

The effect of natural pollen exposure on eosinophil apoptosis and its relationship to bronchial hyperresponsiveness in patients with seasonal allergic rhinitis

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Background: Limited data suggest that there is increased eosinophilic inflammation in the airways of patients with seasonal allergic rhinitis (SAR) during pollen season even if they do not have asthma.

Objective: To investigate the effect of natural pollen exposure on inflammatory cells and apoptosis of eosinophils and its association with bronchial hyperresponsiveness (BHR) during and out of pollen season in SAR patients sensitized to only grass pollens.

Methods: Forty SAR patients and 10 patients with nonallergic rhinitis (NAR) from Ankara, Turkey, were recruited to participate in the study. Two induced sputum samples were taken from SAR patients during pollen season (May-June) and out of pollen season (November-January), but only 1 induced sputum sample was taken from NAR patients. Slides of induced sputum were evaluated by 2 cytologists with the use of light microscopy after cytocentrifuged and dyed with May-Grünwald-Giemsa stain. Induced sputum samples were sufficient for differential cell counts in 14 SAR and 7 NAR patients.

Results: Eosinophil counts in SAR patients were statistically higher in pollen season ($19.4\% \pm 16.2\%$) compared with out of season ($4.6\% \pm 6.9\%$, $P = .003$) and with NAR patients ($4.7\% \pm 9.5\%$, $P = .01$). The apoptotic eosinophil counts in SAR patients were statistically higher out of pollen season ($3.0\% \pm 4.5\%$) than in pollen season ($0.38\% \pm 0.80\%$, $P = .02$) and higher than those of NAR patients ($0.14\% \pm 0.26\%$, $P = .005$). The apoptotic ratio was statistically higher after pollen season compared with pollen season ($0.720\% \pm 0.394\%$ vs $0.044\% \pm 0.116\%$, $P = .002$). Blood eosinophil counts of SAR patients were increased during the pollen season ($364 \pm 187/\text{mm}^3$) compared with out of season ($278 \pm 219/\text{mm}^3$, $P = .04$) and with NAR patients ($85 \pm 54/\text{mm}^3$, $P = .001$). The number of SAR patients who had BHR during the pollen season (7/14) was higher than the number who had BHR out of season (2/14, $\chi^2=4.2$, $P = .04$).

Conclusion: Our data indicate that changes in eosinophil counts and eosinophil apoptosis may be related to the changes of natural pollen exposure and seasonal changes of BHR in SAR patients.

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INTRODUCTION

Eosinophils play an important role in the development and maintenance of acute and chronic allergic inflammatory events.¹ Eosinophilic inflammation has been well established in the airways of patients with asthma.² They can induce the tissue damage by their granular contents, causing airway inflammation when activated.³ Apoptosis is described as programmed cell death.⁴ Eosinophils die by apoptosis and are ingested by macrophages when cultured in vitro.⁵ This en-

vironment prevents the release of eosinophilic granules and tissue necrosis. In recent years, delayed apoptosis of eosinophils has been shown in asthma and atopic dermatitis, suggesting its role in the persistence of allergic inflammation.^{6,7} In addition, Vignola et al⁸ have demonstrated that the absolute apoptotic eosinophil counts in bronchial biopsy specimens from asthmatic patients were inversely correlated with their asthma severity. Bronchial hyperresponsiveness (BHR) is a hallmark feature of asthma and a consequence of the underlying airway inflammation⁹; however, BHR is not confined to asthmatic patients only. Patients with allergic rhinitis (AR) also show some degree of BHR even if they are free of asthma symptoms. AR, particularly associated with BHR, is considered a risk factor for asthma development.^{10,11} Although the mechanism of BHR in AR is not explained exactly, only a few studies performed with induced sputum samples have suggested a role of eosinophilic inflammation.¹² BHR is increased during pollen season in seasonal allergic rhinitis (SAR), suggesting increased airway inflammation due to natural pollen exposure.¹³ Despite data regarding the

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presence of eosinophilic inflammation in the airways of AR patients and its relation with natural pollen exposure, to the best of our knowledge no study has examined the effect of natural pollen exposure on apoptosis of these eosinophils, as a control mechanism of tissue eosinophilia, in the airways of AR patients. In this study, by using induced sputum, we aimed to study the effects of natural pollen exposure on inflammatory cells, including eosinophils, and on apoptosis of eosinophils and whether there was a relationship between these parameters and BHR to methacholine during and out of pollen season in patients with SAR.

METHODS

Study Patients

We selected 2 groups of patients. Forty patients (age range, 17–46 years) with a history of seasonal allergic rhinitis (SAR) to grass pollen only constituted the patient group. Ten patients (age range, 20–40 years) with nonallergic rhinitis (NAR) were considered the control group. Patients were restricted to those living in Ankara, the capital city of Turkey, to exclude possible effect of varying severity of pollen concentration on inflammatory parameters. Patients had to meet the following criteria to participate in the study: historical data defined by nasal itching, rhinorrhea, sneezing, and nasal obstruction for at least 2 years, physical examination that produced positive skin prick test results to grass pollen only, and no sensitization to perennial allergens. NAR patients had perennial symptoms such as nasal itching, rhinorrhea, sneezing, and nasal obstruction for at least 2 years, but none of them had any allergic sensitivity determined by either historical data or skin prick testing.

At the onset of the study, all patients were carefully questioned about even vague lower respiratory tract symptoms to eliminate the patients with hidden asthma from our study group. None of the patients had ever experienced any wheezing, dyspnea, chronic cough, or sputum production. None of the patients reported an upper or lower respiratory tract infection during the previous 6 weeks or within the study period. None of the patients were smokers. During the study period, patients were not permitted to use nasal or inhaled corticosteroids because they can influence bronchial reactivity, but they were allowed to use nasal antihistamine spray if necessary. None of the patients with SAR had previously been treated with immunotherapy. The study was approved by the local ethics committee, and informed consent was obtained.

Evaluation of Atopy

Skin prick tests were performed by using a common panel, including *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, grass, tree, and weed pollens, cat, dog, *Alternaria*, *Cladosporium*, and cockroach allergen extracts (Allergopharma, Stockholm, Sweden). Positive and negative controls were histamine (10 mg/mL) and phenolated glycerol saline, respectively. A mean wheal diameter of 3 mm or greater than that obtained with control solution was considered positive.

Sputum Induction

Sputum induction was performed between 9 and 10 AM according to the method proposed by Pizzichini et al.¹⁴ and slightly modified and adapted according to Pavord et al.¹⁵ Before inhalation of hypertonic saline solution, all patients inhaled 2 puffs of salbutamol (200 µg) and underwent spirometry 10 minutes later. Then an aerosol of sterile 3% saline solution was generated by an ultrasonic nebulizer (particle size, 0.5 to 6 µm; output, approximately 3 mL/min; oscillation frequency, 1.63 MHz) and inhaled for 7 minutes through a mouthpiece without a valve or nose clip. If the patient could not expectorate at this step, the concentration of saline was increased gradually according to the decrease in forced expiratory volume in 1 second (FEV₁) measurements from baseline. In case of a less than 10% decrease in FEV₁, the concentration of saline was increased from 3% to 4% and then to 5%. These procedures were repeated sequentially with each concentration of saline for a total of up to 7 minutes of inhalation, until a sputum sample was obtained or a decrease in FEV₁ of 10% or more occurred. After each period of inhalation, patients were asked to rinse their mouths and throats carefully, swallow the water, and blow the nose to minimize contamination with saliva and postnasal drip. Then they were instructed to try to cough sputum into a container.

Sputum Examination

Sputum was processed as soon as possible within 15 minutes according to the recommended method.¹⁵ It was collected into a Petri dish, and all portions that macroscopically appeared free of salivary contamination were placed in a 15-mL polystyrene tube using a straight blunted 115-mm forceps. This selected portion was treated with 4 times the volume of dithiothreitol freshly diluted to 0.1% by the addition of distilled water to dissociate the disulfide bonds of the mucus. The mixture was vortexed for 15 seconds and gently aspirated in and out of a Pasteur pipette to ensure mixing. To stop the effect of dithiothreitol on the cell suspension, a further 4 volumes of Dulbecco phosphate-buffered saline was added. The suspension was filtered through 48-mm nylon gauze to remove cell debris and mucus. The resulting clear suspension was centrifuged at 790g for 10 minutes. The pellet was resuspended in a volume of 200 to 600 µL of Dulbecco phosphate-buffered saline. Seventy-five microliters of the cell suspension adjusted to 1.0×10^6 /mL was placed into cups of Shandon III cytocentrifuge, and 2 coded Cytospins were prepared at 450 rpm for 6 minutes. The slides were air-dried and stained with May-Grünwald-Giemsa stain. Slides were coded and cell counts were performed by 2 expert pathologists who had no knowledge about the clinical characteristics of the patients.

Pulmonary Function Tests and Nonspecific Bronchial Provocation Test

Pulmonary function tests (SensorMedics, Yorba Linda, CA) were performed before sputum induction to determine baseline FEV₁. The FEV₁ value also was determined after the

induction to determine if there was any bronchoconstricting effect of inhaled saline. Nonspecific bronchial provocation testing using methacholine was performed according to the method described by Cockcroft et al¹⁶ between 8:30 and 10:30 AM. In the method, a Wright nebulizer that delivered aerosols was connected directly to a mouthpiece, a nose clip was worn, and the aerosol was inhaled through the mouth by tidal breathing for 2 minutes. After inhalation of physiologic saline, patients inhaled doubling concentration of methacholine from 0.25 to 8 mg/mL diluted in physiologic saline. A single determination of FEV₁ was taken 60 to 90 seconds after each methacholine concentration. The challenge was stopped when the FEV₁ decreased by more than 20% from the postphysiologic saline level or when the highest concentration of methacholine had been administered. The result was expressed as methacholine PC₂₀ (provocative concentration required to produce a 20% decrease in FEV₁). The methacholine PC₂₀ was calculated from the log concentration-response curve by linear interpolation of the 2 last points. A PC₂₀ of more than 8 mg/mL was accepted as the cutoff point.¹⁷

Study Design

SAR patients. Induced sputum was taken both within the pollen season (between May and July 2001) and out of season (between November 2001 and January 2002) according to the data regarding peak and out of grass pollen season for Ankara.¹⁸ Gramineae pollen counts were 103, 169, 475, 1,554, and 410 grains/m³ in March, April, May, June, and July, respectively. No Gramineae pollen was available in Ankara from October 2001 to March 2002. Pulmonary function tests and NSBP tests were performed during and out of pollen season. Eosinophils in the peripheral blood were also determined during both seasons.

NAR patients. Sputum induction, pulmonary function tests, nonspecific bronchial provocation tests, and peripheral blood samples for eosinophil counts were performed once for the control group. Nasal symptoms, rhinorrhea, nasal blockage, and sneezing were recorded by the patients during a period of 4 weeks. SAR patients recorded symptom scores during pollen season. The scores were graded on a scale of 0 to 3, according to the severity and considering the whole day (0 indicates no symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms). Mean nasal symptom

scores were calculated as the sum of scores divided by the number of days recorded for each patient. SAR patients were allowed to take topical nasal azelastine and ophthalmic cromolyn as required during pollen season. Drug use was stopped 24 hours before the study during pollen season.

Inflammatory cell counts and apoptotic eosinophil counts in induced sputum. Three hundred cells were counted in each slide, and inflammatory cells (macrophages, neutrophils, lymphocytes, and eosinophils) were determined as percentages of total cells using light microscopy. Apoptotic eosinophils were evaluated according to their morphologic changes, such as presence of cell shrinkage and nuclear coalescence and shift from bilobed to monolobed nucleus in preparations from sputum samples based on the previous studies and expressed as percentages of total cells.^{19,20} The apoptotic ratio, as another index of eosinophil apoptosis, was determined using the following formula: [(% apoptotic eosinophils)/(% apoptotic + normal, bilobed eosinophils)]. Slides for differential cell counts and apoptosis of eosinophils were examined by 2 expert cytologists (S.E. and O.A.) who were unaware of the clinical characteristics of the patients.

Statistical analysis. Between-group comparisons were performed by Mann-Whitney *U* test, χ^2 test, and *t* test for unpaired data when appropriate. The first and second values of SAR patients were compared using Wilcoxon signed rank test. We considered *P* < .05 statistically significant. Correlations were determined using a Spearman correlation matrix. Results are reported as mean $\pm$ SD. The personal computer program SPSS for Windows (version 6.0; SPSS Inc, Chicago, IL) was used for all calculations.

RESULTS

Twenty-eight of the 40 SAR patients and 10 of the NAR patients provided sputum samples during the pollen season. Patients tolerated all of the methods. Eighteen of the samples from SAR patients and 7 from NAR patients were found to be sufficient for differential cell counts. Fourteen of the 18 SAR patients whose sputum samples were accepted as sufficient in the pollen season also provided sputum samples out of pollen season. We compared the samples of these 14 SAR and 7 NAR patients. General demographic characteristics of the 14 SAR and 7 NAR patients are given in Table 1. All patients had an FEV₁ of more than 90% of the predicted FEV₁. Blood

Table 1. Demographic Characteristics of 14 SAR Patients and 7 NAR Patients

Characteristics	SAR (n = 14)	NAR (n = 7)	P value
Male/female	6/8	5/2	.21
Age, mean $\pm$ SD, y	27.4 $\pm$ 9.7	30.8 $\pm$ 7.7	.25
Duration of disease, mean $\pm$ SD (range), y	5.5 $\pm$ 2.4 (2–10)	5.3 $\pm$ 6.6 (2–20)	.09
FEV ₁ , mean $\pm$ SD, % of predicted	107.3 $\pm$ 19.2	101.3 $\pm$ 13.9	.41
BHR, yes/no	7/7	2/5	.34
Blood eosinophils, mean $\pm$ SD, /mm ³	364 $\pm$ 187	85 $\pm$ 54	.001

Abbreviations: BHR, bronchial hyperresponsiveness; FEV₁, forced expiratory volume in 1 second; NAR, nonallergic rhinitis; SAR, seasonal allergic rhinitis.

eosinophil counts of SAR patients were increased in pollen season ($364 \pm 187/\text{mm}^3$) compared with out of season ($278 \pm 219/\text{mm}^3$, $P = .04$) and with NAR patients ($85 \pm 54/\text{mm}^3$, $P = .001$). The inflammatory cell counts and apoptotic eosinophil counts of SAR and NAR patients are given in Table 2. Figure 1 shows the features of normal (Fig 1A) and apoptotic eosinophils (Fig 1B) in a sputum sample from one of our AR patients. The eosinophil count in the sputum sample of the SAR patients was higher in pollen season than out of season ($P = .003$, Table 2). Figure 2 shows individual eosinophil counts in sputum samples of SAR patients during and out of pollen season. Although the eosinophil counts decreased after pollen season, both apoptosis and apoptotic ratio of these cells increased out of pollen season ($P = .02$ and $P = .002$, respectively; Table 2). Individual apoptotic eosinophil counts in the sputum of SAR patients during and out of pollen season are shown in Figure 3.

Seven of 14 SAR patients had nonspecific BHR to methacholine during pollen season. Sputum eosinophil and apoptotic eosinophil counts were not different between those patients with and without BHR ($P = .90$ and $P = .70$, respectively). In addition, sputum eosinophils were not correlated with the degree of BHR in the 7 patients with BHR during pollen season ($P = .19$). Changes of individual methacholine PC_{20} values in SAR patients during and out of pollen season are shown in Figure 4. The number of SAR patients who had nonspecific BHR to methacholine during pollen season (7/14) was significantly higher than those out of pollen season (2/14) ($\chi^2 = 4.2$, $P = .04$). Although this finding did not reach statistical significance, the apoptotic ratio of eosinophils was higher in those patients without BHR ($0.07\% \pm 0.16\%$ vs $0.02\% \pm 0.04\%$), and eosinophil counts were higher in those with BHR ($23.7\% \pm 23.4\%$ vs $18.6\% \pm 16.9\%$) during pollen season. In addition, following the increase of eosinophil apoptosis and the decrease in eosinophil counts, the number of patients with BHR decreased from 7 to 2 patients out of season. Although statistical analysis was not convenient due to the small number of patients, the mean apoptotic ratio of eosinophils was lower ($0.34\% \pm 0.47\%$ vs

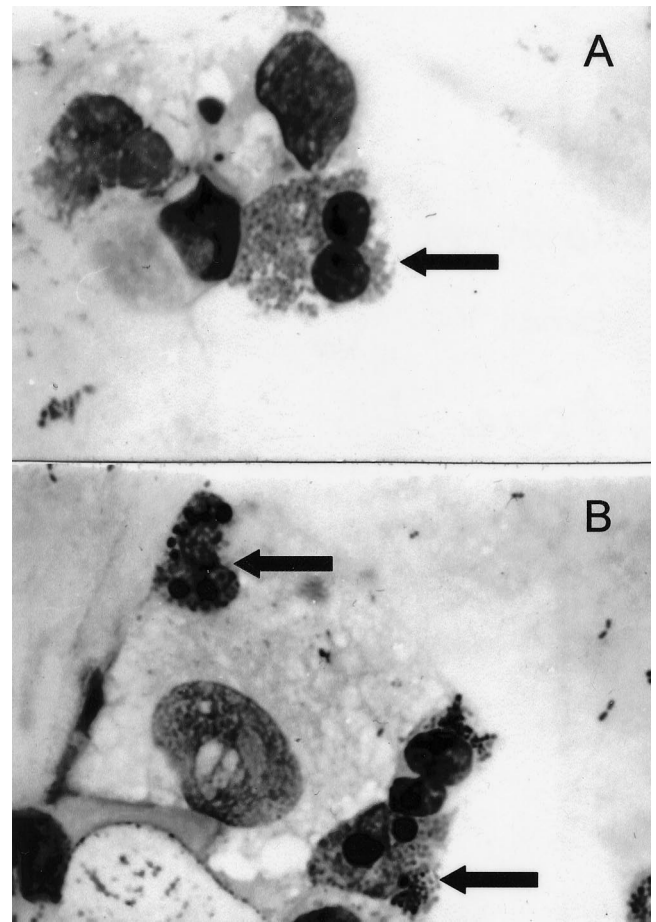


Figure 1. Normal (A) and apoptotic (B) eosinophils in patients with seasonal allergic rhinitis (May-Grünwald-Giemsa stain, original magnification $\times 1,000$).

$0.80\% \pm 0.36\%$) and eosinophil counts were higher ($2.5\% \pm 3.5\%$ vs $1.4\% \pm 2.7\%$, respectively) in these 2 patients with BHR than those 12 patients without BHR out of season.

Table 2. Comparisons of Inflammatory Cells and Apoptotic Eosinophils Between Groups

Parameters	SAR (n = 14)		NAR (n = 7)
	Pollen season	Out of season	
Eosinophils, mean $\pm$ SD, %	$19.4 \pm 16.2^*$	4.6 ± 6.9	4.7 ± 9.5
Apoptotic eosinophils, mean $\pm$ SD, %	0.38 ± 0.80	$3.0 \pm 4.5^\dagger$	0.14 ± 0.26
Apoptotic ratio, mean $\pm$ SD, %	0.044 ± 0.116	$0.720 \pm 0.394^\ddagger$	0.046 ± 0.080
Macrophages, mean $\pm$ SD, %	52.2 ± 15.4	61.7 ± 18.3	61.4 ± 20.2
Neutrophils, mean $\pm$ SD, %	27.0 ± 16.1	33.6 ± 13.6	27.8 ± 21.3
Lymphocytes, mean $\pm$ SD, %	1.8 ± 1.8	0.4 ± 0.7	1.0 ± 1.8
PC_{20} methacholine <8 mg/mL, No.	7	2	2

Abbreviations: NAR, nonallergic rhinitis; PC_{20} , provocative concentration required to produce a 20% decrease in forced expiratory volume in 1 second; SAR, seasonal allergic rhinitis.

*Significantly higher during pollen season when compared with out of season ($P = .003$) and with patients with NAR ($P = .01$).

† Significantly increased after pollen season ($P = .02$).

‡ Higher than those within the pollen season ($P = .002$).

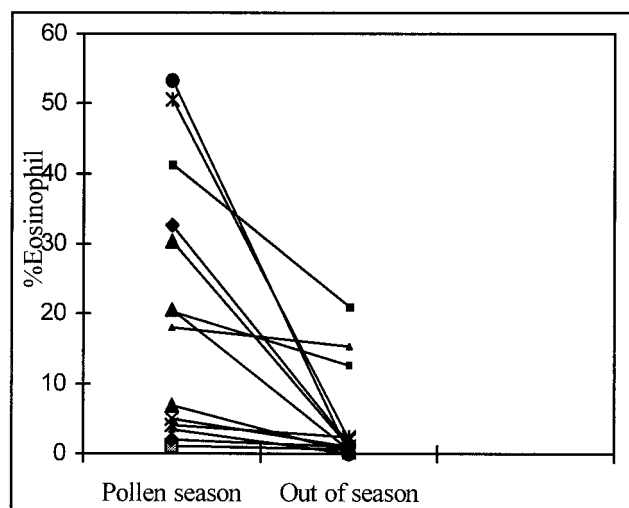


Figure 2. Individual values of percentages of sputum eosinophils in patients with seasonal allergic rhinitis in and out of pollen season ($P = .003$)

Mean values of nasal symptom scores recorded by SAR patients during pollen season were 1.57 ± 0.55 (range, 1–3). Scores of nasal symptoms were not correlated with sputum eosinophil counts ($r = -0.17$, $P = .31$) or apoptotic eosinophil counts ($r = 0.43$, $P = .28$). When we consider SAR patients during pollen season, nasal symptom scores were not different between those patients with BHR and without BHR (1.51 ± 0.42 vs 1.62 ± 0.69 , $P = .95$).

DISCUSSION

The present study shows that the number of eosinophils in induced sputum is increased in patients with SAR during

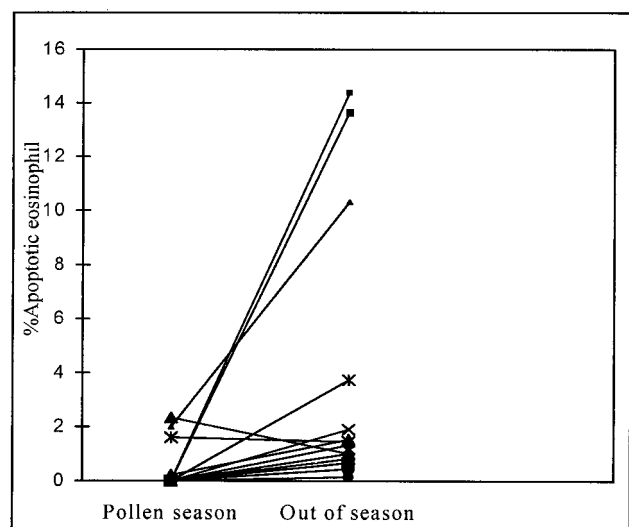


Figure 3. Individual values of apoptotic eosinophil percentages in sputum of patients with seasonal allergic rhinitis in and out of pollen season ($P = .02$).

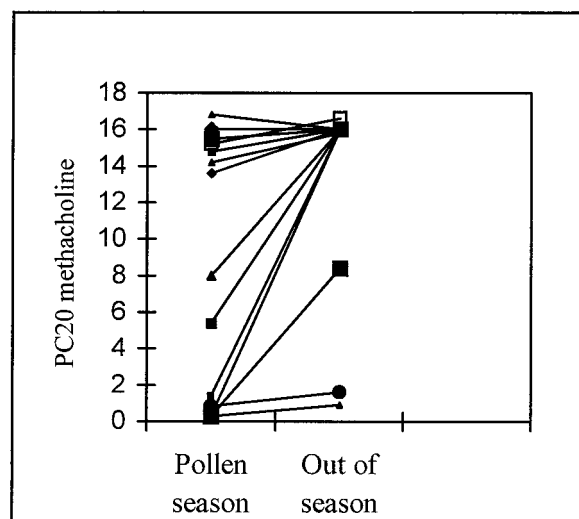


Figure 4. Changes in provocative concentration required to produce a 20% decrease in forced expiratory volume in 1 second (PC_{20}) values in patients with seasonal allergic rhinitis between seasons ($P = .04$).

grass pollen season compared with out of season and with patients with NAR. Contrary to these changes in pollen season, apoptosis and apoptotic ratio of eosinophils are increased in patients with SAR out of pollen season compared with peak pollen season. Although the number of patients with BHR was significantly higher during pollen season compared with out of season, no correlation between PC_{20} to methacholine and eosinophil numbers and apoptosis was observed.

Eosinophils are major effector cells of allergic inflammation. Eosinophil migration to the site of inflammation is the result of several sequential steps and depends on the expression of cytokines and adhesion molecules.²¹ The presence and activation of eosinophils have been demonstrated in bronchial biopsy and induced sputum samples from patients with asthma.^{5,8} Further, significant correlations between eosinophil counts and the severity of asthma and the degree of BHR have been reported.^{5,22} Several studies have shown that nasal exposure to allergen causes a systemic reaction, including eosinophil release from the bone marrow to circulation and migration into airway mucosa.^{23,24} In keeping with that finding, bronchoalveolar lavage and bronchial biopsy samples have revealed that patients with SAR may demonstrate inflammatory process in lower airways with eosinophil predominance.²⁵ Recent studies have proposed induced sputum as a noninvasive method to evaluate airway inflammation. However, a few induced sputum studies are available that have assessed the presence of inflammation in the lower airway from patients with SAR and its relations with natural pollen exposure and seasonal changes of BHR. Two studies^{13,20} have reported that eosinophils are present in induced sputum from patients with SAR even outside pollen season. Another study²⁶ showed not only the presence of inflamma-

tory cells, including eosinophils and basophils, but remarkably changes of these cells during natural pollen exposure. In accordance with these results, increased numbers of eosinophils were found in both peripheral blood and induced sputum of patients with SAR during peak pollen season, and these numbers were decreased to the same level as patients with NAR after pollen season. Other inflammatory cells in induced sputum samples of our patients did not show any significant changes with the changes of pollen exposure, indicating the importance of eosinophils as effector cells of allergic inflammation.

The mechanisms that determine persistence or resolution of eosinophilic inflammation in tissue are not entirely understood. Apoptosis as a physiologic form of cell death seems to play a role in the removal of inflammatory cells from the inflammation site.²⁷ Apoptotic eosinophils are intact and ingested by macrophages, leading to the prevention of the release of toxic eosinophil granules. It is therefore possible that eosinophil apoptosis plays a major role in the resolution of both acute and chronic allergic inflammation. The relation of eosinophil apoptosis with the airway inflammation was mostly studied in patients with asthma.^{6,8,19} Several studies demonstrated that apoptosis of airway cells was related to the clinical severity of asthma.^{8,19} Further, apoptotic eosinophils have been detected in the sputum samples of patients during asthma attacks, and their numbers significantly increased after treatment with inhaled corticosteroids.¹⁹ However, the data regarding eosinophil apoptosis in patients with AR are limited. The only study performed, by Foresi et al,²⁰ reported apoptotic eosinophils in induced sputum samples of patients with SAR during natural pollen exposure. Furthermore, the increase of eosinophil apoptosis was associated with an increase of PC₂₀ to methacholine level. The study did not cover the changes of inflammatory cells and apoptosis, depending on pollen exposure and their relations to seasonal changes of BHR in this particular patient group. To the best of our knowledge, there is no study based on seasonal changes in eosinophil apoptosis and its relationship with BHR in patients with SAR. In our study, the apoptotic eosinophil counts were not different in patients with SAR during natural pollen exposure compared with those of patients with NAR; however, apoptotic eosinophils and apoptotic ratio were significantly increased out of season, indicating their possible roles in the resolution of the inflammation in bronchial mucosa.

BHR, a major feature of asthma, has also been observed in patients with AR even if they have no evidence of asthma, like our patients.²⁸ Data strongly suggest a relationship between eosinophilic bronchial inflammation and BHR in asthmatic patients,²⁹ but the pathogenesis of BHR in AR is not fully understood. There are different reports about the correlation of BHR with airway eosinophilia in AR. Foresi et al¹² provided evidence for the contribution of eosinophils to BHR in SAR by showing a correlation between methacholine PC₂₀ and the number of eosinophils in induced sputum. Furthermore, Leone et al²⁶ reported that changes in inflammatory cells during pollen exposure are more evident in patients with

a significant increase in BHR to methacholine. Contrary to methacholine provocation, some studies speculate that adenosine monophosphate provocations may be a more direct marker of allergic airway inflammation in patients with AR than methacholine provocation.^{30,31} In line with this speculation, Polosa et al³¹ failed to show a significant association between airway eosinophilia and methacholine responsiveness in patients with allergic rhinitis. We were not able to find any significant correlation between the number of airway eosinophils and methacholine PC₂₀ in our patients; however, the small size of the study group and methacholine provocation as an evaluation of BHR may have a role in this discrepancy. Seven of our 14 patients had BHR to methacholine in pollen season, and there were no correlations among airway eosinophilia, eosinophil apoptosis, and methacholine PC₂₀. It could be speculated that the severity of AR affected BHR in the airways, but we did not find any statistically significant difference in nasal symptom scores between those patients with and without BHR during pollen season. Although this finding did not reach statistical significance, the apoptotic ratio of eosinophils was higher in those patients without BHR and eosinophil counts were higher in those with BHR during pollen season. In addition, following the increase of eosinophil apoptosis and decrease of eosinophil numbers, the number of patients with BHR decreased from 7 patients to 2 patients out of season. Although statistical analysis was not convenient because of the small number of patients, the mean apoptotic ratio of eosinophils was lower and eosinophil counts were higher in the 2 patients with BHR than the 12 patients without BHR out of season, confirming the role of apoptosis in the resolution of inflammation in bronchial mucosa. It is, therefore, tempting to speculate that a defect in eosinophil apoptosis could play a role in the persistence of BHR out of pollen season.

The major limitation of our study is the method used to determine apoptosis. When cells undergo apoptosis, they show some characteristic findings, such as cell shrinkage, chromatin condensation, and formation of apoptotic body.³² Considering these structural characteristics of apoptotic cells, we used visual inspection of microscopy samples from dispersed induced sputum, just as previous studies had done.^{19,20} However, more specific apoptosis methods, including a multiparametric analysis by flow cytometry and image cytometry and annexin V/PI labeling, have gained more interest in recent years.³³ Also, the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling technique is used to detect eosinophil apoptosis, determining DNA fragments in induced sputum of asthmatic patients.³⁴ The lack and/or the costs of the other methods were major contributors that drove us to use light microscopy.

In conclusion, our data indicate that changes in the eosinophil counts and eosinophil apoptosis in induced sputum samples are associated with the changes of natural pollen exposure and may have a role in the seasonal changes of BHR in patients with allergic rhinitis. However, further studies

with more patients and more specific methods for the evaluation of apoptosis are needed to elucidate these relationships.

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