

The effects of natural pollen exposure on inflammatory cytokines and their relationship with nonspecific bronchial hyperresponsiveness in seasonal allergic rhinitis

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ABSTRACT

The exact mechanism of bronchial hyperresponsiveness (BHR) is not clear in allergic rhinitis (AR); an increase of BHR in pollen season suggests that natural pollen exposure causes airway inflammation in seasonal AR (SAR). This study was designed to investigate the effects of natural pollen exposure on inflammatory cytokines and their relationship with BHR. Sixty-six SAR patients with grass pollen sensitivity and 26 nonallergic rhinitis (NAR) patients were included. Peripheral blood samples for cytokine levels were taken and a nonspecific bronchial provocation test was performed during pollen season between May and August. The same measurements were repeated off-season between November and February. These measurements were done in NAR patients once. During the pollen season, SAR patients had significantly more increased levels of IL-13 than NAR patients (11.45 ± 12.54 versus 5.19 ± 4.02 ; $p = 0.005$). Blood eosinophil numbers were higher in those patients with BHR during pollen season than those without BHR (399.0 ± 255.8 versus $278.9 \pm 193.2 \text{ mm}^{-3}$; $p = 0.046$). Blood eosinophil numbers during off-season were not different in those with and without BHR (respectively, $261.4 \pm 202.3 \text{ mm}^{-3}$ versus $205.9 \pm 116.9 \text{ mm}^{-3}$; $p = 0.53$). IL-10 levels were higher in the patients without BHR ($n = 28$) than those patients with BHR ($n = 22$) during off-season (8.12 ± 13.1 versus 3.28 ± 0.37 ; $p = 0.04$). Having higher levels of IL-10 than threshold value was more frequent in SAR patients without BHR than those patients with BHR during off-season (7/28 versus 1/22; $\chi^2 = 4.34$; $p = 0.04$). IL-10 has a role in the continuation of BHR during off-season in SAR patients.

(Allergy Asthma Proc 31:126–131, 2010; doi: 10.2500/aap.2010.31.3325)

Key words: Allergic rhinitis, bronchial hyperresponsiveness, bronchial provocation test, cytokine, pollen, rhinitis, seasonal allergic rhinitis

Bronchial hyperresponsiveness (BHR) is a characteristic feature of asthma and a consequence of the underlying airway inflammation.¹ However, BHR is not confined to asthmatic patients only. Seasonal allergic rhinitis (SAR) patients have also BHR even if they have no asthma symptoms. Although the mechanism of BHR in AR is not explained exactly, presence of BHR in SAR is considered as a risk factor for asthma development.^{2,3} The increase of BHR during pollen season strengthens the thought that airway inflammation in SAR is because of natural pollen exposure.^{4,5} Although the seasonal increase of BHR during pollen season is well known in SAR patients, mechanism of BHR in SAR is not explained exactly. We previously suggested that changes in the number of eosinophils and eosinophil apoptosis in induced sputum samples were asso-

ciated with natural pollen exposure and might have a role in the seasonal changes of BHR in patients with AR.⁵ During the process of airway sensitivity, lymphocytes accumulate and send stimulus to eosinophils.⁶ Cytokines play a crucial role in modulating these allergic and asthmatic inflammatory reactions. During the course of airway inflammation a variety of cytokines including IL-4, IL-5, IL-13, IL-6, TNF- α , and granulocyte macrophage colony-stimulating factor have been shown to play a role.^{7–10} IL-1 induces adhesion molecules and activates inflammatory cells.¹¹ Some other cytokines such as interferon γ , IL-10, and IL-12 have a regulatory role; that suppresses the allergic inflammation by inhibiting mainly Th₂-cell differentiation.^{12,13} In this study, we aimed to investigate the effect of natural pollen exposure on inflammatory cytokines in peripheral blood and whether there was a relationship between these parameters and BHR during and out of off-season in patients with SAR.

METHODS

Patients

Two groups of patients were selected: 66 SAR patients (25 were male patients), aged 18–51 years old

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Supported by Eskisehir Osmangazi University Fund (No. 200511027)

The authors had no conflicts of interest regarding this study

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(30.2 ± 9.5 years), and 26 nonallergic rhinitis (NAR) patients (9 were male patients), aged 20–60 years old. Only SAR patients with pollen allergy have been included in the study. The inclusion criteria for SAR patients were having historical data defined by nasal itching, rhinorrhea, sneezing, and nasal obstruction for at least 2 years; positive physical examination finding with positive skin-prick tests (SPTs) 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 SPTs. Patients were restricted to those living in Eskisehir to exclude possible effect of varying severity of pollen concentration on inflammatory parameters.

At the beginning of the study, the patients were asked for asthmatic symptoms and patients thought to be asthmatic by medical story were excluded. None of the subjects reported any upper or lower respiratory tract infection or use of nasal or oral steroids during the previous 6 weeks or within the study period. During the study, none of the patients used nasal or oral corticosteroids that might affect BHR. Intranasal azelastine spray was allowed when necessary to suppress nasal symptoms during pollen season. None of the SAR patients had previously been treated with immunotherapy. None of the subjects were smokers. The study was approved by the local ethics committee and informed consent was obtained from the patients.

Evaluation of Atopy

SPTs were performed by using a common panel including *Dermatophagoides pteronyssinus*, *Dermatophagoides farinae*, grass, tree and weed pollens, animal dander (cat and dog), animal feathers, and mold (including *Alternaria*, *Cladosporium*, *Aspergillus*, and *Penicillium*) allergen extracts (Allergopharma, Reinbeck, Germany). Positive and negative controls were histamine (10 mg/mL) and phenolated glycerol saline, respectively. A mean wheal diameter of 3 millimeter or greater than that of obtained with control solution was considered as positive.

Bronchial Provocation Test (BPT)

Methacholine bronchial challenges were performed by a modification of the method described by Chai *et al.*¹⁴ A dosimeter (Zan 200; Zan, Oberthulpa, Germany), triggered by a valve set to remain open for 0.6 seconds, was used to deliver the aerosol. After inhalation of physiological saline, subjects inhaled doubling concentration of methacholine from 0.06 to 8 mg/mL diluted in buffered saline. Forced expiratory volume in 1 second (FEV₁) was measured with a spirometer (Al-

pha III; Vitalograph, Buckingham, England), and the largest value of triplicate FEV₁ after 60–90 seconds of each inhalation was adopted for analysis. The challenge was stopped when the FEV₁ dropped by >20% from the postphysiological saline or when the highest concentration of methacholine had been administered. The result was expressed as methacholine PC₂₀ (provocative concentration require to produce a 20% fall in FEV₁). The PC₂₀ was obtained from the log concentration–percent fall in FEV₁ curve by linear interpolation of the last two points. Subjects were considered to have BHR if they showed a decline in FEV₁ of ≥20% at a concentration of methacholine of <8 mg/mL. At the end of the challenge, patients received 200 µg of salbutamol from a metered-dose inhaler to relieve airway obstruction.

Cytokine Detection

The peripheral blood samples were kept at room temperature at least 30 minutes to separate the clot. After that, the blood was centrifuged at 1000 × g for 10 minutes, the serum collected by this procedure was kept at –80°C. The levels of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-8, IL-10, IL-12 (p70), IL-13, interferon γ, granulocyte macrophage colony-stimulating factor, and TNF-α in serum were analyzed using a multiplexed bead array system read by Luminex xMAP following the manufacturer's instructions (Linco Research, Inc., St. Louis, MO). Values (expressed as pg/mL) that fell below the limits of detection for the assay were assigned a value equal to the lowest limit of detection for each assay.

Study Design

SAR Patients. Peripheral blood samples to determine eosinophil and IgE levels and to detect cytokine levels have been obtained during the pollen season between May and August and the off-pollen season between November and February. These seasonal intervals were selected according to the data regarding peak and off-grass pollen season in Eskisehir.¹⁵ BPT with methacholine was also measured in both seasons.

NAR Patients. BPT and peripheral blood samples have been obtained once in the control group at any time regardless of the season.

Nasal symptoms, rhinorrhea, nasal blockage, and sneezing were recorded by the patients over a period of 4 weeks. SAR patients recorded symptom scores during the pollen season. The scores were graded from 0 to 3 according to the severity considering the whole day (0 = 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

Table 1 Comparisons of nasal symptom scores, FEV₁ values, blood eosinophils, total IgE levels, and presence of bronchial hyperresponsiveness (BHR) of seasonal allergic rhinitis (SAR) patients during the pollen season and the off-season and of nonallergic rhinitis (NAR) patients

	SAR (n = 50)		p*	NAR (n = 26)	p#
	Pollen Season	Off-Season			
Nasal symptom score, mean ± SD	1.44 ± 0.54			1.2 ± 0.5	0.06
FEV ₁ , mean ± SD (% of predicted)	91.9 ± 11.8	92.6 ± 10.6	0.08	90.8 ± 6.7	0.96–0.44
Blood eosinophils, mean ± SD (mm ⁻³)	353.4 ± 239.3	230.2 ± 160.5	<0.0001	245.8 ± 182.4	0.04–0.72
Total IgE (IU/dL), mean ± SD	214.5 ± 270.4	212.1 ± 291.0	0.73	58.8 ± 62.6	0.006–0.01
BHR (yes/no)	31/19	22/28	0.004	6/20	0.001–0.07

*Statistical significance between in- and off-pollen season findings of SAR patients.

#Statistical significance between SAR and NAR patients. The first p value compares the in-season result of SAR patients with the results of NAR patients; the second p value compares off-season results of SAR patients with the results of NAR patients. Significant findings are set in bold face.

each patient. SAR patients were allowed to take topical nasal azelastine and ophthalmic olopatadine as required during pollen season. Drugs were stopped 24 hours before the study during the pollen season.

Statistical Analysis

The results were presented as mean ± SD. Comparisons between groups were made using the Mann-Whitney U test, χ^2 -test, and parametric and nonparametric types of t-test for unpaired data when appropriate. The pollen season and off-pollen season values of SAR patients were compared using Wilcoxon paired t-test, McNemar test, or Kruskal-Wallis analysis. A value of $p < 0.05$ was considered statistically significant. Spearman test was used for the correlation analysis. SPSS for Windows (Version 10.0; SPSS, Inc. Chicago, IL) was used for the calculations.

RESULTS

Samples from 50 (21 male patients) of 66 SAR patients were provided also during off-season. Sixteen patients were removed from the study because of moving to another city or unwillingness for the second samples. Mean age (30.2 ± 9.2 years old versus 34.2 ± 11.0 years old; $p = 0.12$) and duration of disease (4.7 ± 3.2 years versus 4.7 ± 3.0 years; $p = 0.86$) were not different between the 50 SAR patients and 26 NAR controls. Nasal symptom scores, FEV₁ values, blood eosinophils, total IgE levels, and presence of BHR of 50 SAR patients during pollen season and off-season and of 26 NAR patients are shown in Table 1. The number of patients who have BHR during pollen season is higher in SAR patients when compared with NAR patients. IgE value in SAR patients in both during and out of off-season is higher than NAR patients. Blood eosinophil numbers were higher in SAR patients dur-

ing pollen season when compared with off-season and NAR patients.

The mean values of cytokine and chemokine levels in SAR during pollen season and off-season and NAR patients are in Table 2. During pollen season, SAR patients had significantly increased levels of IL-13 when compared with NAR patients (11.45 ± 12.54 pg/mL versus 5.19 ± 4.02 pg/mL; $p = 0.005$).

The number of SAR patients who had nonspecific BHR to methacholine during pollen season (31/50) was significantly higher than those in the off-season (22/50; $p = 0.004$; Table 1). Those 22 patients with BHR during the off-season were considered as having permanent BHR. All cytokine levels except IL-10 were not different in those patients with and without BHR during the off-season. IL-10 levels were higher in the patients without BHR ($n = 28$) than those patients with BHR ($n = 22$) during the off-season (8.12 ± 13.1 pg/mL versus 3.28 ± 0.37 pg/mL; $p = 0.04$). Having higher levels of IL-10 than threshold value were more frequent in SAR patients without BHR than those patients with BHR during the off-season (7/28 versus 1/22; $\chi^2 = 4.34$; $p = 0.04$). In the pollen season, IL-7 levels were higher in SAR patients without BHR than those with BHR (10.8 ± 18.4 pg/mL versus 3.2 ± 0.0 pg/mL; $p = 0.008$). There was no such relationship between all other cytokines and BHR during the pollen season and the off-season ($p > 0.05$).

Blood eosinophil numbers were higher in those patients with BHR during the pollen season (399.0 ± 255.8 mm⁻³ versus 278.9 ± 193.2 mm⁻³; $p = 0.046$). Blood eosinophil numbers during the off-season were not different between those with and without BHR (respectively, 261.4 ± 202.3 mm⁻³ versus 205.9 ± 116.9 mm⁻³; $p = 0.53$). Serum IgE levels during both seasons were not different between those patients with and without BHR, respectively; 180.0 ± 182.4 IU/mL ver-

Table 2 The mean levels of inflammatory cytokines in seasonal allergic rhinitis (SAR) patients during the pollen season and the off-season and nonallergic rhinitis (NAR) patients (pg/mL)

	SAR (n = 50)			NAR (n = 26)	p#
	Pollen Season	Off-Season	p*		
IL-1 β (pg/mL)	4.62 $\pm$ 6.64	4.01 $\pm$ 4.67	0.50	4.91 $\pm$ 8.70	0.38–0.72
IL-2 (pg/mL)	5.60 $\pm$ 10.48	5.10 $\pm$ 11.5	0.50	5.42 $\pm$ 10.39	0.74–0.78
IL-4 (pg/mL)	43.0 $\pm$ 123.28	36.89 $\pm$ 111.28	0.21	129.64 $\pm$ 323.82	0.91–0.63
IL-5 (pg/mL)	3.26 $\pm$ 0.46	3.33 $\pm$ 0.78	0.11	3.75 $\pm$ 1.64	0.06–0.37
IL-6 (pg/mL)	6.75 $\pm$ 10.02	5.89 $\pm$ 8.93	0.08	17.35 $\pm$ 33.75	0.31–0.28
IL-7 (pg/mL)	6.09 $\pm$ 11.78	4.25 $\pm$ 5.57	0.07	3.33 $\pm$ 0.68	0.46–0.66
IL-8 (pg/mL)	6.17 $\pm$ 5.73	9.71 $\pm$ 17.62	0.33	8.90 $\pm$ 13.26	0.65–0.59
IL-10 (pg/mL)	9.11 $\pm$ 16.10	5.99 $\pm$ 9.99	0.43	4.45 $\pm$ 3.59	0.34–0.62
IL-12 (pg/mL)	4.84 $\pm$ 6.79	4.71 $\pm$ 7.35	1.00	3.20 $\pm$ 0.00	0.11–0.32
IL-13 (pg/mL)	11.45 $\pm$ 12.54	10.19 $\pm$ 12.37	0.17	5.19 $\pm$ 4.02	0.005 §–0.07
IFN- γ (pg/mL)	11.91 $\pm$ 39.30	10.77 $\pm$ 42.57	0.36	7.52 $\pm$ 22.05	0.23–0.27
GM-CSF (pg/mL)	5.01 $\pm$ 4.82	5.07 $\pm$ 6.37	0.90	5.63 $\pm$ 6.81	0.71–0.78
TNF- α (pg/mL)	5.29 $\pm$ 3.83	5.72 $\pm$ 4.06	0.33	5.03 $\pm$ 3.25	0.93–0.39

*Statistical significance between in- and off-pollen season findings of SAR patients.

#Statistical significance between SAR and NAR patients. The first p value compares the in-season result of SAR patients with the results of NAR patients; the second p value compares off-season results of SAR patients with the results of NAR patients.

§During pollen season, SAR patients have more increased levels of IL-13 than NAR patients.

IFN = interferon; GM-CSF = granulocyte macrophage colony-stimulating factor.

Significant finding is set in bold face.

sus 281.4 $\pm$ 388.3 IU/mL (p = 0.77) during the pollen season and 142.2 $\pm$ 150.3 IU/mL versus 275.9 $\pm$ 368.9 IU/mL (p = 0.35) during the off-season.

When we considered SAR patients during the pollen season, there was positive correlation between severity of rhinitis obtained by nasal symptom scores and the number of blood eosinophils (r = 0.42; p = 0.002). Nasal symptom scores were not different between those patients with BHR and without BHR in the pollen season (1.44 $\pm$ 0.59 versus 1.43 $\pm$ 0.49; p = 0.38).

DISCUSSION

The present study showed that SAR patients had asymptomatic BHR even in the off-season, which was associated with low IL-10 levels. SAR patients had higher IL-13 levels when compared with NAR patients during the pollen season although it was not different between the two groups during the off-season.

IL-13 and IL-4 have similar effects in allergic inflammation. After the antigenic stimulus, IL-13 together with IL-4 causes IgE secretion from B lymphocytes by increasing the major histocompatibility complex group II antigen and CD23 presentation.¹⁶ Recent studies emphasized that IL-13 secretion is increased in AR patients with pollen allergy and this is important for the continuation of immediate-type hypersensitivity.¹⁷ IL-13 is also required for the development and continuation of BHR in the asthmatic airways. Eosinophil accumulation and BHR development has been shown

after injection of IL-13 to the trachea.¹⁸ Our results are in accordance with previous studies that confirmed the relationship between allergic stimulus and IL-13 secretion, which appeared during the pollen season.

Although BHR is a characteristic feature of asthma, it can be seen in SAR patients without asthma symptoms as in the present study.^{5,19} The relationship between BHR and eosinophilic airway inflammation in the asthmatic patients is well known; however, the pathogenesis of BHR in AR patients is not clear.²⁰ The proposed mechanisms for BHR in AR patients are contraction of bronchial smooth muscle by mediators releasing from inflammatory cells reaching to the bronchi directly or by blood circulation and eosinophilic inflammation of the airways.^{5,21,22} In the present study BHR was associated with higher blood eosinophil numbers during the pollen season, indicating that allergenic stimulus causes BHR through eosinophil-related mechanisms. Among the cytokines IL-7 levels were higher in SAR patients without BHR than those with BHR during the pollen season. IL-7 has been less studied in allergic diseases. Previous studies showed that IL-7 has regulatory action on T-cell functions.²³ IL-7 has cytotoxic and suppressive effects mediated by CD8⁺ T cells and CD56⁺ cells.^{24,25} This cytokine has been shown to cause increased Th₁ activation that is higher than Th₂ activation as a response to allergen challenge in SAR patients, suggesting a suppressive effect in allergic inflammation.²⁶ We might suggest that IL-7 has suppres-

sive effects on the development of BHR in SAR patients during pollen season; however, it is hard to be certain because of the low numbers of patients having high levels of IL-7 in the pollen season (four patients). This clue needs further investigation. However during off-season, BHR was only correlated with IL-10 levels. Continuation of BHR in SAR patients during the off-pollen season in which there is no allergen exposure is probably related to IL-10-dependant regulatory mechanisms on airway inflammation. Showing that BHR also continues during the off-season confirmed that BHR occurs as a result of systemic effects of allergic inflammation rather than rhinitis or its severity. This opinion was also supported by the result that there was no relation between BHR and the severity of rhinitis, which was determined by symptom score during the pollen season.

In recent years, regulator cells in allergic inflammation have become important as reducing or suppressing inflammatory response. These regulatory cells mainly express transforming growth factor β and IL-10.²⁷ Regulatory cell functions as detected by IL-10 secretion has an important role in the management of allergic diseases, such as in allergen immunotherapy.²⁸ IL-10 is an important regulatory cytokine on the suppression of allergic inflammation.²⁹ It has been shown that exogenous IL-10 suppresses cytokine and chemokine secretion from both Th₁ and Th₂ cells²⁹ and suppress airway hyperresponsiveness after allergen-induced challenge.¹³ In our study, low levels of IL-10 were associated with BHR during the off-season, indicating that IL-10 may have an important role in continuation or resolution of BHR, which occurred in the pollen season. Other cells also participate to suppress the airway inflammation through IL-10. In an experimental study, airway hyperresponsiveness has been shown to decrease after induction of IL-10-treated dendritic cells, suggesting that IL-10 has an important role on the continuation of BHR.³⁰ Previous experimental studies also supported that IL-10 is an important mechanism that prevents BHR by decreasing airway inflammation in the airways.^{31,32} In addition, IL-10 suppresses antigen-presenting cell functions so that it has a suppressive effect as a response to allergen.³³ All patients with BHR during the off-season in the present study also had BHR during the pollen season. It is not known why BHR does not resolve in some of the SAR patients after pollen exposure ceased. It is probable that there is personal predisposition of having defect in suppressive mechanisms such as IL-10 on airway hyperresponsiveness in SAR patients. Such personal propensity has been reported in asthma showing that IL-10 polymorphisms are associated with asthma.^{34,35}

The pathogenesis of allergic airway inflammation is complex, involving multiple cell types and variety of

cytokines and chemokines. SAR patients show a higher degree of BHR during the pollen season although they do not have asthmatic symptoms. BHR in some of these patients improves during the off-season when they do not expose pollen allergen. Our study supported that IL-10 has a role in improvement of BHR that occurred during the pollen season in SAR patients. We need further studies on the propensity of IL-10 response in SAR patients.

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