the only specific treatment that can be offered to patients with allergies besides allergen avoidance. In clinical practice, however, the decision whether to start immunotherapy will often depend on the comparison of the clinical efficacy with that of a drug treatment. Juniper et al²⁷ compared a continuous therapy with the topical steroid budesonide with a preseasonal treatment of a glutaraldehyde-modified tyrosine-adsorbed ragweed allergen preparation in a double-blind study. In their study the continuous treatment with budesonide showed a markedly higher efficacy than the preseasonal treatment with 4 injections of the allergoid in the ragweed season. Because of large local or systemic reactions, 14 of 30 patients discontinued the treatment with the allergoid before having reached the maximum dose. With the molecular standardized unmodified allergen preparation of 7 preseasonal injections that we have used in our study, the therapy was discontinued only in 2 patients and only in one patient as the result of a systemic allergic reaction. The dose was modified in 7 patients: in 4 patients because of local or mild systemic reactions and in 3 patients for other reasons, but all of these patients reached the maximum concentration by additional injections. In our study patients in the drug-treated group have not taken topical steroids continuously but applied vari-

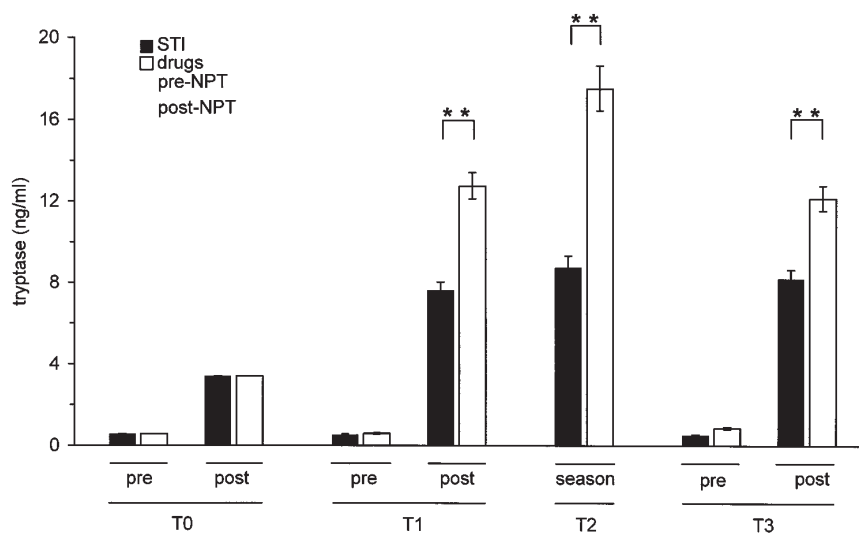


FIG 2. Geometric means ($\pm$ SEM) of tryptase levels in nasal secretions before (*pre*) and after (*post*) NPT for the STI group and the drug-treated group at the control investigations before treatment (*T0*), before season (*T1*), and after season (*T3*) and of levels in season (*T2*) without NPT. Values for the STI and the drug-treated group were adjusted to *T0* levels. Statistical analysis was performed by means of ANCOVA for the repeated measurement model; ** $P < .001$.

ous antiallergic drugs according to their needs, as did the patients treated with STI. The situation, that a drug treatment according to the patients' needs is given in addition to immunotherapy during the season, seems to be common according to our experiences. For the clinical decision about the appropriate treatment alternative, it is therefore important to know whether a preseasonal STI with additional drug treatment according to the patients' needs during the season is superior to an exclusive treatment with symptomatic drugs. The most frequently used drugs in our study were topical and systemic antihistamines, whereas topical steroids were less frequently used. Under these conditions, patients in the STI group had a significantly lower level of nasal and overall symptoms and were using fewer amounts of concomitant medication.

The mechanism of immunotherapy is not understood. Studies on mechanisms of immunotherapy have previously focused on changes in serum antibodies, including blunting of seasonal rises in specific IgE and an increase in specific IgG antibodies. Recent results suggest an effect of immunotherapy on T lymphocytes, leading to a switch from a predominant T_H2 response (IL-4, IL-5) in favor of a T_H1 response (INF- γ). The switch of cytokine profile to a predominant, prevailing INF- γ response results in inhibition of the IL-4 dependent IgE production, reinforced by a decrease in the production of IL-4 from T_H2 cells.

A reduction in the activation of the effector cells of the allergic inflammatory reaction may be of major relevance to explain the treatment efficacy of immunotherapy.⁷ In the current study we used the approach of measuring cell-activation markers to evaluate this activation. The markers that we chose for this purpose, namely tryptase for mast cells and ECP for eosinophils, are highly specific for the respective cell population and therefore reflect the

involvement of these cells in the allergic process.²⁸

Mucosal mast cells are responsible for most of the symptoms in the immediate hypersensitivity reaction of allergic rhinitis. Mast cell bound-specific IgE will induce degranulation if the antigen is presented to the cell in allergic rhinitis. Mast cell-derived mediators, such as histamine, prostaglandin D2, leukotriene D4, and others, create the classic symptoms of nasal blockade, runny nose, sneezing, and itching.

An important recent finding is the reduction in the number of mast cells in nasal epithelial brushings after successful immunotherapy in patients who are sensitive to mites¹³ and the decrease in mast cell numbers in the skin after grass pollen immunotherapy.⁴ Also, a dose-dependent reduction in mast cell-associated mediators has been observed in nasal washings after nasal pollen challenge after immunotherapy.²⁹ The activity of mast cells may be reduced because of a lack of IL-3-dependent activation, a reduced local production of IgE, and a decreased production of histamine-releasing factors.^{1,30} The decreased activity of mast cells results in a decreased release of allergic mediators, thereby diminishing the activity of chemotactic factors such as platelet-activating factor (PAF) and eosinophil chemotactic activity.^{10,12}

Although histamine in nasal fluid seems to be a reliable marker for nasal mast cell activation, it is not useful in the investigation of natural mast cell activation because of its rapid metabolism and the fact that it is not specific for mast cells. Mast cells were reported to also exhibit trypsin-like activity.³¹ This activity is caused by a tryptic proteinase called tryptase.³² The endoprotease tryptase is a major protein component of mast cell granula found in both connective tissue and mucosal mast cells³³ and in trace amounts in basophils but not in other

human cell types.³³⁻³⁵ Because of its longer biologic half-life,³⁶ it is more reliable and easier to determine than histamine. Tryptase is secreted from mast cells during degranulation.²⁸ Tryptase has been shown to be elevated in nasal lavage fluid in patients with rhinitis after allergen challenge³⁷ and during natural allergen exposure.³⁸

In the current study we confirmed and extended these results. Tryptase levels in nasal secretions significantly increased after allergen challenge out of the season. At the intraseasonal visit, tryptase was significantly elevated when compared with the preseasonal prechallenge values. These increased tryptase levels in nasal secretions after both allergen challenge and natural allergen exposure were significantly inhibited by STI.

The second cell population monitored with respect to the activation status after STI was the eosinophil granulocyte with ECP. The ECP is one of the 4 major granule proteins (major basic protein, eosinophil peroxidase, eosinophil-derived neurotoxin, and ECP of the eosinophil).³⁹ These proteins are released in vitro to the extracellular environment on stimulation of the eosinophil by various secretagogues.²⁸ The measurement of ECP in nasal secretions was demonstrated to be a sensitive marker of eosinophil activation either in allergen challenge models⁴⁰⁻⁴² or under natural pollen exposure.⁴² In allergic bronchial asthma, ECP serum levels reflect the severity of the disease measured either as lung function (FEV₁), reactivity to histamine, or the probability to develop allergic late-phase responses or exercise-induced asthma.^{11,43,44}

A characteristic feature of successful immunotherapy is its ability to inhibit allergen-induced late-phase responses.⁷ In this study we found nasal secretion ECP levels after nasal allergen challenge and under natural pollen exposure that were shown to reduce beat frequency of ciliated nasal epithelial cells in an in-vitro model⁴⁵ and that might even cause structural damage and destroy these cells.²⁸ The increase in nasal secretion ECP induced by allergen exposure was significantly inhibited by STI.

An effect of STI on chemotaxis and activation of eosinophils has also been described for other tissues. Varney et al⁴ reported on a trend for reduction in the number of infiltrating activated eosinophils (EG2+) in skin biopsy specimens after dermal grass pollen challenges in a group of patients treated with grass pollen immunotherapy when compared with a placebo-treated group.⁴ Rak et al¹¹ measured ECP levels in blood serum of patients with asthma who were allergic to pollen and found a significant increase during the season in untreated patients but not in patients treated with immunotherapy. In allergic rhinitis, ECP levels in nasal secretions were demonstrated to be better related to disease activity and clinical symptoms compared with serum ECP.^{46,47} So far, no data exist on effects of STI on ECP levels in nasal secretions of patients with seasonal rhinitis. In general, ECP measurements in local body fluids that are sampled close to the particular inflammatory process can be suspected to reflect the process more directly.²⁸ Therefore we regard ECP nasal secretion levels to be more convincing than serum levels to

judge treatment efficacy in patients with allergic rhinitis.

In conclusion, short-term preseasonal immunotherapy with molecular standardized grass and rye allergens is able to reduce ECP and tryptase levels in nasal secretions. Thus STI can be regarded better than symptomatic treatment in reducing allergic nasal inflammatory changes. Symptoms are better controlled, although drug use is less if compared with drug treatment alone.

The authors thank Dr G. Burow, Pharmacia Research Laboratories at Freiburg, Germany, for valuable advice in the ECP and tryptase analyses and for providing external laboratory quality control; M. Reichenbach, MD, Mrs A.K. Olbrich, Mrs S. Föhrenbach, Mrs K. Deibert, and Mr Zimmermann for participating in the clinical investigations; and M. Bollesseu for excellent monitoring.

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