

## Induction of IgE antibodies in mice with recombinant grass pollen antigens

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### SUMMARY

In this study, recombinant *Poa pratensis* (*Poa p*) IX allergens were examined for their *in vivo* allergenicity and antigenicity. Immunization of mice with a fusion protein (FP) comprising  $\beta$ -galactosidase and recombinant KBG8.3 (rKBG8.3) allergen induced high titres of both IgG and IgE antibodies. By contrast, immunization with rKBG60.2, which represents the N-terminal fragment of rKBG8.3, induced only IgG antibodies. The IgE antibody titre specific to Kentucky Bluegrass (KBG) was significantly higher than that to  $\beta$ -galactosidase. Moreover, KBG-specific IgE antibodies showed no apparent decrease in their titres until 60 days after immunization, whereas the  $\beta$ -galactosidase-specific IgE antibodies disappeared after 40 days. The antibodies induced with rKBG8.3 in mice were capable of inhibiting the binding of human IgE antibodies to KBG pollen allergens, which indicated that rKBG8.3-specific murine antibodies recognized epitopes similar to those recognized by human IgE antibodies. Analysis of allergenic cross-reactivities of rKBG8.3 with components from five other species of grass pollens revealed that IgE antibodies induced by this allergen are capable of binding *in vivo* to components from other grass pollens. These results suggest that the mouse may serve as a model for the manipulation of IgE responses to recombinant allergens or their chemically modified derivatives.

### INTRODUCTION

Within the past 4 years, an impressive array of allergens have been cloned and sequenced from mites,<sup>1</sup> grasses,<sup>2,3</sup> weed<sup>4</sup> and tree<sup>5</sup> pollens, and vespid venom.<sup>6</sup> These studies, in turn, have advanced our knowledge of (1) the primary structure of the respective allergen molecules,<sup>1-6</sup> (2) the possible biological functions of some of these allergens,<sup>1,5</sup> and (3) the antigenic cross-reactivities among groups of aeroallergenic proteins.<sup>3,7,8</sup> However, progress on the immunological characterization of recombinant allergens or their fragments has been slow. Most studies to date have comprised determination of their allergenicity by assaying their ability to bind human IgE antibodies either by ELISA or by Western blotting procedures. The studies of T-cell responses to recombinant allergens have primarily comprised the *in vitro* proliferation of murine popliteal lymph node cells<sup>9</sup> or of human peripheral blood mononuclear cells or T-cell lines.<sup>10</sup> To our knowledge, the analysis of *in vivo* immune responses to recombinant allergens has been limited. On the

basis of *in vitro* studies, it is conceivable that recombinant allergens or fragments could be exploited for the preparation of appropriate diagnostic and therapeutic derivatives. It is unlikely, however, that within the framework of the current regulations of the Federal Drug Administration, these derivatives would be permitted to be tested directly in humans. The development of animal models for the above studies is, therefore, critically important.

We have focused our studies on a new group of isoallergens of Kentucky Bluegrass (KBG) pollen, designated as *Poa pratensis* (*Poa p*) IX.<sup>11-13</sup> Synthesis of these allergens by *Escherichia coli* as recombinant proteins has permitted purification of sufficient amounts of individual protein or its peptides for use in *in vitro* studies aimed at definition of B- and T-cell epitopes.<sup>9,11-13</sup> In this report, we present evidence on the IgE and IgG antibody responses to one of these recombinant allergens encoded by cDNA KBG8.3 (rKBG8.3), which represents the common C-terminal region of the *Poa p* IX allergens, and encompasses previously described rKBG7.2.<sup>3,9,13</sup>

### MATERIALS AND METHODS

#### *Isolation of recombinant allergenic peptides*

A cDNA library was constructed in  $\lambda$ gt11 vector using the messenger RNA (mRNA) of KBG pollen and the library was screened with sera from KBG pollen-allergic individuals.<sup>3,11</sup> One

Abbreviations: FP, fusion protein; KBG, Kentucky Bluegrass allergenic proteins; PCA, passive cutaneous anaphylaxis; *Poa p*, *Poa pratensis*; rKBG8.3, recombinant allergenic peptide encoded by cDNA KBG8.3.

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of the IgE binding clones *viz.* KBG8.3 was identified<sup>12,13</sup> and its insert was isolated by treatment of the recombinant  $\lambda$ gt11 phage DNA with restriction enzyme *EcoRI*. The purified insert was ligated in pWR590.<sup>14</sup> vector and expressed in *E. coli* strain JM105. Another cDNA, KBG60.2 was generated by deletion of a 3' fragment using *SalI* enzyme and expressed in *E. coli*. The recombinant peptides encoded by the above clones were isolated and purified as described previously<sup>9</sup> and stored at  $-20^{\circ}$ .

#### SDS-PAGE and Western blotting

The bacteria, which were transformed with the plasmid vector itself or recombinant plasmid containing the cDNA insert of KBG8.3 or of KBG60.2 and expressed the recombinant peptides, were lysed by boiling for 5 min in SDS-PAGE loading buffer.<sup>9</sup> SDS-PAGE was performed on a vertical slab gel apparatus using 10% polyacrylamide gel according to the method of Laemmli.<sup>15</sup> The separated proteins were either stained with Coomassie Blue or electrotransferred<sup>16</sup> onto nitrocellulose membrane. The membrane was then probed with human sera (1:10) followed by incubation with alkaline phosphatase-conjugated goat anti-human IgE antibodies (Tago, Burlingame, CA) as described previously.<sup>8</sup>

#### Grass pollen extracts

The pollen protein extracts were prepared according to the method as described previously.<sup>17</sup> Briefly, the dried pollens (Hollister-Stier Laboratory, Mississauga, ON) were first defatted with ether, and pollen proteins were extracted by stirring the pollen in distilled water overnight. The water soluble components were further dialysed through spectra/pore membrane tubing (m.w. cutoff = 6000–8000; Spectrum Medical Industries, Inc., Los Angeles, CA). The protein concentration was determined with Lowry's method.<sup>18</sup>

#### Immunization of mice with recombinant allergens

To produce murine antisera to rKBG8.3, female B6D2F1 mice (Jackson Laboratories, Bar Harbor, MI), 6–8 weeks old, were immunized with rKBG8.3 protein and dextran sulphate (Sigma, St Louis, MO) as adjuvant.<sup>8,19</sup> Each mouse was primed with 5  $\mu$ g of the recombinant protein and 2.5 mg dextran sulphate. After 23 days the mice were boosted with the same amount of the protein and 1 mg dextran sulphate. Seven days later the mice were bled and the sera were stored at  $-20^{\circ}$ . Murine antibodies to KBG protein extract and  $\beta$ -galactosidase (BRL, Mississauga, ON) were also produced using the same method, except that 20  $\mu$ g KBG protein was used for the immunization.

#### ELISA

KBG- and  $\beta$ -galactosidase-specific IgG antibodies were determined by ELISA. Microtitre plates (Nunc, Roskilde, Denmark) were coated with 1  $\mu$ g KBG pollen extract or 0.1  $\mu$ g  $\beta$ -galactosidase for each well, respectively, in 100  $\mu$ l bicarbonate buffer (0.05 M, pH 9.6) overnight at room temperature. After washing three times with phosphate-buffered saline (PBS)–Tween (PBST) buffer and saturation of the plates with 2% gelatin in PBS buffer, 100  $\mu$ l of serially diluted sera was added to the wells. After 2 hr incubation at room temperature, followed by three washes of 10 min each in PBST, the plates were incubated with alkaline phosphatase-conjugated goat anti-mouse IgG (Zymed, South San Francisco, CA) for 1 hr at room temperature, washed three times and developed.<sup>7</sup> The results

were expressed as ELISA titres; the ELISA was considered positive at optical density (OD) values which were threefold higher than that of the normal mouse sera used as negative control.

Inhibition of human IgE antibody binding to KBG pollen allergens and the full-length recombinant allergen, KBG60, was also performed by ELISA. A human serum pool from 11 individuals allergic to Kentucky Bluegrass pollen<sup>9</sup> was used as the source of specific IgE antibodies. Serially diluted antiserum to rKBG8.3 were added into microtitre plate wells precoated with either 1  $\mu$ g KBG pollen extract or 0.1  $\mu$ g rKBG60 and then blocked with gelatin under conditions described above. After 2 hr incubation at room temperature, the murine serum was discarded and 100  $\mu$ l of human sera (diluted at 1:10) was added. After overnight incubation at room temperature, the antigen-specific human IgE antibodies were detected using goat anti-human  $\epsilon$ -chain conjugated with alkaline phosphatase (Tago).<sup>7</sup> Direct absorption of the human sera with rKBG8.3 was used as control.<sup>8</sup>

#### Passive cutaneous anaphylaxis (PCA)

Antigen-specific IgE antibodies were determined as PCA titres. Hooded rats were sensitized with 50  $\mu$ l of serially diluted mouse sera injected intradermally on the back. Forty-eight hours later, the rats were challenged by the intravenous injection of different antigens, 1 mg for each different grass pollen extract and 0.5 mg for  $\beta$ -galactosidase (BRL), in 1 ml PBS containing 0.5% Evan's blue. The PCA titre was calculated as the highest dilution giving a clearly visible skin reaction (> 5 mm in diameter).

Cross-absorption of antiserum to rKBG8.3 and KBG extract with their respective antigens was performed on solid phase using a method described previously.<sup>7</sup> Briefly, the proteins, 10  $\mu$ g of rKBG8.3 or 100  $\mu$ g of KBG extract, were dotted on to the nitrocellulose and the membrane was air dried for 10 min, followed by incubation in PBS buffer containing 2% bovine serum albumin (BSA) for 1 hr at room temperature. The membrane was then transferred into wells containing the appropriate antiserum. After 2 hr incubation at room temperature, the antiserum was collected and serially diluted for PCA assay.  $\beta$ -galactosidase and bacterial proteins were used as controls.

## RESULTS

### Human IgE recognition of fusion protein rKBG8.3

SDS-PAGE analysis of the bacterial lysate from the transformants containing either the non-recombinant vector or the recombinant plasmid of cDNA insert KBG8.3 clearly showed the enhanced expression of the truncated  $\beta$ -galactosidase or the recombinant protein, respectively (Fig. 1a). The recombinant protein was further analysed in terms of its ability to bind to human IgE antibodies by Western blotting (Fig. 1b) using human sera from KBG-allergic patients. The recombinant protein, but not the truncated  $\beta$ -galactosidase or any other bacterial proteins, were recognized by the human IgE antibodies. The N-terminal peptide of rKBG8.3 (amino acid residues 102–303), designated as rKBG60.2 (amino acid residues 102–188) was similarly synthesized, purified and examined for its binding to IgE antibodies. However, this recombinant peptide exhibited very weak binding to the IgE antibodies (data

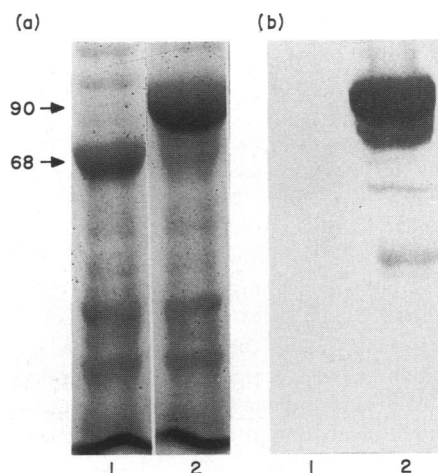


**Figure 1.** SDS-PAGE and Western blot analysis of recombinant protein. Lysate of bacteria expressing either the plasmid vector and recombinant vector of KBG8.3 were analysed. (a) Lysate proteins were separated by electrophoresis on 10% polyacrylamide gel under reduced conditions, and were visualized by staining with Coomassie Blue: (1) proteins of bacteria containing self-ligated plasmid; (2) bacterial lysate containing fusion protein. The arrow indicates the position of the truncated  $\beta$ -galactosidase or the fusion protein. (b) Immunoblot of the gel developed using human IgE antibodies as the primary antibody probe.

**Table 1.** Antibodies induced with rKBG8.3 and rKBG60.2 allergens

| Antisera | KBG (titres)      |                    | $\beta$ -galactosidase (titres) |     |
|----------|-------------------|--------------------|---------------------------------|-----|
|          | IgG               | IgE                | IgG                             | IgE |
| rKBG8.3  | $512 \times 10^2$ | $12.8 \times 10^2$ | $512 \times 10^2$               | 20  |
| rKBG60.2 | $4 \times 10^2$   | Nil                | $128 \times 10^2$               | Nil |

not shown). These two recombinant peptides were used for further studies.

#### Immune response to fusion proteins

Immunization of mice with the fusion protein (FP) containing either rKBG8.3 or rKBG60.2 led to induction of antibodies to both the corresponding KBG pollen allergens (KBG) and to  $\beta$ -galactosidase. The FP containing rKBG8.3 induced high titres of IgG antibodies to both the allergenic peptides and  $\beta$ -galactosidase, and high titres of IgE antibodies to KBG but not to  $\beta$ -galactosidase. The FP comprising the smaller KBG peptide, rKBG60.2, induced high titre of IgG antibodies to  $\beta$ -galactosidase only; the KBG-specific IgG titre was low. Moreover, IgE antibodies to either KBG or  $\beta$ -galactosidase were not detectable (Table 1). No decrease in the titre of IgG antibodies specific to both KBG and  $\beta$ -galactosidase was seen up to 3 months after immunization, contrary to the IgE titres described below.

The possibility of cross-reactivity between KBG and  $\beta$ -galactosidase in PCA assay of IgE detection was ruled out by

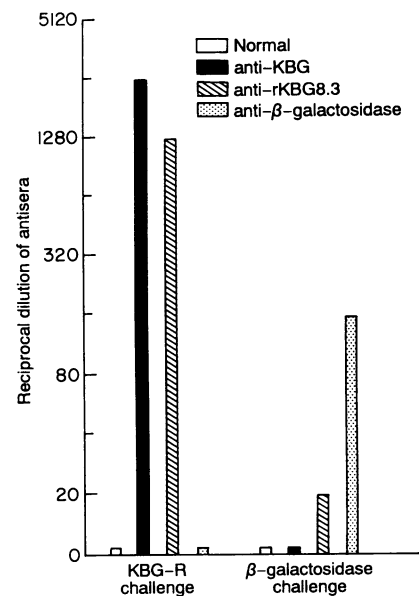


**Figure 2.** Determination of specific IgE antibody titres by PCA assay. Two groups of rats were sensitized with the preimmune serum, antiserum to KBG, FP and  $\beta$ -galactosidase as marked and later challenged with either KBG proteins or  $\beta$ -galactosidase.

**Table 2.** PCA titres of sera preabsorbed with different antigens\*

| Antigens used for absorption | PCA titres |              |
|------------------------------|------------|--------------|
|                              | anti-KBG-R | anti-rKBG8.3 |
| KBG-R                        | 40         | 0            |
| rKBG8.3                      | 640        | 0            |
| $\beta$ -galactosidase       | 2560       | 1280         |
| Non-treated control          | 2560       | 1280         |

\* KBG-R was used as challenge antigen.

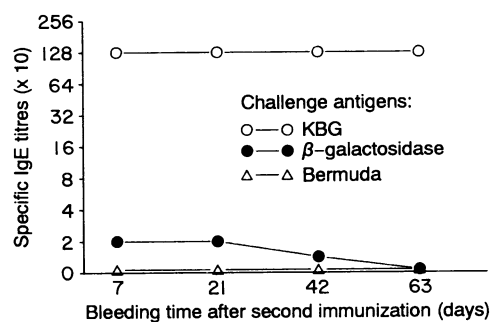


**Figure 3.** Persistent IgE production to KBG proteins and  $\beta$ -galactosidase induced with rKBG8.3. IgE titres were determined using PCA assay at Days 7, 21, 42 and 63 after the second immunization.

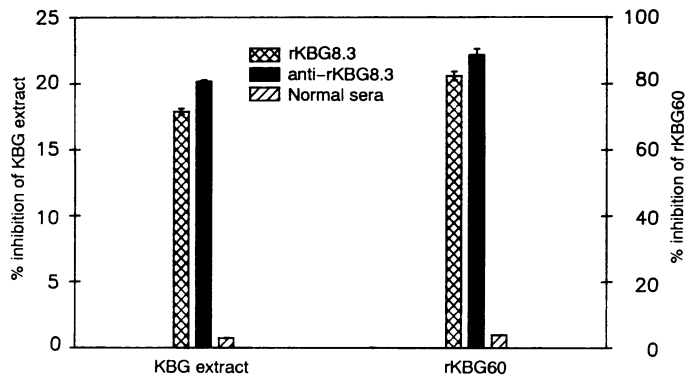


**Figure 4.** Inhibition of human IgE antibody binding to KBG or *Poa p* IX (rKBG60) allergens with murine antiserum to rKBG8.3 as determined by ELISA.

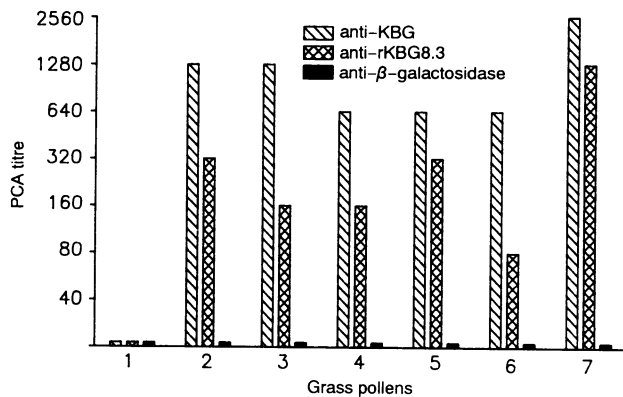


**Figure 5.** Determination of *in vivo* cross-reactivity of rKBG8.3 with allergens in other species of grass pollens by PCA assay. Pollen extracts of KBG and Bermuda grass were employed as positive and negative controls, respectively. Bermuda (1); Canary (2); Orchard (3); Reed Canary (4); tallgras (5); Timothy (6); KBG (7).

employing antibodies to KBG and  $\beta$ -galactosidase (Fig. 2). Rats injected with the antiserum to FP responded to challenge of KBG protein extract and  $\beta$ -galactosidase, although the titres for  $\beta$ -galactosidase were very low (little above the background). Antiserum to KBG pollen extract or to  $\beta$ -galactosidase responded to the corresponding antigens, respectively, and exhibited titres similar to that of the antiserum to FP. These results indicate that there is no cross-reactivity between KBG allergens and  $\beta$ -galactosidase, and suggest that the component antigens of FP differ in their ability to elicit IgE response.

The specificity of the IgE antibodies to KBG allergens was confirmed by absorption of the above sera with different antigens followed by PCA assay (Table 2). KBG pollen proteins, but not  $\beta$ -galactosidase or bacterial proteins, were able to remove IgE antibodies specific to KBG allergens from antiserum to KBG or rKBG8.3 proteins. Similarly, rKBG8.3 protein was capable of absorbing IgE antibodies from antiserum to rKBG8.3 or KBG proteins. The degree of absorption varied depending upon the serum. These results confirmed that rKBG8.3 was capable of inducing KBG-specific IgE antibodies in mice.

### Persistence of IgE antibodies induced with rKBG8.3

Persistence of IgE antibodies induced with the FP was examined using PCA assay. Sera from the same group of three mice were taken at 3-week intervals and tested for the presence of IgE antibody titres (Fig. 3). The KBG-specific IgE antibodies persisted at high level (1:1280), and up to 63 days there was no apparent change in the titres. IgE antibodies to  $\beta$ -galactosidase, on the other hand, became undetectable at Day 42. A grass pollen extract of Bermuda which possesses no cross-reactive epitopes with rKBG8.3<sup>8</sup> was used as negative control.

### Inhibition of human IgE antibody to KBG allergens with murine anti-rKBG8.3 sera

The relationship between murine anti-rKBG8.3 and human IgE antibodies specific to KBG and *Poa p* IX allergens in terms of their recognition of the epitopes was analysed by employing an inhibition ELISA assay. Direct absorption with rKBG8.3 protein to remove the specific subset of IgE antibodies from the human sera in reaction with KBG allergens and the full-length recombinant allergen, rKBG60, demonstrated that more than 18 and 80% IgE antibodies, respectively, were absorbed. Murine anti-rKBG8.3 sera inhibited human IgE antibody binding to the KBG and rKBG60 allergens in a similar manner but to a larger extent than that of direct absorption with rKBG8.3 peptide (Fig. 4). These results suggest that murine antibodies and human IgE antibodies recognize the same or overlapping epitopes.

### *In vivo* cross-reactivity of rKBG8.3 to allergens from other species of grass pollens

Cross-reactivity with allergens from seven different species of grass pollens with rKBG8.3 was determined using PCA assay. After hooded rats were sensitized with antiserum to rKBG8.3 or KBG, they were challenged with different grass pollen protein extracts and PCA titres were determined (Fig. 5). Except for the pollen extract of Bermuda grass, the other five grass pollen extracts possessed some components recognized by IgE antibodies against the epitopes on rKBG8.3 and other allergens in KBG pollen. The antiserum to KBG used as a positive control always gave higher PCA titres than that of antiserum to rKBG8.3. Antiserum to  $\beta$ -galactosidase was used as a negative control. Furthermore, different grass pollen extracts exhibited varying PCA titres. These results demonstrate that IgE antibodies induced by one allergen, rKBG8.3, were able to recognize allergens from five other species of grass pollens *in vivo*.

## DISCUSSION

The main objective of this study was to investigate the ability of recombinant allergen, rKBG8.3, to stimulate KBG-specific IgE antibody production in the mouse. These *in vivo* studies have provided evidence on (1) the immunogenicity of recombinant allergens, (2) the magnitude and nature of the IgE immune response and (3) the cross-reactivity among grass pollen allergens in terms of *in vivo* IgE immune responses. The KBG pollen allergens used in this investigation represented the native Group IX allergens, since it was shown that anti-rKBG8.3

recognized three to five *Poa p* IX isoallergens.<sup>8</sup> However, in view of the possible cross-reactivity between Group IX and Group V allergens,<sup>7</sup> it is likely that antiserum to rKBG8.3 also reacts with Group V specific proteins.

The FP used for immunization comprised a 68,000 MW truncated  $\beta$ -galactosidase fused to rKBG8.3 (20,600 MW) or rKBG60.2 (8900 MW) allergenic peptide of *Poa p* IX allergens. Despite the difference in their molecular mass, rKBG8.3 and the truncated  $\beta$ -galactosidase induced IgG antibodies with similar titres. Moreover, in terms of the IgE response KBG-specific antibody titres were much higher than the  $\beta$ -galactosidase-specific titres. By contrast, rKBG60.2 representing an N-terminal fragment of rKBG8.3, was unable to induce IgE antibodies, and it induced a low titre of IgG antibodies reactive with KBG allergens indicating a weak epitope on this molecule. These results suggested that T- and B-cell epitopes exist on both of these recombinant *Poa p* IX peptides, which is consistent with our previous observation.<sup>9</sup> It is noteworthy that immunization of mice twice with rKBG60.2 FP, followed by two injections with rKBG8.3, led to a decrease in the titres from 1280 to 320 of IgE antibodies to KBG allergens (L. Zhang and S. S. Mohapatra, unpublished results). The non-allergenic fragments of allergens, therefore, have the potential of being exploited for allergen-specific immunotherapy.

Furthermore,  $\beta$ -galactosidase and rKBG8.3 differ in their structure insofar that they induced differential IgE production. In the mouse model, production of IgE antibodies to KBG allergens after secondary immunization was considerably higher, persistent and was not affected by repeated immunization. This is consistent with the clinical situation in atopic patients, whereby the production of IgE antibodies continues for a long time after their secondary exposure to the allergens. Recent studies indicated that the B cells from the patients are capable of spontaneously producing IgE antibodies independent of interleukin-4 (IL-4).<sup>20</sup> This similarity between the human and murine systems suggests that the mouse may serve as a model for the manipulation of ongoing IgE production. By contrast to the allergenic peptide, IgE antibodies specific to  $\beta$ -galactosidase were consistently low, and became undetectable after a short period. Therefore, structure of a particular antigen may be important in the induction and persistence of IgE immune response.

Whether the epitopes of a certain antigen recognized by different species are the same is still questionable. The murine antisera to recombinant allergens proved to be a better reagent for study of B-cell epitopes than human sera which contained low titres of specific antibodies. Moreover, a previous study indicated that epitopes of an *Amba*III allergen were recognized by human, mouse and rabbit antibodies.<sup>21</sup> Our results of inhibition assay also suggested that mouse antibodies bound to the same or similar sites as the human IgE antibodies, although one could not rule out the steric hindrance effect.

There exist extensive cross-reactive components among different species of grass pollens. Previous studies have shown that other species of grass pollen also possessed components that are antigenically and allergenically cross-reactive with *Poa p* IX.<sup>8</sup> This study extended the *in vitro* observations of allergenic cross-reactivity to the cross-bridging of IgE antibodies *in vivo* employing the murine model. The fact that IgE antibodies induced by one allergen can be recognized by other allergenic components has significant implications in terms of future

diagnosis and therapy of allergies using these recombinant allergens.

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