Epsilon mRNA 792bp

AID mRNA 335bp

FIG 2. RT-PCR analysis of the e-chain mRNA and AID mRNA expression in the giant papillae tissues. Nested RT-PCR was carried out with cDNA specimens obtained from patients with AKC/VKC (patients 1, 3, 5, 8, and 10 in Table I). The 2% agarose gel electrophoresis showed positive bands (789 bp for e-chain mRNA and 355 bp for AID mRNA).

(PNAd)-positive high endothelial venules existed near the LYVE-1+ lymphatic endothelial cells (Fig 1, D). Various degrees of TLOs were observed in all of the AKC/VKC tissues (summarized in Table 1). e-Chain mRNA and AID mRNA were detected in the cDNA sample obtained from the giant papilla of a patient with VKC (see patient 1 in Table I) by means of nested RT-PCR (Fig 2), and the sequence of the PCR products was verified by means of PCR direct sequencing. No e-chain mRNA or AID mRNA transcripts were detected in 2 control conjunctiva cDNA samples (samples were obtained during cataract surgery, data not shown).

Ectopic lymphoid neogenesis has been observed in patients with many chronic inflammatory disorders, including rheumatoid synovitis, Sjogren syndrome, and a murine model of asthma. We found the essential structures for ectopic lymphoid neogenesis (clusters of B cells, the T-cell zone, and FDCs) in the giant papillae. PNAd+ high endothelial venules around the TLOs, which are known to supply naive T cells, further suggested ongoing immune responses at the giant papillae of patients with chronic allergic conjunctivitis. The TLOs in the giant papillae might also contribute to regional immune responses against persistent antigens at the ocular surface and thus promote chronic inflammation. Another function of lymphoid neogenesis is immunoglobulin class switching in the B-cell clusters. In addition to e-chain mRNA expression in the giant papillae, we also detected AID mRNA, which encodes an essential enzyme that catalyzes the class-switch recombination process and is expressed only in germinal-center B cells. We also showed IgE plasma cells and IgE+ B cells in the substantia propria of giant papillae (Fig E1). Although we could not exclude the possibility that class-switch recombination in B cells might occur in regional secondary lymphoid organs, our results suggested that direct class-switch recombination in the TLOs of the giant papillae is highly probable, as reported in cases involving nasal polyps. It also should be noted that all of the giant papillae analyzed in this study were treated with topical glucocorticoid eye drops. A previous report showed that glucocorticoid could induce IgE isotype switching in human B cells, and therefore further studies are essential to elucidate the effect of glucocorticoids in giant papillae.

In conclusion, we showed the formation of TLOs and some evidence of local IgE production in patients with severe chronic allergic conjunctivitis (AKC/VKC). Regional therapy, including anti-IgE therapy with eye drops, might be effective for the treatment of AKC/VKC.

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Nasal application of rBet v 1 or non–IgE-reactive T-cell epitope–containing rBet v 1 fragments has different effects on systemic allergen-specific antibody responses

To the Editor:

Allergen-specific IgE antibodies are key elements in allergic inflammation and in the immunoregulation of allergic disease. Mucosal allergen contact induces allergic inflammation and also boosts systemic allergen-specific IgE production, which leads to increased sensitivity of mast cells and basophils, to the upregulation of Fce receptor expression on allergic effector cells and antigen-presenting cells, and thus to increased T-cell activation. Increases in allergen-specific IgE levels are also associated with increased clinical sensitivity and aggravation of symptoms.

Unwanted boosts of allergen-specific IgE production occur during seasonal allergen contact through the respiratory mucosa, which can be reproduced in clinical studies by controlled nasal allergen provocation. However, strong boosts of IgE production are also observed in the course of allergen-specific immunotherapy, reaching up to 5-fold of pretreatment levels, for example, after sublingual immunotherapy when allergens are applied through the mucosa. In immunotherapy studies performed with...
recombinant allergen derivatives, which have been engineered to eliminate IgE reactivity and to preserve specific T-cell epitopes, only mild boosts of allergen-specific IgE production were observed that did not cause clinically relevant sensitization.7,8

Here we conducted a nasal provocation test study with rBet v 1, the major allergen of birch pollen, and 2 non–IgE-reactive rBet v 1 fragments. These fragments have previously been shown to induce proliferation in 9 different T-cell clones specific for 8 different T-cell epitopes spread over the whole Bet v 1 molecule, indicating that the combination of the 2 fragments harbors all T-cell epitopes of the complete Bet v 1 molecule.9 The aim of the present study was to investigate the effects of nasal application of these molecules on systemic allergen-specific IgE production, on allergen-specific IgG, IgA, and IgM antibody levels and on allergic symptoms. We hypothesized that in allergic patients nasal contact with T-cell epitopes from a relevant allergen without concurrent contact with B-cell epitopes from the same allergen would not be sufficient to induce a secondary IgE response.

Nineteen patients with birch pollen allergy were enrolled in the study to receive either nasal Bet v 1 (patient B1-B10) or Bet v 1 fragments (patients F1-F9, see Table E1 in this article’s Online Repository at www.jacionline.org). All patients had symptoms of allergic rhinitis during the birch pollen season, and birch pollen allergy was confirmed by means of standard skin prick testing with birch pollen extract (Allergopharma, Reinbek, Germany) and Bet v 1–specific serum IgE levels of greater than 0.7 kU/L at the screening visit. Patients with unstable lung function, persistent allergy (as defined by the Allergic Rhinitis and Its Impact on Asthma working group, www.whiar.org), or airway infection or those who had used antihistamines 2 weeks before or corticosteroids 3 months before the study were excluded. Subjects were randomly allocated to the 2 study groups, and demographic data, allergic history, and sensitization profiles in the 2 groups were comparable at the beginning of the study (see Table E1). Single-blind nasal provocation was performed on 2 consecutive days (days 1 and 2 of the study). Nasal provocation was followed by anterior rhinomanometry to examine inflammation-induced nasal blockage, and provocation-induced symptoms were evaluated by using a symptom score. Blood samples were obtained on the first day of nasal provocation and 1, 2, 4, 6, and 8 weeks thereafter and were stored at −20°C until use (study protocol, see Fig E1 in this article’s Online Repository at www.jacionline.org). The study was performed in winter outside the pollen season with the approval of the local ethics committee, and written informed consent was obtained from each patient.

Recombinant Bet v 1 was from BIOMAY (Vienna, Austria), and the 2 recombinant fragments (F1 and F2) were expressed in Escherichia coli and purified as previously described.9 For nasal provocation, rBet v 1, F1, and F2 were reconstituted and diluted in sterile 0.9% sodium chloride solution at 1.6 mg/mL. F1 and F2 were then mixed 1:1 to obtain nearly equimolar solutions. Recombinant antigens were administered as previously described,10 yielding a cumulative dose of 1.6 µg of recombinant antigen per study day. Nasal flow was measured at 150 Pa by means of anterior rhinomanometry after measurements had been performed without administration of test substances to determine baseline levels, 10 minutes after application of 0.9% sodium chloride solution to rule out any nonspecific hyperreactivity, and 20 minutes after provocation with the test solutions to determine nasal flow reduction.10 In addition, patients were asked to score their symptoms of nasal itching, nasal congestion, and nasal discharge by using a 4-point scale (0, no symptoms; 1, mild symptoms; 2, moderate symptoms; and 3, severe symptoms) and to count the number of sneezes (0, no sneeze; 1, 1-5 sneezes; 2, 6-10 sneezes; and 3, ≥11 sneezes). The scores were added to calculate a total nasal symptom score with a maximum of 12 points.

In the 10 patients who underwent nasal provocation with 1.6 µg of the rBet v 1 allergen (patients B1-B10), we observed a significant decrease of nasal flow 20 minutes after nasal provocation on both study days (day 1: −49.3% ± 22.0%, P < .001, Fig 1, A; day 2: −48.7% ± 22.3%, P < .001, Wilcoxon signed-rank test). This reduction in nasal flow was observed in all patients and on both provocation days. By contrast, nasal provocation with 1.6 µg of the 2 fragments did not significantly affect nasal flow (day 1: −7.3% ± 16.5%, P = not significant, Fig 1, B; day 2: +14.4% ± 78.5%, P = not significant, Wilcoxon signed-rank test).

Nasal provocation with Bet v 1 led to a significant increase of symptoms (symptom score on day 1: 7.1, Fig 1, B; day 2: 7.0), whereas nasal exposure to the 2 fragments did not cause any relevant symptoms (average symptom scores on day 1: 0.6, Fig 1, B; day 2: 0.7). None of the patients in either study group had late-phase reactions (≥4 hours after nasal provocation). The difference between the effect of Bet v 1 and the 2 fragments was statistically
Our results thus demonstrate that the presence of IgE epitopes is required for the induction of allergen-specific IgE production through the nasal mucosa. Non–IgE-reactive allergen derivatives containing only the allergen-specific T-cell epitopes neither boosted allergen-specific IgE production nor induced de novo IgE responses. For the boost of IgE by allergens, at least 2 explanations are conceivable. First, allergens can directly activate allergen-specific B cells through surface immunoglobulin receptors to produce more IgE. Second, it is possible that the boosts of IgE production through mucosal allergen contact requires IgE-facilitated antigen presentation, whereas antigen presentation without IgE is not sufficient for the boosting of IgE responses.

Besides these mechanistic considerations, the results of this study might also have implications for the therapy and prevention of allergy. Non–IgE-reactive recombinant allergen derivatives might thus be useful for preventative and therapeutic tolerance strategies based on mucosal routes without inducing IgE-mediated side effects, unwanted IgE sensitizations, or boosts of IgE production.

We thank the Allergiezentrum Wien West for patient care and Professor P. Bauer, Department of Medical Statistics, Medical University of Vienna, for his help with the statistical analysis of the data.

**REFERENCES**

Exposure of rye (Secale cereale) cultivars to elevated ozone levels increases the allergen content in pollen

To the Editor:

The increase of air pollution because of climate change and global warming represents a major challenge for humankind in this century. Changes in climate and air pollution have also been suggested to be potential factors behind the increasing prevalence of allergic diseases.1 In this context, it has been shown that particular matter (eg, diesel exhaust particles) can act as carriers and adjuvant for allergens, thereby enhancing the allergic immune response and promoting airway inflammation.2 Furthermore, it has been demonstrated that rising concentrations of CO2 increase the production of ragweed pollen and their major allergen contents.3 Environmental effects caused by the air pollutant ozone (O3) may occur at 2 levels. On the one hand, depletion of ozone in the stratosphere results in increased UV exposure. On the other hand, new low-level ozone is formed by the photochemical dissociation of nitrogen dioxide in the troposphere and has been reported to have adverse effects on human health. The latter ozone, which is a major component of photochemical smog, is particularly produced during the summer months in urban settings. Evidence has been provided that it may increase the content of a particular allergen (ie, group 5 allergen) in rye grass pollen.4 However, a more recent study has suggested that grass pollen exposure to gaseous air pollutants (ie, O3, nitrogen dioxide, sulfur dioxide) decreases IgE recognition of major grass pollen allergens.5

We designed a controlled experimental system to investigate the effects of increased tropospheric O3 levels on the contents of major allergens in pollen from a highly allergenic plant (ie, rye, Secale cereale). Two different rye cultivars, S cereale cv “Motto” and S cereale cv “Rapid,” were grown under controlled conditions in closed-top cabinets of a greenhouse. Two pots of each cultivar were grown in ambient air, and the other 2 were exposed to elevated O3 concentrations (80 ppb) during the day. This concentration corresponds to peak O3 levels measured during the month of May in Vienna, Austria (mean, 2006-2009). O3 was produced from pure oxygen with an O3 generator (Model 502; Fischer, Meckenheim/Bonn, Germany) and was distributed in the greenhouse by 3 fans to which perforated plastic tubes were connected. O3 exposure was carried out daily between 9 AM and 5 PM for up to 107 days during plant growth. The O3 load of the air in the greenhouse was continuously monitored and was kept in a range of 79 ± 13 ppb (mean ± SD) in the O3 cabinet and 22 ± 12 ppb in the ambient air cabinet (control).

Rye pollen was regarded as mature and thus ready to harvest immediately after the color of the anthers had turned from light yellow to dark yellow. Harvest of mature pollen was repeated at 2-day intervals, and pollen collected from 1 pot during this time was pooled at the end of the harvesting period. Pollen extracts were prepared by suspending exactly the same amounts of pollen (10 mg) in 1 mL sample buffer (62 mmol/L TRIS HCl [pH 6.8], 2.3% SDS, 10% glycerol, 0.1% bromophenol blue, 5% 2-mercaptopethanol) and by performing identical ultraturax treatment. Samples were then heated at 95°C for 20 minutes and centrifuged at 10,000 g for 30 minutes. A total of 75 µL of the supernatant, corresponding to 75 µg extracted pollen, was loaded per lane of an SDS-protein gel and subjected to electrophoresis. Fig 1 shows that the protein content of the different preparations as visualized by silver staining was consistently higher for both rye cultivars when plants were grown in an environment with increased O3 levels. To study a potential association between protein and

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**FIG 1.** Total protein content of rye cultivated in ambient air or ozone enriched environment. Two different S cereale L cultivars, “Motto” (S cereale M1 and M2) and “Rapid” (S cereale R1 and R2), were exposed to either ambient air (-) or 80ppb ozone (+). On harvest, proteins from pollen extracts were prepared and separated by SDS-PAGE, and total protein content was visualized by silver staining (A) and quantified by densitometry (B). Extracts from equal amounts of pollen corresponding to 75 µg were loaded per lane. Molecular weights (kd) are displayed on the left.
ELISA MEASUREMENTS

ELISA plates (Nunc Maxisorb, Roskilde, Denmark) were coated with 5 μg/mL rBet v 1 (Biomay, Vienna, Austria) diluted in PBS overnight at 4°C. Plates were blocked with PBS-T-BSA (PBS containing 0.05% vol/vol Tween 20 [PBS-T] and 0.5% wt/vol BSA) for 2 hours at room temperature and 2 hours at 4°C. Sera were diluted 1:5 for the detection of IgE antibodies and 1:50 for the detection of IgG1, IgG2, IgG3, IgG4, IgA, and IgM. Murine mAbs (IgE, clone G7-26; IgG1, clone JDC-1; IgG2, clone G18-21; IgG4, clone JDC-14; IgA, clone G18-1; IgM, clone JDC-15 [all BD PharMingen, San Diego, Calif]; and IgG3, clone HP-6050 [Sigma, St Louis, Mo]) were used for the detection of immunoglobulins. A peroxidase-linked sheep anti-mouse IgG antibody (Amersham Biosciences, Little Chalfont, Buckinghamshire, United Kingdom) diluted 1:1,000 was used as a secondary antibody. The coating step and each incubation step were followed by 5 washing steps with PBS-T. Each ELISA plate included a buffer control, a negative control (serum from a nonallergic subject), and reference sera from patients with birch pollen allergy (positive control subjects) whose Bet v 1–specific IgE levels had been measured with the CAP system. To allow a comparison of OD values from different ELISA plates and to avoid plate-to-plate variability in OD measurements, results for each immunoglobulin isotype were normalized by using the positive control sera as a reference, as described previously. The cutoff level for positive IgE measurement was set at 2-fold the OD value measured for a negative control serum.

REFERENCE

FIG E1. A, Study design (timeline). B, Graphic depiction of Bet v 1 and the 2 rBet v 1 fragments. aa, Amino acid.
FIG E2. Time course of Bet v 1–specific IgE, IgG1, IgG2, IgG4, IgA, and IgM antibody responses in patients after nasal administration of rBet v 1 or fragments. Percentage changes in Bet v 1–specific IgE, IgG1, IgG2, IgG4, IgA, and IgM antibody levels measured by means of ELISA (y-axes) in patients who had received nasal provocation (NP) with Bet v 1 (black diamonds) or fragments (gray circles) are displayed at the time of nasal provocation and at 1, 2, 4, 6, and 8 weeks thereafter (x-axes). SEMs are shown as error bars.
## Table E1. Demographic and clinical data

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (y)</th>
<th>Sex</th>
<th>Allergen*</th>
<th>Symptoms †</th>
<th>Bet v 1 (KUA/L)</th>
<th>Provoking agent</th>
<th>Duration of symptoms after provocation</th>
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<td>r, c</td>
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<td>—</td>
</tr>
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<td>r, c</td>
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<td>—</td>
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<td>b, g, h, c, d</td>
<td>r, c</td>
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<td>r, c</td>
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<td>NK</td>
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<td>16.6</td>
<td>Bet v 1</td>
<td>1-2 h</td>
</tr>
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</table>

F, Female; M, male; NK, not known.

*a, Alternaria species; b, birch; c, cat; cl, Cladosporium species; d, dog; g, grasses; h, house dust mites.

†a, Asthma; c, conjunctivitis; r, rhinitis.

‡F1, Fragment 1; F2, fragment 2.