

ORIGINAL RESEARCH–ALLERGY

Local production of antigen-specific IgE in different anatomic subsites of allergic fungal rhinosinusitis patients

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ABSTRACT

OBJECTIVE: Local production of antigen-specific IgE in allergic fungal rhinosinusitis (AFRS) is likely integral to the expression of allergy. This study examines if there are anatomic variations in local IgE expression or if variations among fungal and nonfungal IgE exist.

STUDY DESIGN: Cross-sectional study.

SETTING: Tertiary medical center.

SUBJECTS AND METHODS: Specimens from 11 AFRS, 8 chronic rhinosinusitis without nasal polyps (CRSsNP), and 9 control patients underwent immunohistochemical localization for IgE and evaluation for antigen-specific IgE by ImmunoCAP testing.

RESULTS: Inferior turbinate (IT) epithelium had greater IgE staining in AFRS than control ($P = 0.013$) and CRSsNP ($P = 0.002$). A significant difference was also found at the IT subepithelial level for AFRS compared with controls ($P = 0.001$) and CRSsNP ($P < 0.001$). Within AFRS, IgE staining was increased in the subepithelium compared to epithelium ($P = 0.003$). ImmunoCAP analysis on IT tissue from AFRS and controls demonstrated increased antigen-specific IgE for 5 of 14 antigens ($P < 0.05$) and total IgE ($P < 0.001$). There were no significant anatomic differences between IT and sinus IgE staining.

CONCLUSION: More fungal and nonfungal IgE is expressed in IT and sinus tissues of AFRS patients, as compared with control and CRSsNP patients.

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Allergic fungal rhinosinusitis (AFRS) is a chronic condition prevalent throughout the humid river basins and coastal regions of the Southeastern United States. Despite recent advances in the field of endoscopic sinus surgery and improvement in the delivery methods of topical drugs and irrigations, AFRS patients continually suffer from the consequences of their disease. Bent and Kuhn¹ have established the classic criteria for defining this entity, which includes a

type I IgE-mediated hypersensitivity reaction, a positive fungal stain/culture, characteristic radiologic findings, the presence of eosinophilic mucin without fungal invasion, and nasal polyposis.

Nasal polyposis is a hallmark of AFRS. A multitude of factors have been proposed as key agents in the development of polyposis, including fungi, enterotoxins, eosinophils, and IgE.^{2–6} Researchers have been recently investigating specific inflammatory markers in various sinus inflammatory conditions. A study by Kim et al² highlighted some of the immunologic and histologic aspects of nasal polyps, with the majority of polyps examined by their study being non-eosinophilic in nature. In addition, they found no true relationship between the levels of serum IgE and presence or absence of eosinophils in nasal polyps. This finding is in contrast to the results of the study by Bachert et al,⁴ in which nasal tissue homogenates from patients with and without nasal polyposis were examined, and a relationship between IgE and eosinophilic inflammation in polyps was identified.⁴ Furthermore, they suggest this IgE could be locally produced, which helps to explain the discrepancy between these two studies.⁴ These studies illustrate the diversity that exists across the vast spectrum of patients and their corresponding inflammatory conditions, possibly due to geographic variations or heterogeneity in the pathophysiology of nasal polyposis.

Recent literature has focused on the role and significance of local IgE production in a variety of rhinologic conditions, including AFRS. KleinJan et al^{7,8} demonstrated local sinonasal mucosal production of allergen-specific IgE in allergic rhinitis patients. Similarly, production of antigen-specific IgE has been demonstrated locally in the bronchoalveolar lavage fluid of asthmatic patients after allergen challenge.⁹ Applications to AFRS were previously limited to the investigation of local IgE presence in nasal lavage fluid and eosinophilic mucin.^{3,10} A recent study completed by our group took this issue a step further by

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demonstrating increased expression of antigen-specific fungal and nonfungal IgE in sinus mucosa of AFRS patients compared with controls.¹¹

The purpose of this study was to expand on our previous results by examining the anatomic differences in location and quantity of local IgE at both sinus mucosa and inferior turbinate subsites in patients with AFRS, as compared with patients with chronic rhinosinusitis without nasal polyposis and those designated as control.¹¹ We previously hypothesized and demonstrated that IgE-bearing cells would be present in greater numbers in AFRS sinus mucosa, compared with the other diagnostic groups. In this study, we further hypothesize that AFRS inferior turbinate tissue will show increased local IgE production over inferior turbinate tissue from other patient groups. From an anatomic standpoint, we hypothesized that IgE-bearing cells would be present in greater concentrations in sinus mucosa specimens compared with turbinate specimens, given the propensity of polyps to form in the sinuses rather than nasal and turbinate tissue. The second aim was to investigate the quantity of both antigen-specific fungal and nonfungal IgE in both sinus mucosa and inferior turbinate specimens of AFRS patients compared with controls.

MATERIALS AND METHODS

Approval was obtained from the Institutional Review Board at the Medical University of South Carolina before this study was initiated. Patients were counseled before surgery about the research protocol, and informed written consent was acquired from each patient who participated in this study. All participants were treated at a single institution, the Medical University of South Carolina.

Twenty-eight patients were enrolled in the study. The patients were divided into three diagnostic groups: 1) patients with AFRS (11 patients); 2) patients with chronic rhinosinusitis without nasal polyposis (CRSsNP) (8 patients); and 3) patients designated as control (9 patients). All AFRS patients were diagnosed with the classic Bent and Kuhn criteria. IgE hypersensitivity to fungi in AFRS patients was demonstrated by either skin testing (MQT method) or in vitro testing of serum. CRSsNP patients were diagnosed through clinical and radiographic examinations that revealed inflammatory sinus disease without the subjective history of atopy. Control patients were those who underwent either tumor extirpation or CSF leak repair and had no subjective or objective history of inflammatory sinus disease. CRSsNP and control patients were not routinely allergy tested as this was not part of their standard treatment. During endoscopic sinus surgery, tissue specimens were taken from the patients at both osteomeatal complex (uncinate and anterior ethmoid tissue) and inferior turbinate subsites. All patients were treated the same pre-operatively, ie, patients who consented to surgery were devoid of both

steroidal and antifungal medications for one month prior to surgery.

Histologic Immunolocalization and Quantification of Total IgE (Immunoperoxidase Staining)

Specimens were collected at the time of surgery, placed in Tissue-Tek O.C.T. medium (Sarkura Finetek, Torrance, CA), and immediately snap-frozen in liquid nitrogen. They were then stored at -80°C until time of processing. A Leica CM 1850 cryostat (Wetzlar, Germany) was used to cut the specimens (6 μm thickness), which were subsequently affixed to poly-L-lysine coated slides. All slides were then thawed for 24 hours before processing.

Immunoperoxidase staining began by subjecting the slides to tissue fixation in ice-cold acetone for 10 minutes at room temperature. Slides were then dried completely, washed in FA (phosphate-buffered saline solution for fluorescent antibody applications) buffer, and subjected to 0.6 percent hydrogen peroxide (a total of 5 treatments) to quench endogenous peroxidase activity. Nonspecific antibody staining was blocked with the Vectastain ABC blocking agent (Vector Labs, Burlingame, CA) for a period of 20 minutes. The slides were then incubated with commercially available antibody to IgE (Invitrogen/Zymed, Carlsbad, CA) for one hour. After this incubation, the slides were again washed in FA buffer and subsequently subjected to biotinylated secondary antibody (Vector Labs) for 30 minutes. A third FA buffer wash was then performed. Next, Vectastain ABC solution was added to the slides for 30 minutes, followed by incubation with AEC (3-Amino-9-Ethylcarbazole, Sigma Chemical Company, St Louis, MO) reagent for 10 minutes. A final FA buffer wash was performed. Slides were then counterstained with Harris hematoxylin (Surgipath Medical Ind., Inc, Richmond, IL) for 45 seconds. Slides were allowed to air-dry overnight, followed by application of aquamount and a coverslip to all specimens. Isotype negative controls were performed simultaneously on all specimens.

A Zeiss Axiovert 200 M microscope (Carl Zeiss, Inc, Germany) was used to take photomicrographs from 5 random epithelial and 5 random subepithelial sections on each slide. The photographer was blinded to patient and condition. Photos were then presented to a panel of 4 blinded graders who quantified the positive stains.

Appropriate statistical analysis was performed with a Kruskal-Wallis nonparametric test to assess group differences in local IgE staining among the three diagnostic groups (AFRS, CRSsNP, and controls) at both the epithelial and subepithelial levels. Follow-up testing was performed with Mann-Whitney U tests to identify significant group differences. Nonparametric Wilcoxon sign rank tests were performed within each diagnostic group to evaluate for significant differences in IgE staining at

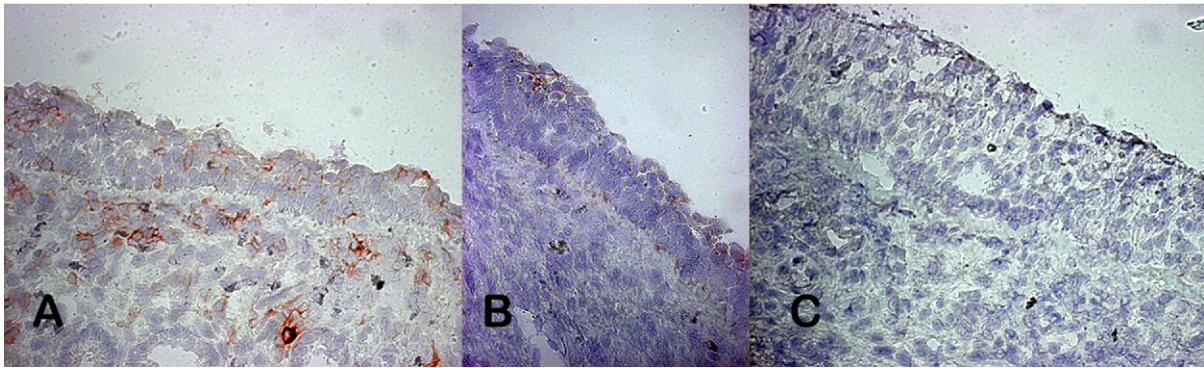


Figure 1 Pictomicrographs of IgE antibody staining in turbinate epithelial representative tissue sections. Within epithelial sections, only the positive staining cells (red) within the epithelium were counted; positive subepithelial staining was not counted on tissue sections designated as epithelial sections: (A) AFRS patient, (B) control patient, and (C) CRSsNP patient.

the epithelial versus subepithelial level and between sinus and turbinate tissue.

Anatomic Subsite Identification of Fungal and Nonfungal Specific IgE (ImmunoCAP Analysis)

At the time of surgery, specimens were placed in sterile conical vials and snap frozen in liquid nitrogen. Only specimens from AFRS patients and controls were stored, as tissue quantity was not adequate for analysis of CRSsNP patients. Before processing, specimens were thawed for one hour and placed in 1 mL of sterile phosphate-buffered saline (PBS) solution. A tissue homogenizer was used to obtain a single cell suspension for each specimen. The suspensions were transferred to 1.5 mL microcentrifuge tubes and centrifuged for 5 minutes at 10,000 rpm (4° C). Supernatants were decanted into 5 mL snap top tubes and placed on ice. All specimens were processed by a standard laboratory homogenization protocol, and slight modifications were made to ensure consistent sample quality for subsequent ImmunoCAP testing.

The single cell suspensions were then converted into lysates with an ultrasonicator. Each specimen was sonicated at 15 second pulse intervals until a total of 7 pulses had been performed. Complete lysis was confirmed by Trypan Blue exclusion. A sample of the mixture was transferred to a hemecytometer to identify whole cells. If whole cells were observed, the sonication process was repeated until whole cells were no longer observed. Total protein levels were determined for the lysates by BCA assay (Thermo Scientific, Rockford, IL).

One milliliter samples of each lysate were submitted for ImmunoCAP (Phadia AB, Portage, MI) analysis through the clinical laboratory at the Medical University of South Carolina. IgE levels determined by ImmunoCAP analysis were standardized for total protein. The following specific antigens were examined: *Cladosporium*, *Aspergillus*, *Alternaria*, Bermuda grass, Timothy grass, white oak tree, red maple tree, cocklebur, plantain, ragweed, dust mite, cockroach, cat, dog, and total IgE.

RESULTS

Histologic Immunolocalization and Quantification of Total IgE

Examination of nonspecific total IgE epithelial staining among the three diagnostic groups revealed statistically significant differences in the turbinate specimens ($P < 0.005$) (Fig 1). Within the turbinate epithelial samples, AFRS tissue demonstrated an increased amount of IgE staining as compared with CRSsNP ($P = 0.002$) and controls ($P = 0.013$) (Fig 2). No difference was seen in epithelial IgE stains between CRSsNP and control patients.

Analysis of subepithelial specimens also demonstrated a statistically significant difference among all diagnostic groups ($P < 0.001$) (Fig 3). In the turbinate subepithelial specimens, AFRS IgE stains were increased as compared with CRSsNP ($P < 0.001$) and controls ($P = 0.001$) (Fig 4).

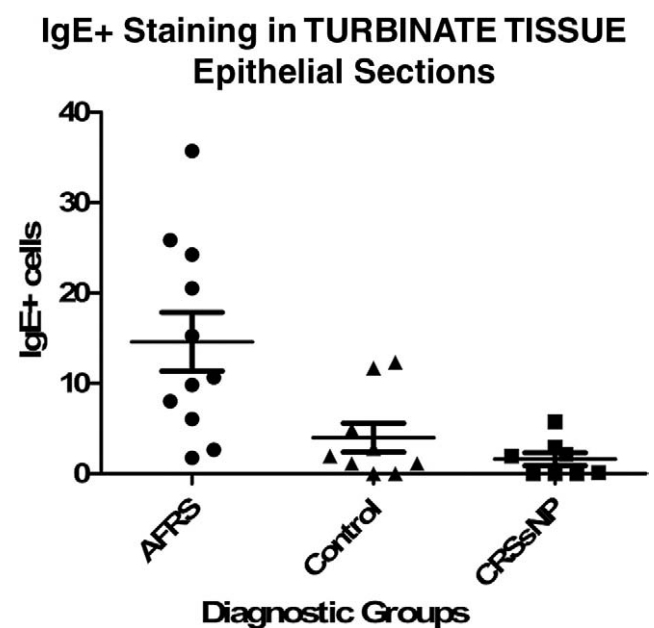


Figure 2 Graph of IgE positive cell counts among various diagnostic groups in the turbinate epithelial specimens.

Table 1
Specific and total IgE in sinus and turbinate specimens: AFRS and control ImmunoCAP analysis¹¹

Specific IgE (mean mUA/mg)	Sinus tissue			Turbinate tissue		
	Control (n = 9)	AFRS (n = 11)	P value	Control (n = 9)	AFRS (n = 11)	P value
Bermuda grass	35.35	138.28	0.146	23.08	179.23	0.203
Timothy grass	32.94	191.48	0.041	25.34	563.77	0.198
White oak	66.68	156.60	0.288	54.66	378.21	0.260
<i>Cladosporium</i>	52.22	324.69	0.044	44.92	394.11	0.031
Red maple	0	96.10	0.045	11.03	68.04	0.075
Dust mite	93.20	1031.27	0.176	63.38	658.44	0.071
Cockroach	6.01	84.53	0.034	21.60	67.41	0.189
Ragweed	0	102.41	0.029	18.46	59.26	0.183
Plantain	29.73	152.92	0.072	26.29	113.60	0.027
Cocklebur	46.60	208.75	0.046	31.28	110.86	0.043
<i>Aspergillus</i>	74.72	581.43	0.004	118.24	380.07	0.031
<i>Alternaria</i>	190.38	1781.12	0.177	123.99	1066.20	0.025
Cat dander	114.65	48.23	0.536	44.90	266.85	0.462
Dog dander	102.19	52.24	0.488	21.23	73.90	0.478
Total IgE (mIU/mg)	1537.34	73204.51	0.004	839.15	20259.29	<0.001

For individual antigens listed above, the numerical values listed represent the mean value obtained from all specimens within each patient category and is expressed as mUA/mg (of total protein). Bold numbering indicates which values are statistically significant.

DISCUSSION

Few would argue that one of the mainstays behind the pathophysiology of AFRS is atopy. This belief is supported by numerous articles and studies, and as a result we have seen a shift in the treatment paradigm for this disease.^{10,12,13} Mabry et al¹⁴ highlighted a successful treatment protocol for AFRS that combined immunotherapy for both fungal and non-fungal antigens with traditional surgical methods. Marple¹³ thoroughly reviewed the history and evolution of the treatment for AFRS and emphasized that the likely successful route for these patients includes a combination of medical and surgical therapies no longer aimed at eradication of an infectious process, but rather an atopic one.

Addressing the upregulated IgE response through immunomodulation stemmed from earlier studies that investigated AFRS patients and their proclivity for systemic reactivity to fungal-specific IgE by skin and in vitro (serum) allergy testing.¹⁵⁻¹⁷ Treatment in the form of immunotherapy has expanded to include nonfungal antigens, as some studies have shown both fungal and nonfungal antigens to be increased in this patient population.^{14,18} Our data from ImmunoCAP analysis of local antigen-specific IgE production in sinus and inferior turbinate tissue support this course of treatment, as we found both fungal and nonfungal specific IgE to be increased in AFRS patients compared with controls.

Nasal polyposis is a hallmark of this disease, and previous studies link polyp development, at least in part, to the role of IgE.²⁻⁵ Early theories of local production of IgE have been supported by recent studies that investigated biopsies of polyps and nasal lavage fluid from patients with allergic rhinitis, where increased levels of IgE were found in local tissues.¹⁹⁻²¹ Bachert et al⁴ suggested that local IgE produc-

tion occurred after finding increased levels of total IgE in nasal polyp tissue compared with inferior turbinate tissue. Our present study supports these findings, as we examined and quantified local IgE expression via immunohistochemical analysis. In AFRS specimens, we found significantly increased levels of IgE staining throughout the epithelium and subepithelium in turbinate tissues of patients with AFRS as compared with the other diagnostic groups. These results and patterns were identical to the previously published increased levels of IgE staining seen in sinus tissues of the same patient cohort.¹¹

As previously mentioned, the upregulation of staining was exaggerated in the subepithelium compared with the epithelium. IgE staining is nonspecific as to site of production and cell of origin. The staining demonstrated in our study may be representative of an increase in a variety of cell types, such as plasma cells, mast cells, or eosinophils, which bear IgE. An examination into which specific cells are responsible and whether tissue IgE is actually produced locally or is simply increased due to local migration after production elsewhere remains an area for future investigation. However, the information is valuable in that it provides insight into the location of the epicenter of the IgE-mediated inflammatory process.

In contrast to the Bachert et al⁴ study, we did not find a significant difference in total IgE between sinus mucosa and inferior turbinate specimens. One possible cause for this discrepancy may be related to the different patient populations studied (AFRS vs CRS with nasal polyps). Our original hypothesis was that IgE-bearing cells would be present in greater concentrations in sinus mucosa specimens compared with turbinate specimens given the propensity of polyps to form on sinus mucosa. The absence of this finding

suggests that local IgE expression and production is likely not the sole factor in polyp formation, but rather one of many contributing factors in their evolution. Furthermore, this negative finding brings into question the previously used practice of including turbinate specimens as control tissues from patients with sinonasal inflammatory conditions. Because IgE is upregulated throughout the sinonasal cavity in AFRS, including polyp-forming and non-polyp-forming tissues, other inflammatory mediators may exhibit similar patterns of expression. If true, the use of different subsites for comparison in the same patient may yield inaccurate information.

CONCLUSION

An increased local expression and accumulation of IgE occurs throughout the nasal cavity of patients with AFRS, and this response appears to be exaggerated in the subepithelium compared with the epithelium. Elevations of antigen-specific IgE to both fungal and nonfungal antigens were detected in our ImmunoCAP analysis. Interestingly, our data demonstrate no anatomic differences with respect to IgE expression, which is surprising when one considers the usual site of polyp formation. This suggests that controlling the type I mediated hypersensitivity reaction that occurs is not the only aspect that should be addressed by otolaryngologists.

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Chadwick N. Ahn, study design, data collection, and writing; **Sarah K. Wise**, study design, writing, data collection, statistics; **Deanne M. R. Lathers**, study design, mentor, writing, statistics; **Ryan M. Mulligan**, data collection, writing; **Richard J. Harvey**, graphics; and **Rodney J. Schlosser**, study design, mentor, writing.

DISCLOSURES

Competing interests: **Deanne M. R. Lathers**, employee, Bristol Myers Squibb; **Richard J. Harvey**, advisory board, Schering Plough; **Rodney J. Schlosser**, consultant, BrainLab, Xomed, Gyru; advisory board, Schering Plough; speaker's bureau, GlaxoSmithKline.

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REFERENCES

1. Bent J, Kuhn F. Diagnosis of allergic fungal sinusitis. *Otolaryngol Head Neck Surg* 1994;111:580–88.
2. Kim JW, Hong SL, Kim YK, et al. Histological and immunological features of non-eosinophilic nasal polyps. *Otolaryngol Head Neck Surg* 2007;137:925–30.
3. Weschta M, Rimek D, Formanek M, et al. Local production of *Aspergillus fumigatus* specific immunoglobulin E in nasal polyps. *Laryngoscope* 2003;113:1798–802.
4. Bachert C, Gevaert P, Holtappels G, et al. Total and specific IgE in nasal polyps is related to local eosinophilic inflammation. *J Allergy Clin Immunol* 2001;107:607–14.
5. Sabirov A, Hamilton RG, Jacobs JB, et al. Role of local immunoglobulin E specific for *Alternaria alternata* in the pathogenesis of nasal polyposis. *Laryngoscope* 2007;118:4–9.
6. Perez-Novoa CA, Kowalski ML, Kuna P, et al. Aspirin sensitivity and IgE antibodies to *Staphylococcus aureus* enterotoxins in nasal polyposis: studies on the relationship. *Int Arch Allergy Immunol* 2004;133:255–60.
7. KleinJan A, Vinke JG, Severijnen LW, et al. Local production and detection of (specific) IgE in nasal B-cells and plasma cells of allergic rhinitis patients. *Eur Respir J* 2000;15:491–7.
8. KleinJan A, Godthelp T, van Toornenbergen AW, et al. Allergen binding to specific IgE in the nasal mucosa of allergic patients. *J Allergy Clin Immunol* 1997;99:515–21.
9. Wilson DR, Merrett TG, Varga EM, et al. Increases in allergen-specific IgE in BAL after segmental allergen challenge in atopic asthmatics. *Am J Resp Crit Care Med* 2002;165:22–6.
10. Collins M, Nair S, Smith W, et al. Role of local immunoglobulin E production in the pathophysiology of noninvasive fungal sinusitis. *Laryngoscope* 2004;114:1242–6.
11. Wise SK, Ahn CN, Lathers D, et al. Antigen-specific IgE in sinus mucosa of allergic fungal rhinosinusitis patients. *Am J Rhinol* 2008;22:451–6.
12. Manning SC, Holman M. Further evidence for allergic pathophysiology in allergic fungal sinusitis. *Laryngoscope* 1998;108:1485–96.
13. Marple BF. Allergic fungal rhinosinusitis: current theories and management strategies. *Laryngoscope* 2001;111:1006–19.
14. Mabry RL, Marple BF, Folker RJ, et al. Immunotherapy for allergic fungal sinusitis: three years' experience. *Otolaryngol Head Neck Surg* 1998;119:648–51.
15. Manning SC, Mabry RL, Schaefer SD, et al. Evidence of IgE-mediated hypersensitivity in allergic fungal sinusitis. *Laryngoscope* 1993;103:717–21.
16. Gourley DS, Whisman BA, Jorgensen NL, et al. Allergic bipolaris sinusitis: clinical and immunopathologic characteristics. *J Allergy Clin Immunol* 1990;85:583–91.
17. Bartynski JM, McCaffrey TV, Frigas E. Allergic fungal sinusitis secondary to dermatiaceous fungi-*Curvularia lunata* and *Alternaria*. *Otolaryngol Head Neck Surg* 1990;103:32–9.

18. Folker RJ, Marple BF, Mabry RL, et al. Treatment of allergic fungal sinusitis: a comparison trial of postoperative immunotherapy with specific fungal antigens. *Laryngoscope* 1998;108:1623–7.
19. Suh KS, Park HS, Nahm DH, et al. Role of IgG, IgA, and IgE antibodies in nasal polyp tissue: their relationships with eosinophilic infiltration and degranulation. *J Korean Med Sci* 2002;17: 375–80.
20. Smurthwaite L, Walker SN, Wilson DR, et al. Persistent IgE synthesis in the nasal mucosa of hay fever patients. *Eur J Immunol* 2001;31: 3422–31.
21. Yoshida T, Usui A, Kusumi T, et al. A quantitative analysis of cedar pollen-specific immunoglobulins in nasal lavage supported the local production of specific IgE, not of specific IgG. *Microbiol Immunol* 2005;49:529–34.