

Multiple B- and T-cell epitopes on a major allergen of Kentucky Bluegrass pollen

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SUMMARY

The B- and T-cell epitopes of a recombinant grass allergen, rKBG60, were delineated using a set of overlapping synthetic peptides. Direct binding by enzyme-linked immunosorbent assay (ELISA) utilizing serum pools led to the identification of 13 murine immunoglobulin-, and nine to 13 human IgG- and five to seven human IgE-reactive overlapping peptides. Of the peptides which bound to human IgE antibodies, all but three peptides bound to human and/or murine IgG antibodies. Furthermore, eight out of 12 synthetic peptides induced antigen-specific antibodies in mice, suggesting that these peptides contained epitopes that recognized and/or induced T cells. These results, in conjunction with cross-recognition of different peptides at the C-terminus of rKBG60 by antibodies to neighbouring or non-overlapping peptides suggest that the C-terminus of this antigen represents a dominant antigenic and allergenic site. Peripheral blood mononuclear cell (PBMC) proliferation studies using these synthetic peptides for 13 grass allergic individuals indicated that seven potential human T-cell epitopes exist on this allergen. Taken together, the results demonstrate that multiple B- and T-cell epitopes exist on this major group of grass allergens, the majority of which are localized at the C-terminus of this antigen.

INTRODUCTION

The development of therapeutic strategies for allergic diseases that would specifically regulate IgE antibody production to defined allergens requires knowledge of the primary structure of individual allergens, in particular the knowledge of their epitopes, that are recognized by antibodies and T cells.¹ Thus, peptides comprising the B-cell epitopes may be exploited for devising epitope-specific diagnosis² and for inducing allergen-specific B-cell tolerance.³ Similarly, T-cell epitopes are considered to be relevant because the structural analogues of the corresponding peptides may be used to manipulate T-cell responses.^{4–7}

The B-cell epitopes may comprise IgG-binding epitopes⁸ and/or IgE-binding epitopes of allergens. These may be sequential or conformational in nature.^{9–13} Evidence gathered so far from epitope mapping of a few allergens indicates that IgG and IgE antibodies recognize the same or similar epitopes on allergens.^{14–18} Expression of various antibody isotypes has been reported to be controlled by lymphokines secreted by different subsets of T

cells.^{19–21} In contrast to the B-cell epitopes, the epitopes recognized by T cells are linear in nature. T-cell epitopes of allergens are pivotal to T-cell activation and may also play a role in differentiation of these T-cell subsets.^{4–6} Moreover, recent studies suggest that T-cell epitopes may be exploited for induction of allergen-specific tolerance *in vivo*.²² Definition of T-cell epitopes, therefore, is not only required for a further understanding of their role in T-cell differentiation, but also essential for designing more effective immunotherapeutic strategies.

The application of recombinant DNA and peptide synthesis technologies has led to the rapid growth of our knowledge of epitopes of allergenic proteins.¹ However, the progress in defining the epitopes of all grass allergens has been slow. We have previously reported molecular cloning and sequencing of a grass allergen, rKBG60, from Kentucky Bluegrass (*Poa pratensis*) pollen.^{23–26} Furthermore, the antibody-binding sites of rKBG60 have been defined using overlapping cDNA clones and peptides synthesized on polypropylin pins with the aid of murine antisera to rKBG60. However, we failed to detect human IgE-binding epitopes using the decapeptides on the pins.²⁵ In the present study, we have extended our analysis of B- and T-cell epitopes utilizing 20-residue long synthetic peptides, each with a 10-residue overlap, for further defining these epitopes in humans and mice.

MATERIALS AND METHODS

Synthesis of 20 residue peptides

For the analysis of human IgE and IgG antibody-binding epitopes,

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a set of 23 peptides, each 20 residues long and with 10 amino acid overlaps, was designed according to the deduced amino acid sequence of the cDNA clone, KBG60.²⁶ The peptides were synthesized using an ABI-model 438 peptide synthesizer based on the methods described by Merrifield.²⁷ After cleavage, the peptides were purified with reverse-phase high-performance liquid chromatography (HPLC). For all experiments peptide concentrations were established by weight.

Analysis of epitopes with the synthetic peptides

The binding of these peptides to the murine anti-rKBG60, and human IgE and IgG antibodies, was examined by enzyme-linked immunosorbent assay (ELISA). Briefly, each well of a Nunc Maxisorb (Life Technologies, Gaithersburg, MD) microtitre plate was coated with 10 µg peptides in 100 µl of 0.05 M carbonate/bicarbonate buffer, pH 9.6, overnight at 4°. The plates were then washed with phosphate-buffered saline (PBS)–Tween buffer three times, and the free sites of the wells were saturated with 2% bovine serum albumin (BSA) in PBS buffer by incubation at room temperature for 2 hr. After washing, the wells were incubated with 100 µl of the murine antisera raised against the rKBG60 at a 1 : 200 dilution. For detection of human IgG- and IgE-binding peptides, 1 : 50 and 1 : 2 diluted human sera were used, respectively. PBS containing 0.5% BSA and 0.1% Tween-20 was used as dilution buffer. The plates were incubated with the antisera at room temperature overnight, and washed three times with PBS–Tween buffer. The bound antibodies were detected with alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Zymed, San Francisco, CA), or goat anti-human IgG (Bio-Rad, Ontario, Canada), or goat anti-human IgE (Tago, Burlingame, CA). All the second antibodies were used at 1 : 2000 dilution. The colour development of the substrate was carried out at 37° for 60 min and the optical density (OD) values were read with an ELISA reader. The OD values were regarded as positive when the reading was more than threefold of the negative control. Each ELISA was repeated at least three times. One of the three representative experiments is shown.

Human sera

The serum pools from 11 grass-allergic patients and five non-grass allergic individuals were used as allergic and control serum, respectively, for the peptide binding studies, as described elsewhere.²⁵

Preparation of murine antibodies

The capacity of each peptide to induce antibodies *in vivo* was examined by immunization of mice with the corresponding peptide. For this purpose, female BDF1 mice, 6–8 weeks old, were immunized with the synthetic peptides. Each mouse received 30 µg of the peptides mixed with 2.5 mg dextran sulphate in 200 µl saline. Mice were boosted three times at three-week interval, with the same amount of peptide and 1 mg dextran sulphate. The blood was collected after 7 days of second, third and fourth immunization. The sera were isolated and stored at –20°. The murine anti-rKBG60 antibodies were also produced according to a method reported elsewhere.²⁸

Detection of the allergen-specific antibodies induced with the synthetic peptides

The specific antibodies induced by the peptides in mice were examined by ELISA. Microtitre plates were either coated with 0.1 µg/well of the rKBG8.3 protein for detection of the reactivities

of the antibodies induced with the peptides to the recombinant allergen, or coated with 10 µg/well of the synthetic peptides used in immunization for detection of peptide-specific antibodies, in 100 µl carbonate/bicarbonate buffer, pH 9.6, overnight at 4°. The plates were washed three times, saturated with PBS buffer containing 2% BSA and incubated with 1:20 diluted murine sera at room temperature overnight. The bound antibodies were detected with goat anti-murine immunoglobulin conjugated with alkaline phosphatase (Zymed) and the enzyme substrate by colour reaction, as described above.

Preparation of peripheral blood mononuclear cells (PBMC) and proliferation assay

Venous blood (20 ml) was collected into sterile plastic syringes containing 250 U/ml of heparin, diluted 1 : 2 with balanced solution in 50-ml sterile centrifuge tubes, and underlaid with Ficoll–Hypaque (Pharmacia, Uppsala, Sweden) at a ratio of 1 : 3, according to manufacturer's instructions. The tubes were centrifuged at 400 g for 30 min. Interphase cells were removed and washed three times, twice with balanced salt solution, and once with RPMI culture medium. These cells were counted and used for proliferation studies. The proliferation assays were performed in 96-well microtitre plates. The PBMC were resuspended to a concentration of 5×10^6 /ml in RPMI medium with 5% human AB⁺ serum. PBMC (5×10^5 /well) were cultured in triplicate in the absence or presence of antigen or peptide. Four days later the culture was pulsed with [³H]thymidine for 16 hr and [³H] incorporation was measured by scintillation counting. The values given are the arithmetic mean d.p.m. of triplicate cultures. The background in proliferation assays varied between 300 and 500 d.p.m.

RESULTS

Binding of human IgG antibodies to synthetic peptides

Several overlapping peptides of rKBG60 were assayed for their IgG binding using a serum pool of patients allergic to KBG pollen. Peptide numbers 6, 9–12, 17, 21, 23, and 28 were capable of binding to human IgG antibodies more than threefold the background, as determined by ELISA, and were therefore inferred as the human IgG binding determinants (Fig. 1a). Assay of these peptides for their ability to bind to antibodies in the control serum pool revealed that two peptides, 10 and 27, had relatively higher binding activities compared to the other peptides (Fig. 1b).

Binding of human IgE antibodies to synthetic peptides

The synthetic peptides were also used to determine the IgE binding epitopes. The serum pool from the allergic patients recognized the peptides 12, 18, 20, 21 and 23–28 (Fig. 2a). These peptides reacted with human IgE antibodies in repeated experiments. The control serum pool showed partial binding to some peptides, such as peptides 9, 23 and 27, and these reactions were regarded as non-specific binding (Fig. 2b). These results demonstrated that IgE-binding epitopes of the rKBG60 were located mainly on its C-terminal region, and 10 peptides representing at least five epitopes were involved in the human IgE antibody binding.

Both human IgG and IgE antibodies from the atopic serum pool recognized at least 10 peptides. The peptides 12, 21, 23, 25 and 28 showed both IgG and IgE binding activity, whereas peptides 18, 20 and 24–27 were found to be recognized exclusively by the human

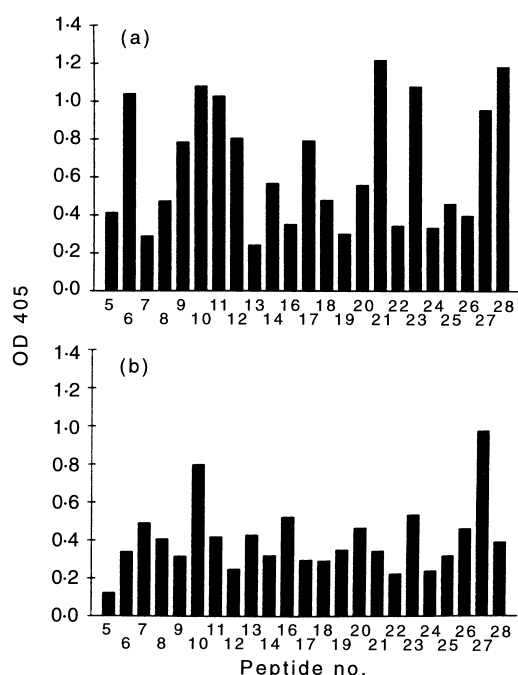


Figure 1. Determination of human IgG-binding epitopes on rKBG60 allergen. The reactivity of the overlapping synthetic peptides (Table 1) with the IgG antibody of the serum pools of the KBG-allergic (a) and non-KBG allergic (b) individuals was determined by ELISA.

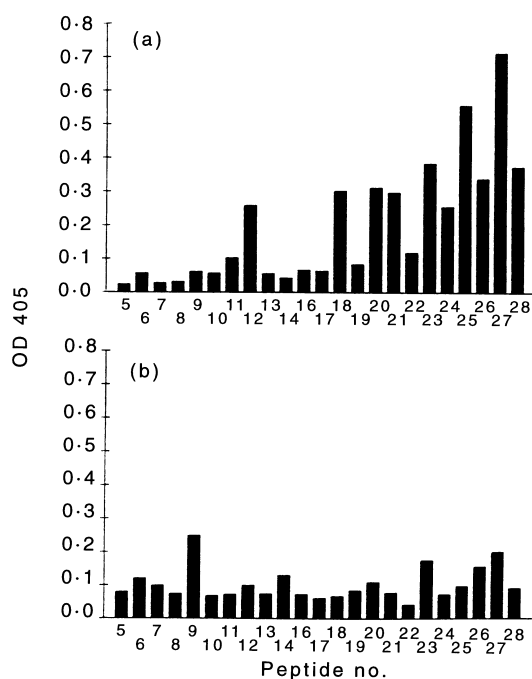


Figure 2. Determination of human IgE-binding epitopes on rKBG60 allergen. The IgE-binding epitopes were identified by the reactivity of overlapping synthetic peptides (Table 1) with IgE antibodies in serum pools of the KBG-allergic (a) and non-KBG allergic (b) individuals using an ELISA.

IgE antibody from the sera. Peptides 6, 9–11 and 17 showed no binding activities to human IgE antibodies. Taken together, these results indicated that most but not all human IgG and IgE antibodies from the same serum pool recognized the same epitopes of the rKBG60.

Recognition of synthetic peptides by murine anti-rKBG60 sera

For comparison of epitopes recognized by human IgE and IgG antibodies with the epitopes defined by the murine antibodies, the synthetic peptides were also analysed for their reactivity with murine anti-rKBG60 antisera. The results presented in Fig. 3a indicate that peptides 7, 12–19, 21, 23 and 26–28 bound to anti-rKBG60 antibodies, whereas the control sera showed no detectable reactivity (Fig. 3b). These results indicated that the peptides 7, 12–19, 21, 23 and 26–28 contained epitopes recognized by murine B cells.

Binding of synthetic peptides to antibodies of humans and mice

A comparison of antibody-binding sites of rKBG60 allergen is shown in Table 1. Murine antisera recognized 13 peptides in total, whereas human IgG and human IgE antibodies recognized nine and 10 peptides, respectively. Comparison of peptide binding of human versus murine IgG antibodies indicated that the patterns were different, only five peptides bound to IgG of both of these species. The human IgE antibody positives showed most restricted patterns. Three peptides (20, 24 and 25) were recognized by human IgE but not by the murine or human IgG antibodies. This comparison led to the inference that there might be subtle differences among murine antibody-, human IgG- and human IgE-binding peptides, although most of the human IgG and IgE antibody-binding epitopes were found on the same or adjacent to the murine antibody-binding peptides.

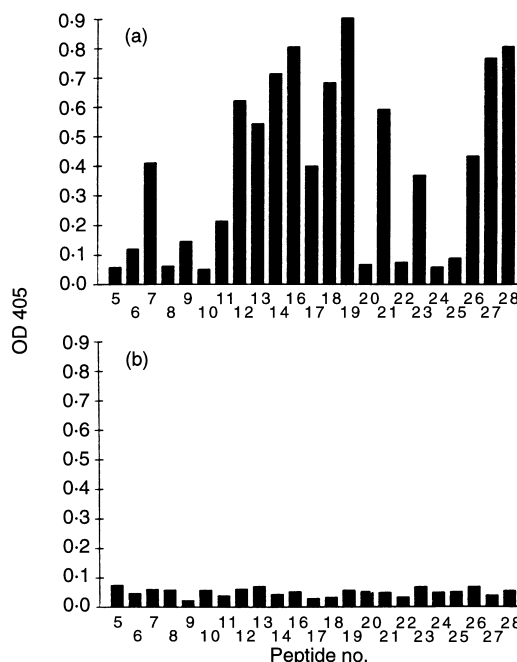


Figure 3. Analysis of murine antibody-binding epitopes on rKBG60 allergen. The murine antibody-binding peptides were defined by the reactivity of the synthetic peptides (Table 1) with anti-rKBG60 (a) and pre-immune (negative control) sera (b) by ELISA.

Table 1. Amino acid sequences of the 20-residue peptides and summary of their reactivities with the mouse anti-rKBG60 and human IgG and IgE antibodies

Peptide no.	Antibodies			Positions on the rKBG60	Amino acid sequence
	mIg	hIgG	hIgE*		
6	—	+	—	29–48	APKATTDEQKMIKINVGFK
7	+	—	—	39–58	MIEKINVGFKAAVAAAGGVP
9	—	+	—	59–78	AANKYKTFVATFGAASNKAF
10	—	+	—	69–88	TFGAASNKAFAEALSTEPKG
11	—	+	—	79–98	AEALSTEPKGAAVDSSKAAL
12	+	+	+	89–108	AAVDSSKAALTSKLDAAAYKL
13	+	—	—	99–118	TSKLDAAAYKLAYKSAEGATP
14	+	—	—	109–128	AYKSAEGATPEAKYDDYVAT
16	+	—	—	129–148	LSEALRIIAGTLEVHGVKPA
17	+	+	—	139–158	TLEVHGVKPAEEVKATPAG
18	+	—	+	149–168	AEEVKATPAGELQVIDKVDA
19	+	—	—	159–178	ELQVIDKVDAAFKVAATAAN
20	—	—	+	169–188	AFKVAATAANAAPANDKFTV
21	+	+	+	179–198	AAPANDKFTVFEEAFNDAIK
23	+	+	+	199–218	ASTGGAYQSYKFIPALEAAV
24	—	—	+	209–228	KFIPALEAAVKQSYAATVAT
25	—	—	+	219–238	KQSYAATVATAPAVKYTVFE
26	+	—	+	229–248	APAVKYTVFETALKKAITAM
27	+	—	+	239–258	TALKKAITAMSQAQKAAPPA
28	+	+	+	249–268	SQAQKAAPPAATGTATAA

* mIg, murine antibody; hIgG, human IgG; hIgE, human IgE.

Induction of antibodies to synthetic peptides

To examine the possibility that T-cell and B-cell epitopes may coincide on the same peptide, nine of these peptides, i.e. peptides 14, 16, 18, 19, 21, 23 and 26–28 that bound to the murine antibodies and three peptides, i.e. 5, 22 and 25, that did not bind to the murine antibodies, were immunized to induce the antibody production in mice. After secondary immunization, the sera of mice were tested and found to have no detectable peptide-specific antibodies; however, the antibodies were detected only after tertiary immunization. Peptides that showed no antibody induction after tertiary immunization failed to induce antibodies after further immunization. Eight out of 12 peptides used in immunization induced antibodies that were cross-reactive to rKBG8.3 (Fig. 4). Peptide 25, which bound neither to murine antibodies nor to human IgG but to human IgE, induced high titres of peptide-specific antibodies. Non-immune and pre-immune sera used as controls showed no reactivity to rKBG8.3. From the reactivities of the peptide-induced antisera, it is inferred that peptides 16, 18, 19, 23 and 25–28 are immunogenic and represent putative T-cell epitopes in BDF1 mice.

Cross-recognition of peptides by peptide-induced antibodies

The capacity of anti-peptide serum to bind to itself and to the other five peptides as negative controls, is illustrated in Fig. 5. The first set consisted of peptides 5, 18, 19, 21, 22 and 23 (Fig. 5a). The sera raised against peptides 19 and 23 were found to react with the corresponding peptides, whereas the sera against peptides 5, 21 and 22 did not. The latter finding was consistent with our previous finding that peptides 5, 21 and 22 were non-immunogenic. Furthermore, the sera against peptides 18 and 22 bound to the adjacent peptides 19 and 23, respectively, but not to the peptide

that was used as immunogen or to other peptides. As shown in Fig. 5b, the sera against peptides 26 showed binding to the peptide 26 as well as the adjacent overlapping peptides 25 and 27; a similar finding was made for sera against peptide 27. Sera against peptide 25 showed binding to self-peptide and the overlapping peptide 26 as well as the non-overlapping peptide 27. Furthermore, anti-peptide 28 serum showed binding to the adjacent peptides 25–27 but not to itself. Taken together, these results suggest that the C-terminus of rKBG60 not only represents the most antigenic and allergenic site of this allergen but may comprise a discontinuous or topographic epitope.

Stimulation of human T cells by synthetic peptides

The T-cell epitopes of rKBG60 were examined by determining the capacity of synthetic peptides to stimulate directly PBMC from

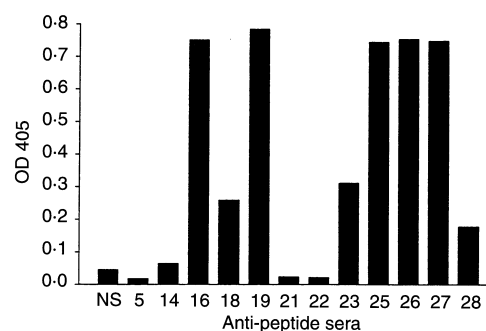


Figure 4. Allergen-specific antibodies induced by the synthetic peptides. The antisera from the mice immunized with the peptides were assayed by ELISA to determine their binding to the rKBG8.3. A pool of non-immunized mouse sera (NS) was used as negative control.

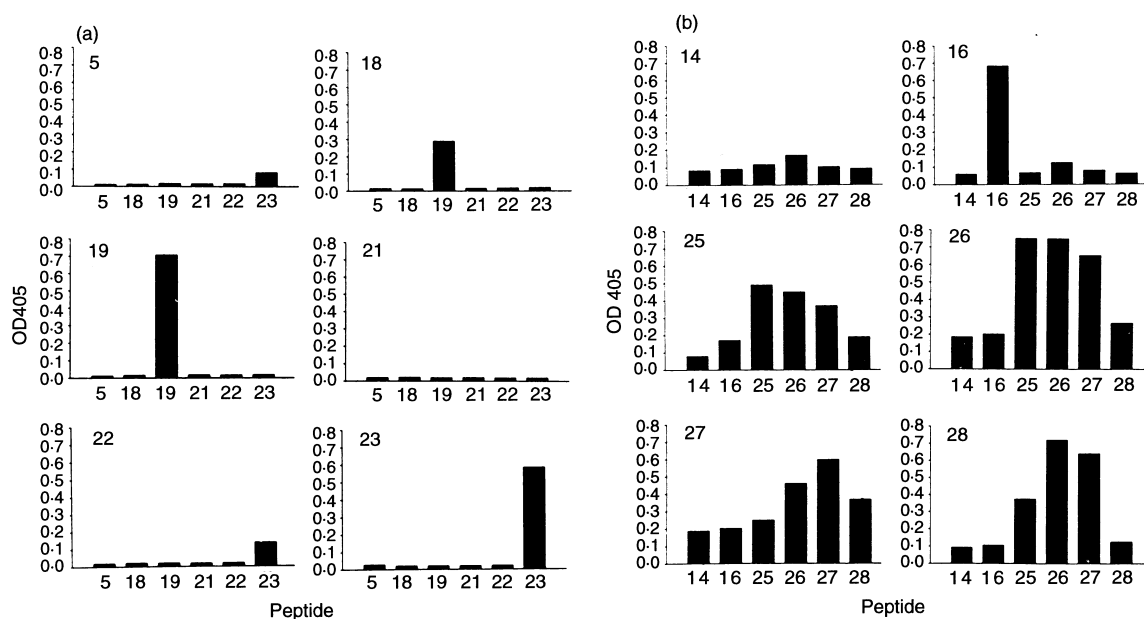


Figure 5. Reactivities of the synthetic peptides with the antibodies induced by the same or other peptides. Each of the peptides used for immunization was coated onto the ELISA plate to determine its ability to bind to the anti-peptide sera. Two sets of experiments were performed with six peptides used as coating antigens in each set; (a) set A included peptides 5, 18, 19 and 21–23, and (b) set B included peptides 14, 16 and 25–28.

grass pollen-allergic patients. Initially, using peptides 13 and 26, a dose–response curve was established; the maximal proliferative response was obtained at a peptide concentration of 100 $\mu\text{g/ml}$ (data not shown). In total, PBMC proliferation was examined in 13 patients using a number of overlapping peptides; the data for eight

patients with selected peptides are illustrated in Fig. 6, and the stimulation index for each peptide, shown in order of their reactivity as measured by proliferative responses, is summarized in Table 2. Results indicated that the PBMC of different individuals had varied levels of proliferative response to different peptides. For each individual, the mean proliferative response with at least three peptides showing the minimal d.p.m. value was considered as background. Except for three individuals, the rest of the group responded to peptide 13, while eight of nine subjects showed a response to peptides 25 and 26. Some individuals also responded to peptides 18, 23, 27 or 28. It is inferred from these results that the rKBG60 allergen consists of several potential T-cell epitopes.

DISCUSSION

A majority of antigens recognize antibodies in a 3-dimensional (3-D) complementary fashion; however, the lack of a 3-D structure of the rKBG60 allergen makes the search for its discontinuous or topographical epitopes impossible at present. The method commonly used for epitope determination by synthetic peptides is quantitative inhibition of monoclonal antibodies (mAb) to putative surface epitopes. The specificity of mAb, in turn, is related by competition to natural antibodies. However, it is difficult to interpret such results in the context of the allergy because of the variations in affinity of diverse antibodies, and the presence of anti-idiotypic and specific IgG antibodies *in vivo*. As our previous study using 10-residue peptides had suggested that some of the epitopes on the rKBG60 may be sequential,²⁵ we examined the immunoglobulin-binding property of a complete set of 20-residue, 10-overlapping synthetic peptides representing rKBG60. We elected to take a polyclonal approach and employed an ELISA-based assay to examine the antibody binding ability of these peptides. It is therefore assumed that ELISA readings are proportional to the amount of antibodies bound by each peptide irrespective of their affinities.

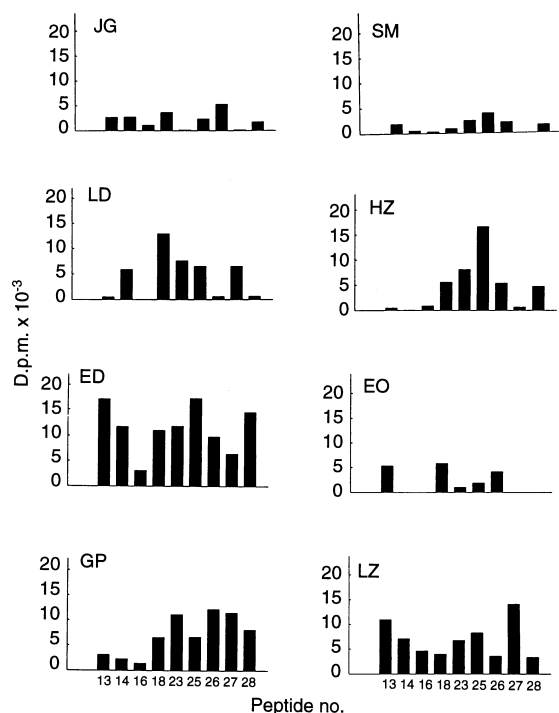


Figure 6. Proliferative responses of PBMC. 5×10^5 PBMC were cultured in the presence of different peptides at 100 $\mu\text{g/ml}$ for 5 days. The values given are the arithmetic mean d.p.m. of [^3H]thymidine incorporation in triplicate cultures with the background subtracted.

Table 2. Summary of PBMC proliferation studies with synthetic overlapping peptides of rKBG60

Individuals	Peptides	
	With SI = 3	Order of peptide reactivity*
SM	25	25 > 23 > 26 > 28 > 13
EO	13, 18, 26	18 > 13 > 26 > 25 > 23
LD	14, 18, 23, 25, 27	18 > 23 > 25 = 27 = 14
LZ	13, 25, 27	27 > 13 > 25 > 23 = 14
JG	26	26 > 18 > 14 = 13 > 25 > 28
ED	13, 25, 28	25 > 13 > 28 > 23 > 18
HZ	25	25 > 23 > 18 = 26 > 28
GP	23, 26, 27, 28	26 > 27 > 23 > 28 > 18 = 25 > 13 = 14
SJ	13, 25	13 > 25 > 14
EH	5, 13, 18, 23	13 > 5 > 14 > 18 = 19 = 22 = 23 > 6 = 10 = 25 > 17
CD	13, 17, 20, 25	20 > 13 = 14 > 17 = 25 > 28 = 19 = 18 > 9 > 5
KM	13	13 > 21 = 23 > 14 = 9 > 12 > 26
TK	13, 27, 19	27 > 13 > 20 = 19 > 6 = 9 > 10 = 23

* Peptides other than those listed in column 2 showed a stimulation index (SI) between 2 and 3.

Our results revealed that a number of peptides were capable of binding to human and murine antibodies suggesting strong antigenicity and allergenicity of the molecule, as illustrated schematically in Fig. 7. In contrast to the human and murine IgG antibodies, human IgE antibodies recognized peptides predominantly from the C-terminus of the molecule (Table 1). A major finding from this study is that some peptides that bound to human IgE antibodies did not bind to human or mouse IgG antibodies. However, in most cases peptides recognized by human IgE antibodies were adjacent to those bound by murine or human IgG antibodies. The basis for this isotype-associated recognition of allergen epitopes is unknown at present. This may be due to the induction of isotype-specific antibodies resulting from the

exposure to varying doses of the antigen.²⁹ Furthermore, some epitopes may induce IgE antibodies because of a specific V-gene usage.³⁰

Another noteworthy finding involved the comparison of the peptides recognized by human and murine antibodies. Our results indicated that the peptides recognized by human and murine IgG antibodies differed. Human antibodies recognized the N-terminal part of the molecule, whereas murine antibodies predominantly recognized the central core of the antigen. These differences in epitope recognition between mouse and human may have resulted from variations in the mode, frequency and/or route of immunization, and in the major histocompatibility complex (MHC).

We also examined immunogenicity of peptides in B6D2F1

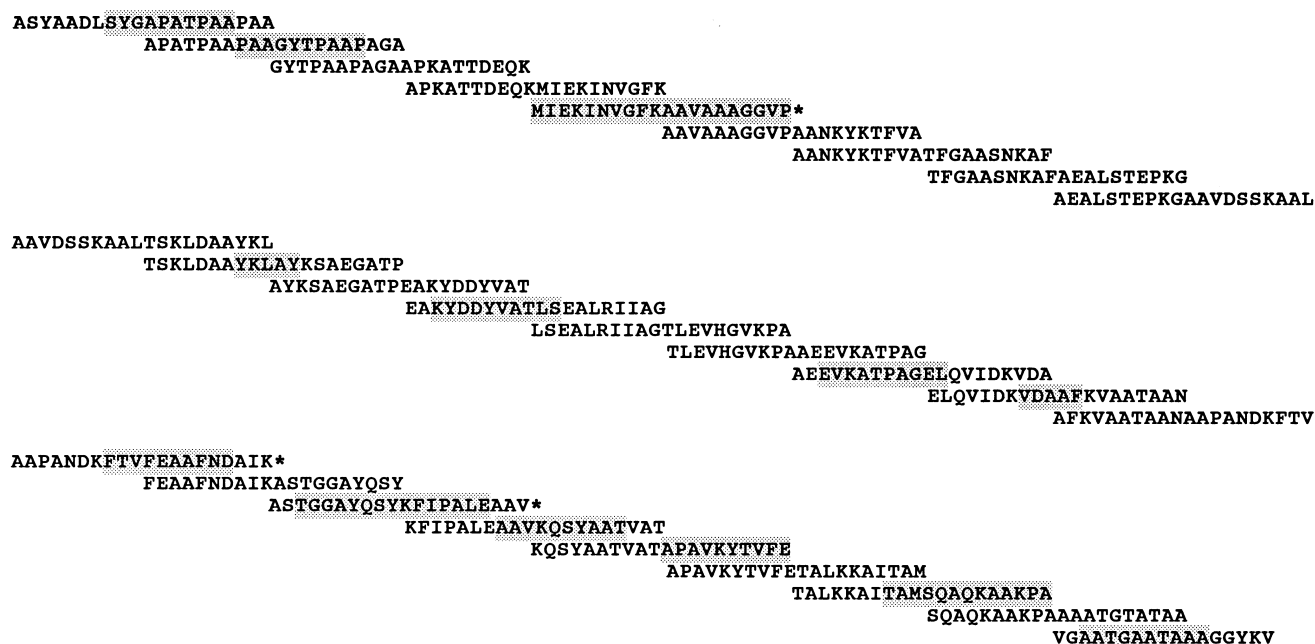


Figure 7. Schematic illustration of the epitopes present on rKBG60 allergen. The shaded regions represent the putative epitopic residues recognized by antibodies and T cells.

mice, which represent the high IgE-responder among several strains examined (M. Yang, L. Zhang, Y. Wong, P. Chong & S. S. Mohapatra, manuscript submitted for publication). This approach is based on the principle that a short peptide can elicit antibody production only if it possesses three sites, i.e. sites recognized by B-cell surface immunoglobulin, class II MHC molecule, and T-cell receptors.^{31–33} Accordingly, the antibody-binding peptides that elicited allergen-specific antibodies without coupling to carrier macro molecules, are considered to possess T-cell epitopes. T-cell epitopes from foreign proteins provide help to the B-cell epitopes to induce antibodies that recognize the corresponding native proteins when the two kinds of epitopes are linked.^{34,35} Thus eight out of 12 synthetic peptides used in this study induced antibodies that not only recognized the peptides used as immunogen but also the intact antigen, which is consistent with previous studies.^{14,36} However, the antibodies induced by each of the peptides 18, 22 and 28 bound weakly or not at all with the corresponding peptide but reacted with an adjacent overlapping peptide. Furthermore, the antibodies induced by peptides 25–28 were cross-reactive with each other, which is consistent with our previous finding that this hydrophobic part of the molecule is highly immunogenic.²⁵

To elucidate peptides recognized by human T cells, we used a PBMC proliferation assay rather than T-cell lines or clones. The results demonstrated that rKBG60 allergen contained a number of T-cell epitopes that varied with each individual. Nevertheless, certain epitopes were recognized more frequently by T cells of different individuals than others. These results are consistent with recent studies with other pollen allergens.³⁷ Moreover, the peptides differed in terms of their ability to stimulate PBMC. Interestingly, our results demonstrated that the T-cell epitopes determined by PBMC proliferation and by immunization of mice were localized on the same synthetic peptide, which suggest that the human and murine T-cell epitopes may be similar.

From therapeutic standpoints, T-cell epitopes are presently being considered by some laboratories as potential immunotherapeutic regimens, in particular the peptides of *Fel d I*, which constitutes the most dominant allergen of the cat dander.²² As *Fel d I* contains only four T-cell epitopes, it is likely that a combination of T-cell epitopes may substitute the crude extract for therapeutic purposes. However, the situation is more complex with the grass pollens, which contain at least three major groups of allergens. Our study with rKBG60, representing one of the three major grass allergens, indicates that the epitope recognition profiles by grass-allergic individuals are more diverse. Therefore, for immunotherapeutic purposes, the design of an appropriate combination of T-cell epitopes may be difficult, if not impossible. Furthermore, some of the T-cell epitopes of this grass allergen are capable of inducing antibody response. Thus, therapeutic peptides have to be thoroughly curtailed to avoid the IgE binding ability of these peptides and consequently the possibility of systemic reactions.

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